STUDIES ON MITOZOLOMIDE (CCRG 81010), A NEW ANTINEOPLASTIC AGENT.

BY

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To Mom and Dad

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STUDIES ON MITOZOLOMIDE (CCRG 81Ø1Ø), A NEW ANTINEOPLASTIC AGENT.

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Submitted for the degree of Doctor of Philosophy, at Aston University, March 1985.

The rational for the synthesis of the novel 1,2,3,5-imidazotetrazinone ring system and in particular for that of mitozolomide [8-carbamoyl-3-(2-chloroethyl)imidazo [5,1-d][1,2,3,5]tetrazin-4-(3H)-one] is discussed. The antitumour activity of the triazenes, the chemistry of the 1,2,3-benzotriazinones and the imidazo [1,2,4]-triazinones which contributed to the hypothesis that mitozolomide might possess antitumour activity are reviewed. Various mechanisms of action are proposed. Chiefly via the production of the metabonates 2-chloroethyl isocyanate, Diazo-IC (5-diazo imidazole-4-carboxamide) and MCTIC (5-[3-(2-chloroethyl) triazen-1-yl] imidazole-4-carboxamide).

No evidence of isocyanate generation by mitozolomide could be found using the inhibition of enzymes sensitive to the effects of carbamoylation. It was concluded that 2-chloroethyl isocyanate and Diazo-IC production did not occur in intact cells. In precursor incorporation experiments mitozolomide and MCTIC were shown to have similar effects. The generation of MCTIC was further supported by the finding that [^{14}C]-imidazo mitozolomide decomposed to produce [^{14}C]-AIC, (4-aminoimidazole-5-carboxamide) which is an accepted metabonate of MCTIC. BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea) a known antitumour agent produces a chloroethydiazo moiety, which is fundamental to its mode of action. If mitozolomide does generate MCTIC which is also a chlorodiazo species it is possible that both mitozolomide and BCNU possess a common mechanism of action.

Flow cytometry of Lewis lung carcinoma cells treated <u>in vivo</u> and <u>in vitro</u> with mitozolomide revealed a marked G_2/M block. Such a result would be expected of an alkylating agent. Again the effects of MCTIC were similar to those of mitozolomide.

The production of O^6-chloroethylguanine adducts in DNA by mitozolomide was found to be an important feature of mitozolomide toxicity using methyl excision repair proficient and deficient cell lines. The stability of mitozolomide and its analogues was investigated, but no obvious correlation with antitumour activity existed.

In conclusion, mitozolomide generates MCTIC and hence alkylates DNA in a manner analogous to that of the nitrosoureas. A number of DNA lesions are presumably produced, of which the O^6-chloroethylguanine adduct may be particularly important.

Key words: Carbamoylation, Alkylation, Triazenes, Nitrosoureas, Imidazotetrazinones.

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A. INTRODUCTION

A.1 Cancer Chemotherapy.

Modern cancer chemotherapy stemmed from observations on the effects of a chemical weapon, sulphur mustard gas (Gilman 1946). It was noted that this produced a depression in the number of bone marrow cells which prompted the use of similar agents - nitrogen mustard and tris-chloroethylamine - in the treatment of bone marrow malignances (Gilman 1963). The array of compounds used in cancer chemotherapy today has expanded rapidly since 1946 but most have one feature in common, they are all extremely cytotoxic compounds with little or no intrinsic selective toxicity to tumour cells.

Most antineoplastic compounds may be designated as antimitotic agents, that is, they exhibit the greatest toxicity to rapidly dividing cells. Such drugs interfere with cell division in a number of different ways: they affect the ability of DNA to act as a template (eg. cross-linking and intercalating agents such as the mustards and adriamycin); they interupt the supply of precursors of DNA synthesis (eg. the anti-metabolites such as methotrexate); or they damage the mitotic spindle (eg. the spindle poisons such as vincristine).

Originally it was thought that tumour cells were the most rapidly dividing cells in the body and therefore antimitotic agents were believed to provide a basis for selective toxicity. Unfortunately tumour kinetics are more complex than then appreciated and it is now known that the

rate of proliferation of tumour cells may cover a vast range, overlapping with that of normal dividing tissue such as the cells of the bone marrow and the gastrointestinal tract. Even if all actively dividing cells are destroyed repopulation of the tumour mass can often occur by recruitment of previously quiescent cells into a new dividing fraction.

There are many characteristics of cancer that make it a difficult chemotherapeutic problem. In particular "cancer" encompasses a number of distinct diseases each with potentially different chemotherapeutic requirements. The similarity of cancer cells to their normal precursors has also defied efforts to define an unambiquous target around which a selective antitumour agent could be designed. The heterogenous nature of tumour cells, the poor vascularization of solid tumours and the ability to metastasize are some of the characteristics of cancer which provide such a formidable enemy for the chemotherapist.

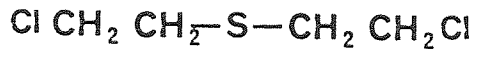
Despite these significant difficulties successes against certain cancers have been acheived, for example the response of choriocarcinoma, Hodgkin's disease and childhood lymphatic leukeamia are dramatic and often complete. There are also notable failures, carcinoma of the lung and kidney have an abysmal response rate and when a response does occur it is often partial and brief. Development of new antineoplastic agents is therefore essential. Since "biochemical targets" are ill-defined and

often represent quantitative differences between normal and tumour cells, such as the level of an enzyme rather than unique enzymes or metabolic pathways, development of new drugs mainly depends on the efforts of the synthetic chemist.

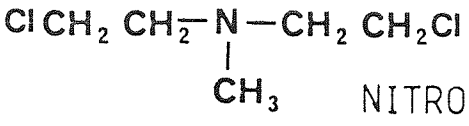
Synthetic programs have been based around molecules that have already enjoyed success in the clinic or the synthesis and testing of novel, reactive compounds. A combination of these two strategies may be said to account for the eventual synthesis of mitozolomide. It is a novel structure but one it was hoped would possess antitumour activity after consideration of the clinically used agents, BCNU and DTIC. Investigation of related chemical structures also predicted that mitozolomide would have a chemical reactivity conducive to antitumour activity.

FIG.1 The structures of some antineoplastic agents

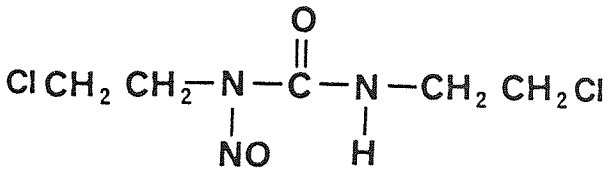
Alkylating agents



SULPHUR MUSTARD

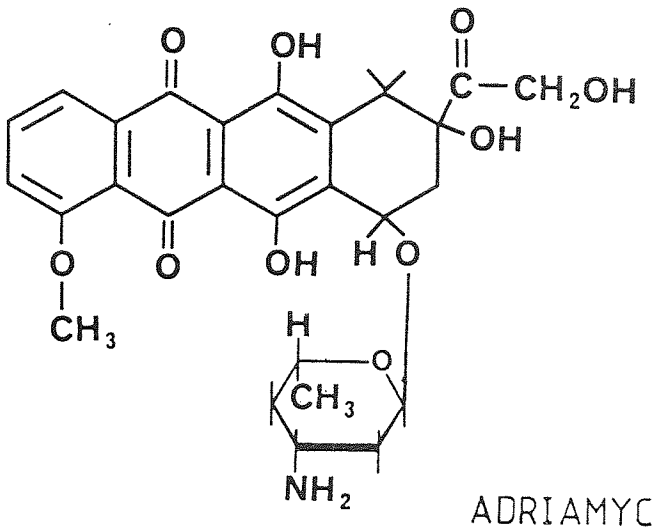


NITROGEN MUSTARD



BCNU

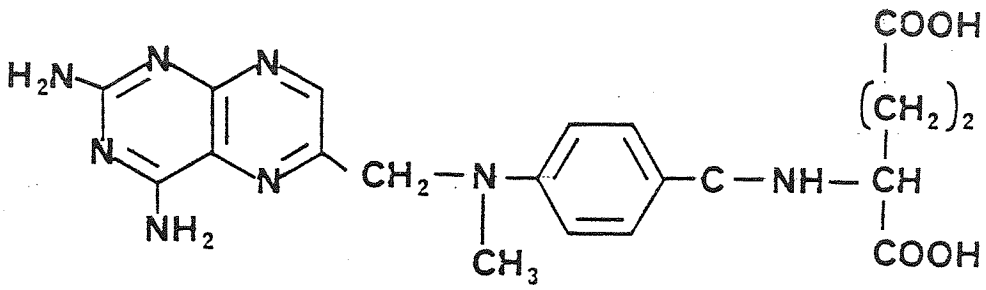
Intercalating agents



ADRIAMYCIN

FIG. 1 Cont.

Antimetabolites



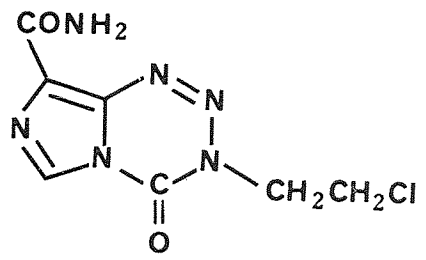
METHOTREXATE

A.2. The rationale for the synthesis of Mitozolomide.

Although a novel compound mitozolomide, (8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one) (Fig 2), was synthesised with regard to known antitumour agents. This is well illustrated by the incorporation of the "NNN" link and the 2-chloroethyl side-chain into its structure. Both these functionalities have long associations within cancer chemotherapy.

The chemical skeleton of mitozolomide, the 1,2,3,5-tetrazinone ring system fused to an imidazole ring is the structural feature which justifies the classification of mitozolomide as a "novel compound", since it had not previously been synthesised. The belief that imidazotetrazinone ring system could possess antitumour properties was based not only on the presence of the "NNN" link but also on the anticipation that it would be very reactive chemically. The considerations which lead to the synthesis of mitozolomide are discussed below.

FIG 2



Mitozolomide

A.2.1. The imidazo[5,1-d]-1,2,3,5-tetrazinone ring system.

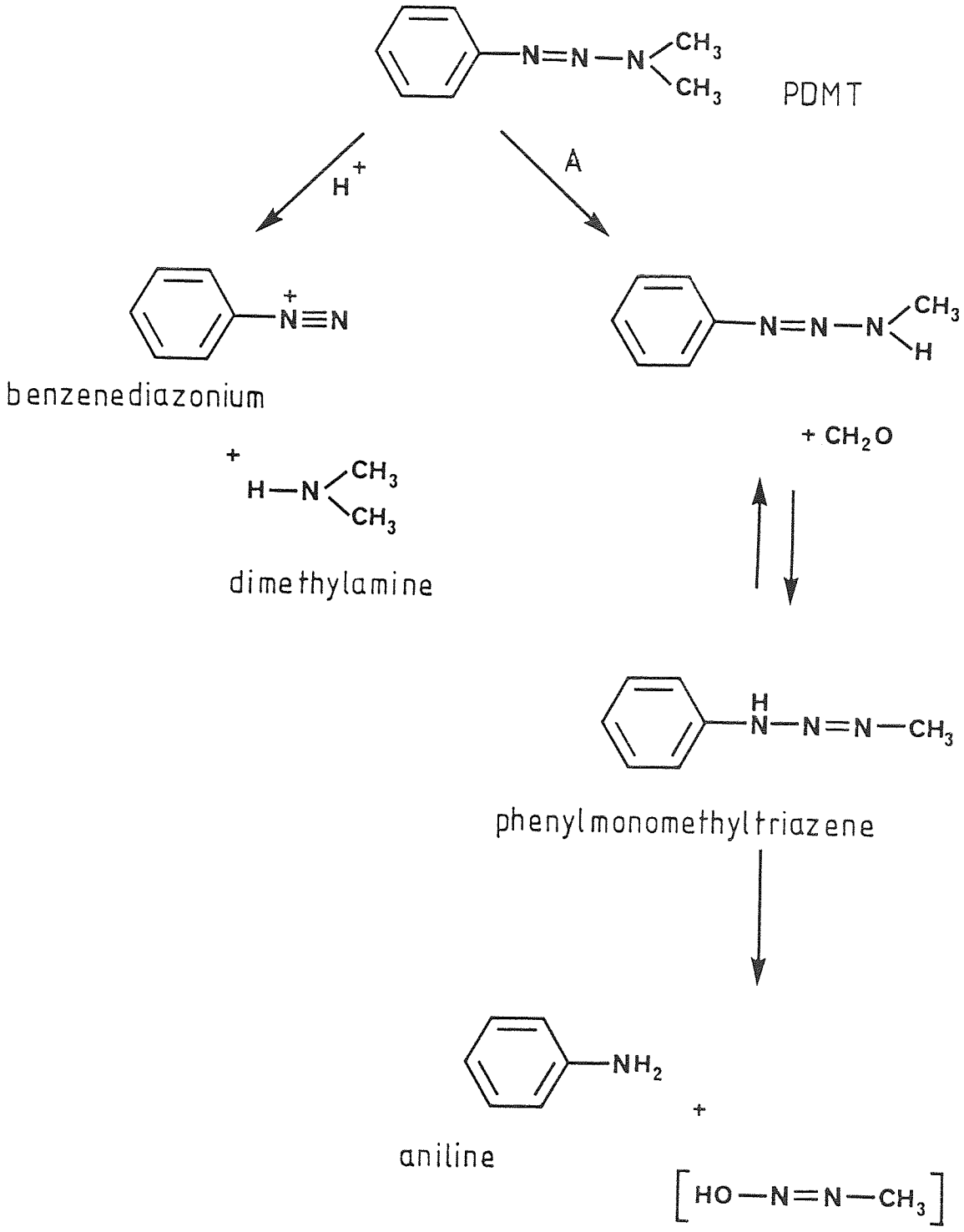
A.2.1.1. The "NNN" linkage.

The "NNN" linkage in either acyclic (eg. triazene) or cyclic (eg. 1,2,3,-triazine) arrangements is the only common feature of an extensive range of compounds that have been of interest in cancer chemotherapy since 3,3-dimethyl-1-phenyltriazene (PDMT) was shown to be active against mouse sarcoma 180 by Clarke et al. (1955). The activity of PDMT prompted the synthesis of further triazenes including many phenyl derivatives (Lin et al. 1972), benzene analogues (Shealy et al. 1971) and the imidazotriazenes which were reviewed by Shealy (1970). Many of these compounds were active against murine tumours.

A number of theories were advanced in an attempt to account for the antitumour activity of PDMT. Clarke et al (1955) suggested that the activity of PDMT was due to the formation of the reactive benzenediazonium ion which then inhibited cell proliferation. However Preussmann et al (1969) demonstrated PDMT metabolism by rat liver microsomes in vitro and proposed that triazenes might act as alkylating agents in vivo through carbonium ions produced by this mechanism (Fig 3).

The suggestion that the diazonium ion had antitumour activity lead to the diazotisation of 5-amino-4-carboxamide (AIC) by Shealy et al (1961) and the production of 5-diazo-imidazole-4-carboxamide (Diazo-IC). The ribotide of AIC occupies a central position in the de novo purine

FIG. 3 The possible reactive species generated by PDMT



A = Oxidative metabolism

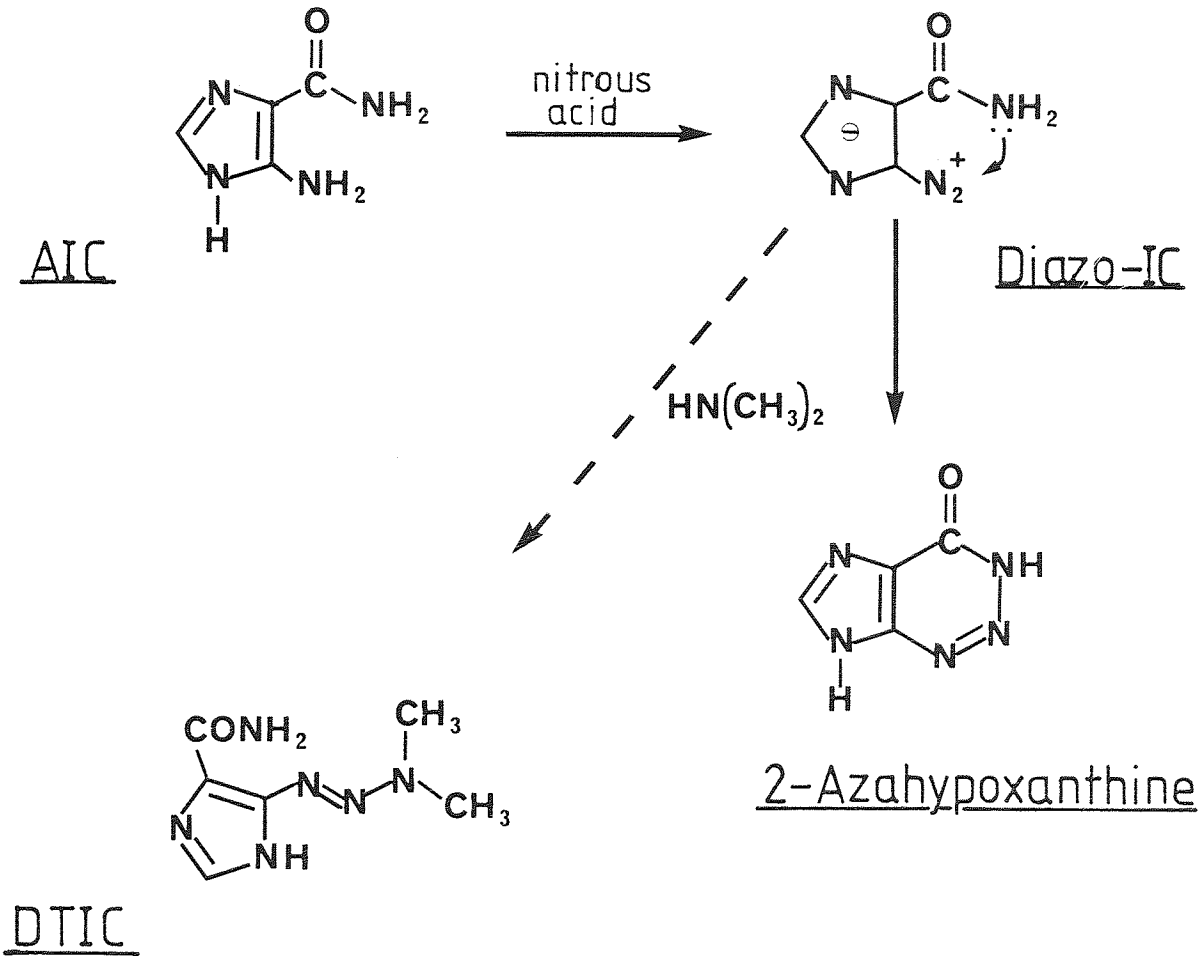
synthetic pathway. It was proposed that Diazo-IC, possessing the reactive properties of the diazonium ion and being structurally related to AIC was a potential anti-metabolite of the de novo synthetic pathway.

The possibility of selective toxicity to tumour cells arose since AIC had been found to be more rapidly utilized by tumour cells than normal tissue (Hano and Akashi 1964). Diazo-IC was found to exhibit antitumour activity both *in vivo* and *in vitro* (Shealy et al. 1961 and Hano et al. 1968).

The antitumour activity of Diazo-IC was limited by a tendency to cyclise in aqueous conditions to 2-azahypoxanthine which is inactive as an antitumour agent (Shealy et al. 1961). In an attempt to provide a more stable preparation of Diazo-IC Shealy coupled Diazo-IC with secondary amines (Shealy et al. 1962a). The most notable result of this synthetic strategy was the production of 5-(3,3-dimethyltriazen-yl)imidazo-4-carboxamide; DTIC (Fig 4).

FIG. 4

Diazotisation of AIC and synthesis of DTIC



DTIC possessed marked antitumour activity (Shealy et al. 1962b) and is at present a clinically used agent, mainly in the treatment of malignant melanoma (Carter and Friedman 1972 and Comis 1976). However the mechanism of action of DTIC against human disease is now believed to have little to do with the production of Diazo-IC (Kreis 1977) or the inhibition of purine biosynthesis (Skibba et al.197Ø).

The success of DTIC in the clinic is by no means complete. Although it is the most used agent against melanoma it only produces temporary responses in 19% of patients treated (Luce et al. 197Ø). Also it is not active against many tumour types and has serious side-effects. The search for improved agents fuelled further chemical synthetic efforts in the triazine and triazene field (Lin and Loo 1978, Giraldi et al. 198Ø). The study of the chemical properties of the 1,2,3-benzotriazinones (Siddiqui and Stevens 1974 a,b and Stevens 1976) is one area of investigation that has eventually lead to a novel compound with excellent activity against murine tumours:- mitozolomide.

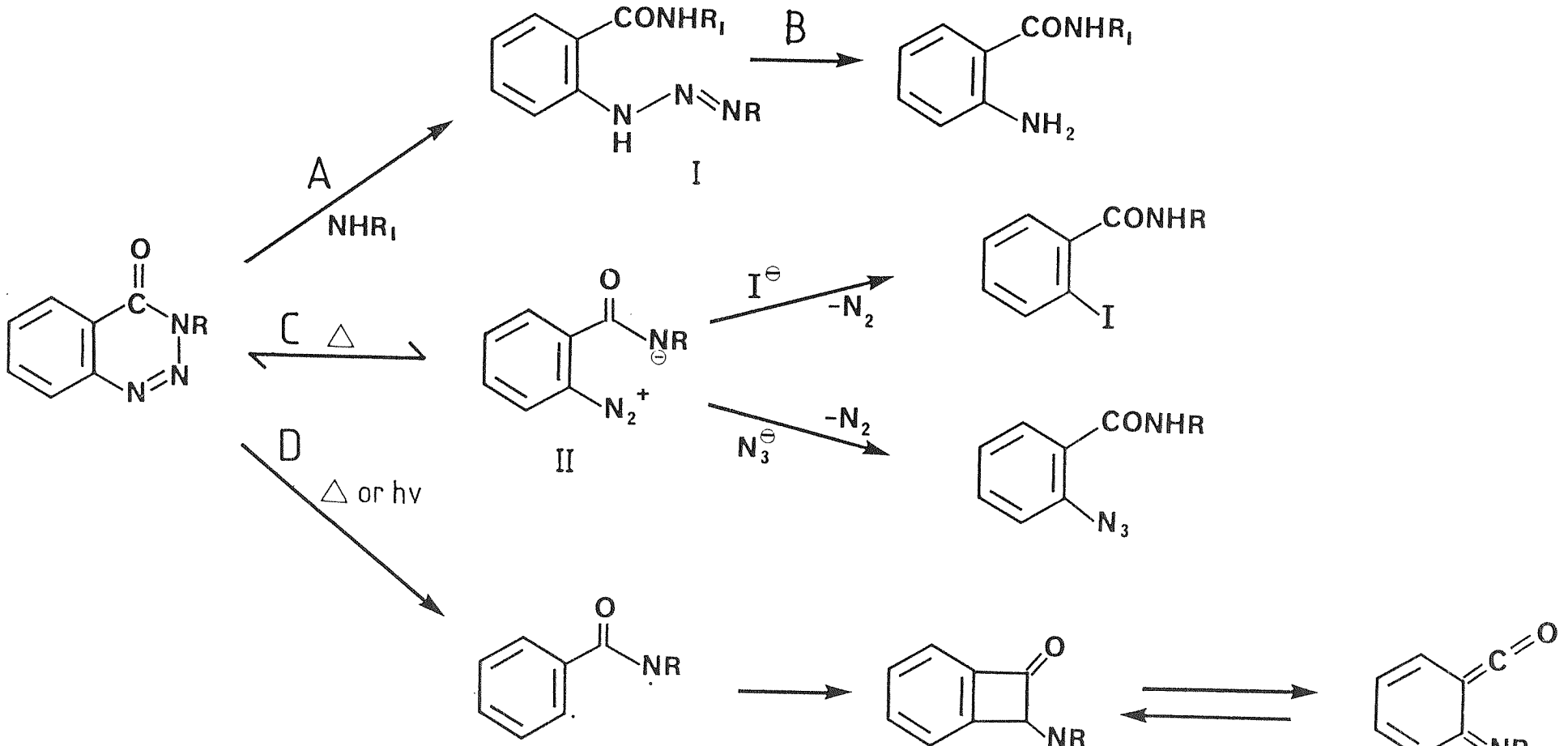
A.2.1.2 Projected reactivity of theimidazo[5,1-d]-1,2,3,5-tetrazinone ring system.

The 1,2,3,-benzotriazinones have a very complex chemistry (Stevens 1976). The ring fission reactions undergone by particular benzotriazinones are generally governed by the N3 substituent (Gescher et al. 1977). A summary of the ring fission reactions known to occur is given in Fig.5.

Nucleophilic attack by amines at C4 as shown in pathway A produces the triazene (I) (Siddiqui and Stevens 1974a). The heterolytic pyrolysis of benzotriazinones (pathway B), is a property of the unsubstituted N-aryl derivatives and is most readily acheived by boiling in acetic acid. Heterolysis proceeds by cleavage of the N2-N3 bond to produce the betaine (II) (Siddiqui and Stevens 1974b) which may be trapped by iodide and azide to yield iodo- and azido- benzamides, respectively (Gescher et al. 1977).

Benzotriazinones have also been shown to undergo homolytic fission in both photolytic (Huisgen 1968) and thermolytic conditions. The reaction proceeds via a diradical intermediate (Bashir and Gilchrist 1973, Burgess and Milne 1966) to produce the benzazetinone and its imino-valence tautomer.

FIG.5 The ring fission reactions of the 1,2,3-benzotriazinones

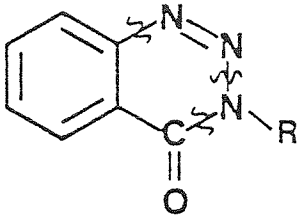


Pathway A : R = 3 alkyl, 3 araalkyl, 3 aryl
Pathway B : R = H
Pathway C : R = unsubstituted N-aryl

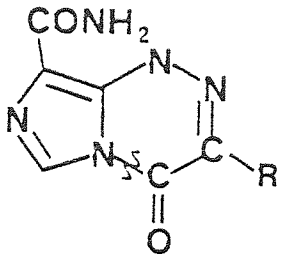
Pathway D : R = N-aryl or unsaturated group

Despite its reactivity and relationship to proven antitumour agents in the triazene series no benzotriazinone has yet displayed similar antitumour activity (Stevens 1976). Investigation of compounds in which a bridgehead nitrogen was incorporated with the triazine system to produce the 1,2,3,5-tetrazine ring was considered an interesting progression from the study of the benzotriazinones since the resulting molecule might possess a great flexibility in its chemistry (Stevens et al. 1984). Such a structure is theimidazo[5,1d-]-1,2,3,5-tetrazin-4(3H)-one ring system. This can be considered as a combination of the 1,2,3-benzotriazinone and the 3-substituted imidazo[5-1d]-1,2,4-triazin-4(3H)-ones. The imidazotriazinone ring cleaves in one position only, the 4-5 bond (Baig et al. 1982). and as the benzotriazinones cleave in three positions it was possible that the imidazotetrazinones would ring open in a total of four different modes (Fig. 6). Such a decomposition could produce an array of reactive fragments which might be responsible for antitumour activity.

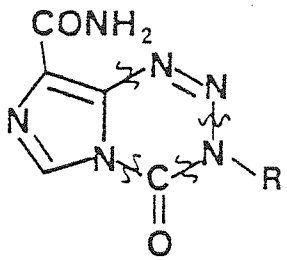
FIG. 6 Ring cleavage reactions of the benzotriazinones, the imidazotriazinones and possible reactions of the imidazotetrazinones.



BENZO-1,2,3-TRIAZINONE



IMIDAZO-1,2,4-TRIAZINONE



IMIDAZO-1,2,3,5-TETRAZINONE

A.2.1.3. The choice of ring substituents.

The derivative believed to be the best prospect to realise the hopes that the imidazotetrazinone ring system would provide the basis of an antitumour agent was 8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one, mitozolomide. The preferred peripheral substituents of 8-carbamoyl and 3-(2-chloroethyl) were arrived at by the consideration of the structural features of the clinically used antitumour agents, DTIC and the nitrosourea 1,3-bis(2-chloroethyl)-1-nitrosourea, BCNU (Fig 7).

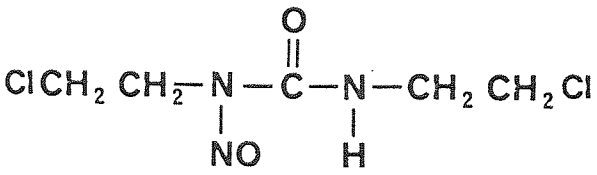
Lin et al. (1972) had shown that the 8-carbamoyl group of DTIC was not crucial to its activity, an observation also true for the aryl substituent of its counterpart, the phenyl triazenes (Rondestvedlt and Davis 1957). Although not crucial the carbamoyl group is certainly permissive to antitumour activity and also produces a molecule with an imidazo ring directly analogous to that of DTIC.

BCNU has been used as a single agent in the treatment of Hodgkin's disease and non-Hodgkin's lymphoma but its main use is in the treatment of head and neck tumours (Gottlieb et al. 1981, Levin 1981). In common with the 2-chloroethylamine type alkylating agents the nitrosoureas possess a 2-chloroethyl moiety which enables the alkylation of DNA, an action believed to be fundamental to their anti-

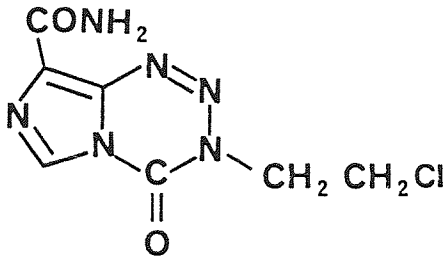
tumour activity (Kreis 1977). The importance of the 2-chloroethyl group to so many active agents recommended its incorporation onto the imidazotetrazinone ring system at an early stage in the gestation of the synthetic project.

Further support for the synthesis of mitozolomide was the fact that the necessary starting materials were Diazo-IC and 2-chloroethyl isocyanate. It has already been mentioned that Diazo-IC is an antitumour agent per se: 2-chloroethyl isocyanate is also a reactive moiety and is produced on the decomposition of BCNU (Colvin and Brundrett 1981). It was believed that the bridge-head nitrogen could facilitate a retro-cycloaddition reaction. that is a reversal of the chemical synthesis of mitozolomide. Such a reaction would produce Diazo-IC and the isocyanate intracellularly, possibly resulting in antitumour activity. This suggestion will be discussed further in section A.5.1.

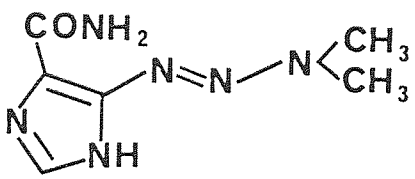
FIG. 7 The structures of BCNU, mitozolomide and DTIC.



BCNU



Mitozolomide



DTIC

A.3 Chemical synthesis of the imidazotetrazinones.

A.3.1 The synthetic reaction.

Ege and Gilbert (1979) reported the cycloaddition reaction of diazoazoles and isocyanates (Fig. 8A) and the production of the previously unknown pyrazolo-[5,1-d]-1,2,3,5-tetrazin-7(6H)-one. Until its synthesis the 1,2,3,5-tetrazine system had been believed to be inherently unstable (Wiley 1978). In the directly analogous cycloaddition reaction of diazo-IC and an isocyanate Stevens et al. (1984) were able to produce derivatives of the imidazo[5,1d]-1,2,3,5-tetrazin-4(3H)-one ring system (Fig. 8B) in high yield.

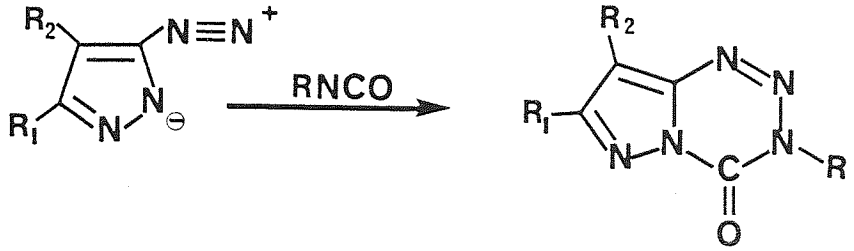
The products were cream or pastel coloured powders which were soluble in DMF, DMSO, 1-methy-2-pyrolidinone and sparingly soluble and unstable in alcohols. The imidazotetrazinone ring system was found to be unstable in protic solvents but when stored under dry, dark conditions showed no detectable deterioration for several months. Melting points ranged between 138 and 210°C and were associated with violent decomposition and effervescence (Stone 1981).

Although a large number of imidazotetrazinone derivatives have been synthesised there are some limitations to the reaction, preparation of the unsubstituted imidazotetrazinone has so far failed and cycloadducts are not formed with cyclohexyl, n-butyl, t-butyl, n-tridecyl, and n-pentadecyl isocyanates. Similarly phenyl-p-tolyl-

isothiocyanate and NN'-diphenyl-,NN'-di-p-tolyl, and NN'-dicyclohexyl-carboiimides also failed to participate in the reaction (Stone 1981).

FIG. 8

A. Synthesis of the pyrazolotetrazinones



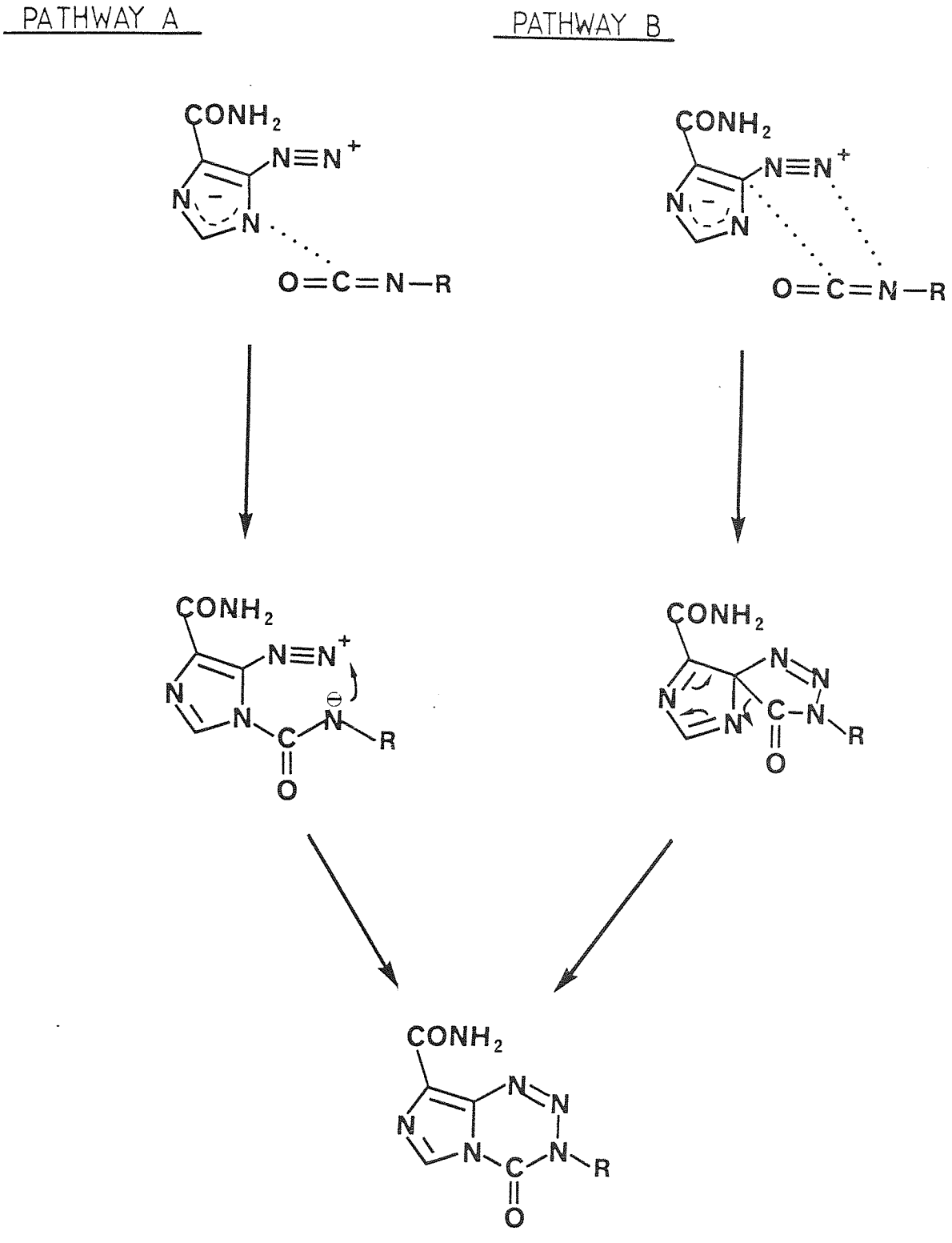
B. Synthesis of the imidazotetrazinones.

A.3.2. The reaction mechanism.

The mechanism of cycloaddition between diazoazoles and electron deficient heterocumulenes such as the isocyanates has not yet been proven but two possibilities exist, concerted or stepwise addition. The concerted addition pathway was considered unlikely by Ege and Gilbert (1979), a view supported by Gilchrist and Storr (1979) who believed that heterocumulenes did not normally participate in such reactions. Eqe and Gilbert (1979) proposed two alternative for a stepwise mechanisms (Fig. 9). Pathway B has precedents, Padua and Kumagai (1981) showed that the reaction of heterocyclic diazo compounds with added dipolarophiles proceeds via an initial 1,3-dipolar cyclo addition followed by a subsequent rearrangement. Stone (1981) has also suggested pathway B to be the most likely candidate for the reaction mechanism.

FIG. 9

Reaction mechanism for the synthesis of the imidazotetrazinones.



A.4. Experimental antitumour activity of mitozolomide.

Mitozolomide was screened aqainst a range of murine tumours and found to be as active as many clinically used agents (table 1). Pronounced antitumour activity was achieved against a number of advanced solid murine tumours (table 2). The Lewis lung carcinoma was particulary sensitive, both the primary and the metastases being inhibited. Activity aqainst this tumour is thought to be the most predictive of the mouse tumours of activity in man (Venditti 1975).

Against the human tumour xenografts the results were more variable. The LX-1 type lung xenograft responded well to mitozolomide , the MX-1 mammary xenograft was less sensitive and the CX-1 colon xenograft was completly resistant (table 3).

TABLE 1 Comparative activity of mitozolomide in the NCI tumour panel.

DRUG	TUMOUR				
	L1210 i.p	P388 i.p	B16 i.p	L.L i.v	C38 s.c
MITOZOLOMIDE	+ +	+ +	+ +	+ +	+ +
BCNU	+ +	+ +	+ +	+ +	+
DTIC	+ +	+	–	+	+
BCTIC	+ +	+ +	+ +	+ +	+
CIS-PLATINUM	+ +	+ +	+ +	+ +	+
CYCLO-PHOSPHAMIDE	+ +	+ +	+ +	+ +	+ +
METHO-TREXATE	+ +	+ +	–	+	–

++, +, –, N.C.I activity criteria
Goldin and Venditti (1980)

Table 2. Activity of Mitozolomide against murine solid tumours

Experiment Number	Tumour	Screening Centre	Inoculum		Treatment			Dose mg/kg/day	W[b] (g)	Tumour[c] Volume Index	Percent Inhibition
			Route	Cell Number	Route	Days of injection	Vehicle[a]				
1	LL	B	I.M.	5×10^5	I.P.	1-4	I	20	-2.0	15	100
								20	+0.1	33	100
								5	+0.7	71	81
								2.5	+1.0	82	68
2	C38	C	S.C.	fragment	I.P.	7,14	III	50	-4.7	0	
								25	-0.4	0	
								12.5	+0.2	21	
								6.25	0	41	
3	M5076	A	I.M.	10^6	I.P.	1-17	I	8	-0.4	0	
								4	+2.0	0	
								2	+2.4	5	
4	ADJ/PC6A	A	I.M.	10^6	I.P.	14	I	20	+1.1	0	
								10	-0.3	0	
								5	0	0	
								2.5	+1.1	0	
5	$CD8F_1$	C	S.C.	Homogenate	I.P.	1	III	1.25	+0.1	57	
								50	-2.2	41	
								25	-1.6	45	
								12.5	-1.2	76	

Table 2 continued

a - For injection vehicles see Table 1.

b - Change in mean body weight between 1st and 2nd weight evaluatin days were as follows:

Experiment 1 (days 0 and 8)
Experiment 2 (days 2 and 20)
Experiment 3 (days 1 and 17)
Experiment 4 (days 14 and 19)

c - Days for evaluation of tumor volume indes were Experiment 1 (day 21); Experiment 2 (20); Experiments 3 and 4 (24); Experiment 5 (28).

Positive controls were employed in some experiments:

Experiment 1 (DTIC; 200 mg/kg/day; T/C = 78%; 22% inhibition of metastases)
Experiment 3 (CCNU; 40 mg/kg; T/C = 0
Experiment 4 (CCNU; 40 mg/kg; T/C = 0

TABLE 3 Activity of mitozolomide against tumour xenografts.

TUMOUR	Schedule	Activity
S.r.c. CX-1 Colon Tumour	s.c. Q4D, days 1-14	−
S.r.c. LX-1 Lung Tumour	s.c. Q4D, days 1-9	+ +
S.r.c. MX-1 Mammary Tumour	s.c. Q4D, days 1-9	+

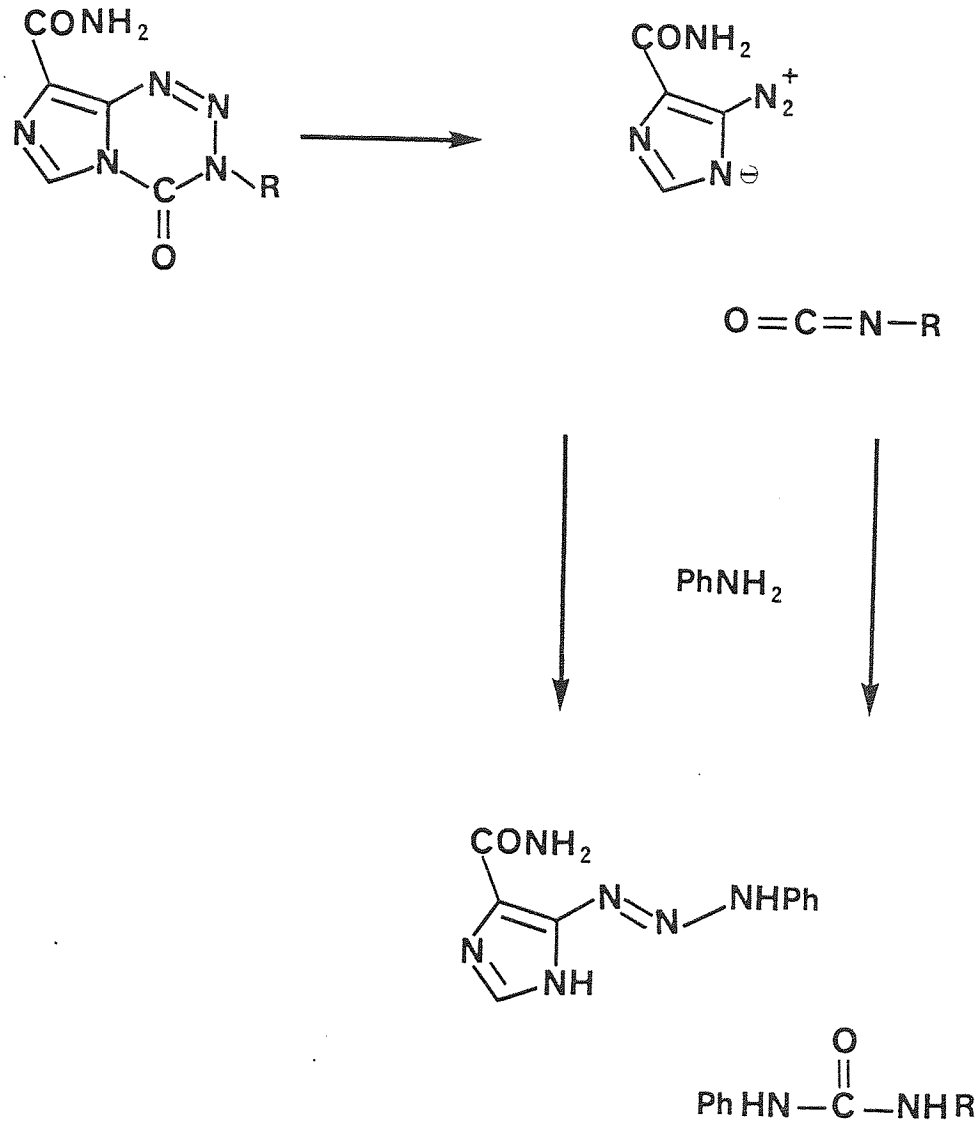
A.5. Possible mechanism of action of mitozolomide.

A.5.1. Diazo-IC and 2-chloroethyl isocyanate production.

As previously mentioned it is possible that mitozolomide might undergo a retro-cycloaddition reaction to produce Diazo-IC and 2-chloroethyl isocyanate. There is some chemical evidence in support of this hypothesis; the imidazotetrazinones decompose in protic solvents and it is possible to trap Diazo-IC and isocyanate with aniline (Stone 1981) (Fig. 10).

The bridgehead nitrogen might facilitate this decomposition and the reaction of Diazo-IC and the isocyanate with protic reagents could provide the "driving force" to disturb the equilibrium to the right. The imidazotetrazinones are stable in non-protic solvents.

FIG. 10 The retro-cycloaddition reaction of mitozolomide.



A.5.1.1. Diazo-IC as an antitumour agent.

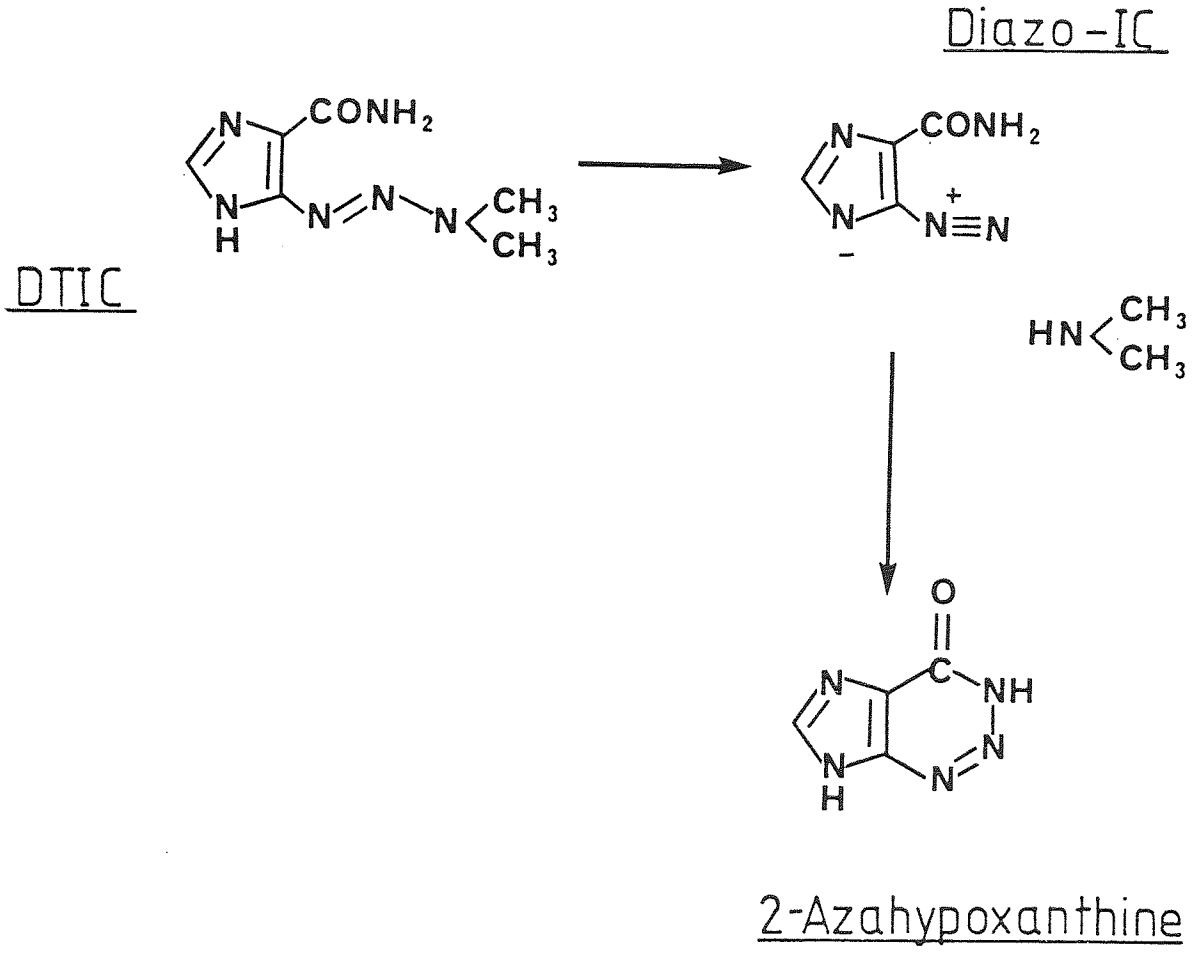
DTIC was designed as a pro-drug of Diazo-IC. Under the influence of light and at low pH DTIC splits to produce Diazo-IC which cyclises to 2-azahypoxanthine (Beal et al 1975) (Fig. 11). The photodecomposition of DTIC is quite complex; it is very sensitive to pH and can lead to the generation of other compounds not shown (Stevens and Peatey 1978, Horton and Stevens 1981 a and b). The pathway illustrated is that most important to this discussion and a full review of competing reactions is given by Stevens (1983).

Diazo-IC has been shown to inhibit human epidermal carcinoma (H.Ep.2) in tissue culture and in vivo is active against Ehrlich ascites carcinoma and Walker 256 carcinoma (Shealy 1961 and Hano et al. 1968). 2-Azahypoxathine is not active against the H.Ep.2 test system and is less toxic than Diazo-IC, but it is not entirely without biological activity being a potent inhibitor of the enzyme xanthine oxidase (Iwata et al. 1969).

Although active per se Diazo-IC cannot explain the tumour activity of DTIC, since in physiological conditions in vivo photodecomposition of DTIC is negligible and so consequently is the generation of Diazo-IC (Kreis 1977). It is now believed that DTIC undergoes oxidative N-demethylation producing an active methylating agent which is the effective antitumour species (Skibba et al. 1970a and b) (Fig. 12). The precise identity or (identities) of the

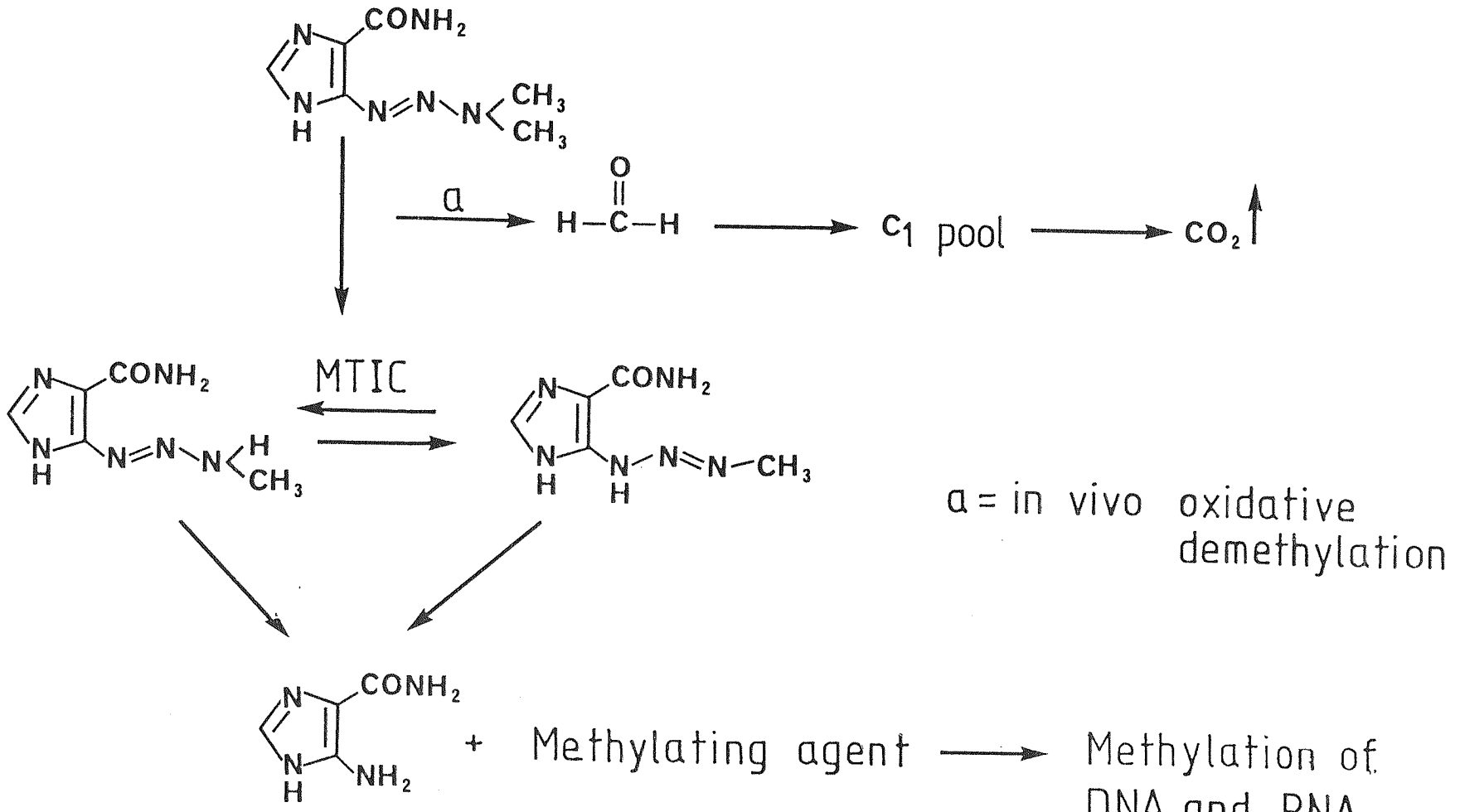
selective cytotoxic species is /are not yet known.

FIG. 11 The production of Diazo-IC from DTIC



2-Azahypoxanthine

FIG. 12 The metabolic activation of DTIC



Much of the work that supported the contention that DTIC did exert its effect via Diazo-IC was carried out *in vitro*, in conditions that favoured the photodecomposition of Diazo-IC. In such experiments it was shown that Diazo-IC was responsible for the cytotoxicity of DTIC to bacteria, chinese hamster ovary and human malignant melanoma cells (Gerulath and Loo 1972, Saunders and Chao 1974, Saunders and Schultz 1970).

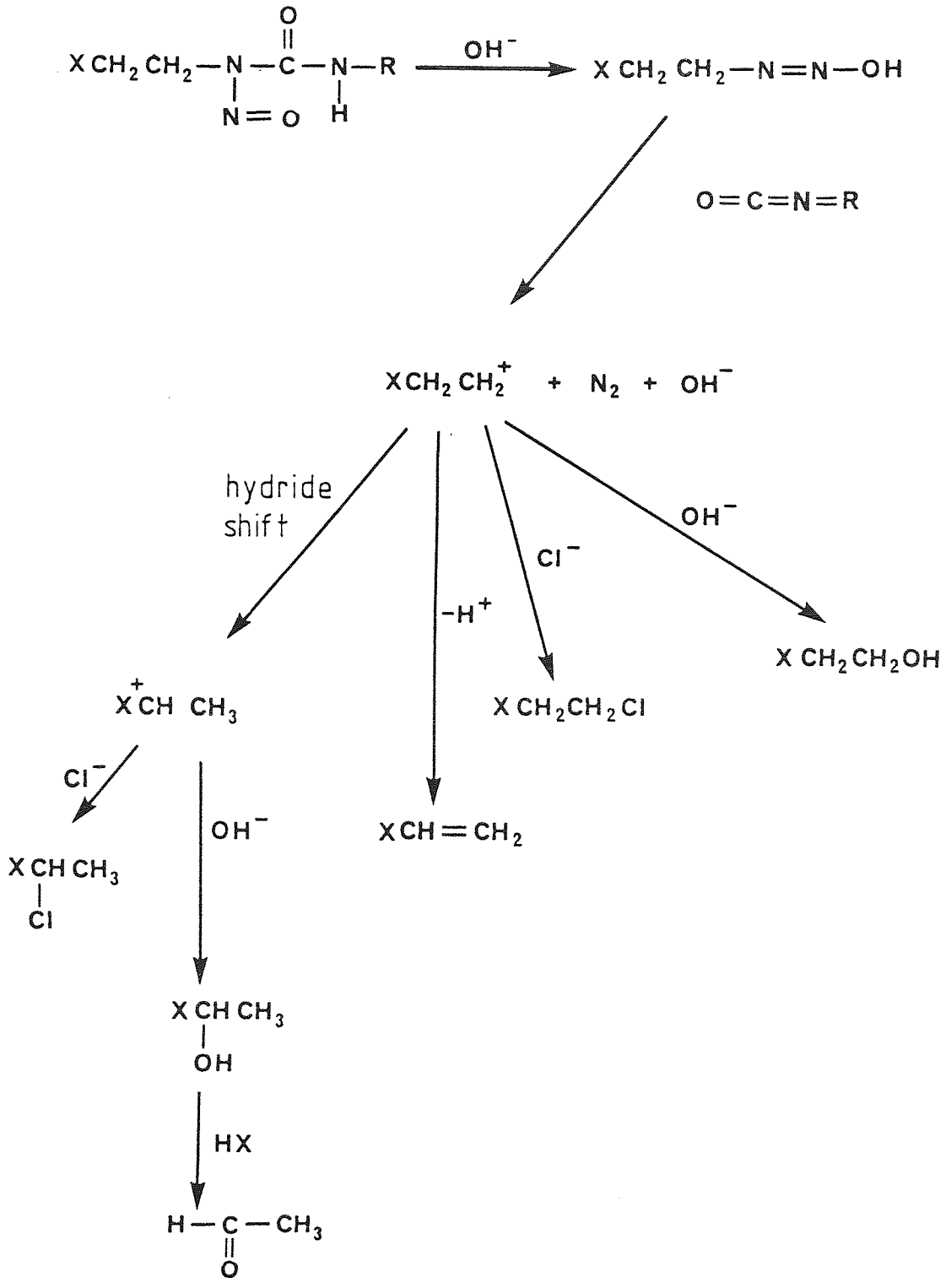
Diazonium compounds are extremely reactive and couple readily under mild conditions with the histidyl, tyrosyl, and lysyl residues of proteins (Howard and Wild 1957, Higgins and Harrington 1959, Tabachnick and Sabatka 1960, and Harinishi et al. 1964). They also react with the sulphydryl groups of cysteine and reduced glutathione (Iwata et al. 1968) and with purines such as theophylline, xanthine and guanine (Stevens 1976). As a diazonium compound Diazo-IC therefore possesses the ability to react with many biological molecules. Addition of exogenous cysteine or reduced glutathione protects bacteria from the effects of Diazo-IC (Yamamoto 1969, Saunders and Schutlz 1970). It has been suggested that Diazo-IC interferes with the function of biological SH groups causing inhibition of DNA synthesis as observed in bacteria (Yamamoto 1969), so producing its cytotoxic and antitumour effects.

A.5.1.2. Chloroethyl isocyanate as an antitumour agent.

In physiological conditions the major decomposition reaction of the 2-haloalkylnitrosoureas appears to be spontaneous cleavage to liberate equivalent ammounts of the alkylating agent 2-haloethyldiazohydroxide and an isocyanate capable of carbamoylation (Weinkam and Lin 1979) (Fig. 13). The decomposition pathway is pH sensitive giving a range of compounds in different conditions. This had led to the suggestion of a number of different pathways and reactive intermediates to account for these various products of decomposition (Chatterjai et al. 1978, Montgomery 1976, Brundrett 1980). While the production of the alkyldiazohydroxide and the isocyanate as illustrated in Fig. 13 is the dominant feature of halonitrosourea decomposition, the existence of minor reactive intermediates cannot be ignored (Lown and Chauhan 1981, Chatterjai et al. 1978). Tong et al (1982a) have postulated that certain DNA adducts produced by the haloethylnitrosoureas might be due to such intermediates.

Isocyanate production is a constant feature of 2-haloalkylnitrosoureas decomposition schemes, its presence at physiological pH being unquestioned. The nitrosourea, BCNU, produces 2-chloroethyl isocyanate which it is proposed might also be generated by mitozolomide.

FIG. 13 The decompostion reactions of the 2-haloalkynitrosoureas.



X = Cl, F

R = H, XCH_2CH_2, cyclohexyl

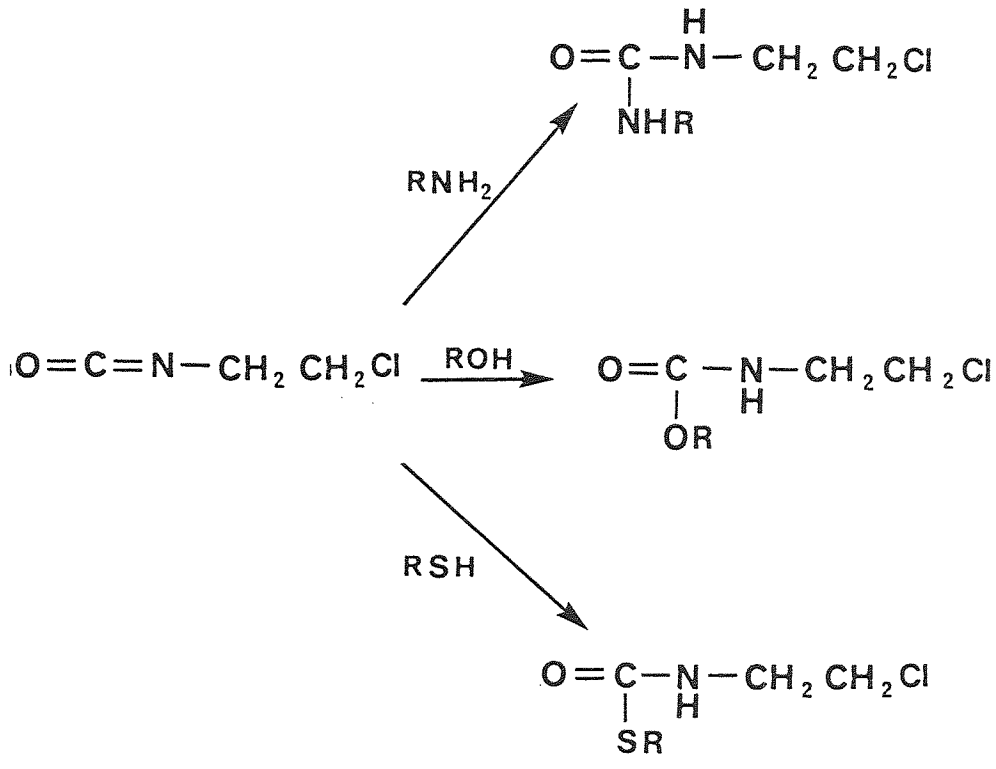
TAKEN FROM COLVIN AND BRUNDRETT (1981).

Isocyanates are very reactive being able to carbamoylate a number of biologically important groups, (Fig. 14). They react mainly with proteins (Bowdon and Wheeler 1971), specifically the α-amino groups of amino acids, terminal amino groups of peptides and proteins and the ϵ-amino groups of lysine (Wheeler et al. 1975). This ability has produced a range of biochemical effects: isocyanates have been shown to inhibit the repair of DNA damage (Kann et al. 1980 a and b), DNA polymerase II activity is also inhibited (Baril et al. 1975, Wheeler and Bowdon 1968) as is the activity of a number of other enzymes including; glutathione reductase (Babson and Reed 1978), α-chymotryspsin (Babson et al. 1977, Brown and Wold 1973), and the polymerisation of tubulin (Brodie et al. 1980).

Many of the above effects of isocyanates have been elucidated in vitro and their relevance in vivo is in dispute (Weinkam and Lin 1982). The role of the isocyanate in the toxicity and/or the antitumour activity of the nitrosoureas therefore remains largely speculative; their antitumour effect is generally ascribed to the ability of the chloroethyldiazohydroxide moiety to alkylate then cross-link DNA (Kohn et al. 1981). Initially it was thought that the isocyanate could be responsible for the toxic side-effects of the nitrosoureas, such as the dose limiting toxicity to the bone-marrow (Wheeler et al. 1974). This supposition was based on the qualitative analyses of a number of nitrosoureas by Wheeler et al.(1974) and as the

correlation co-efficients were poor the case was not proven. Further studies also failed to demonstrate a significant correlation between carbamoylation and lethal toxicity or granulocyte suppression (Panasci et al. 1977, Heal et al. 1979a).

FIG. 14 Carbamoylation reactions of 2-chloroethyl isocyanate.

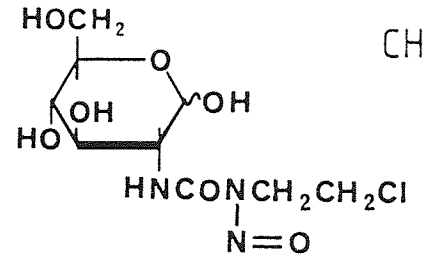


A number of recent developments argue against the role of carbamoylation in chloroethynitrosourea toxicity or antitumour activity. Chlorozotocin is an effective anti-tumour agent that has little carbamoylating activity and relatively low bone-marrow toxicity (Panasci et al. 1977, Anderson et al. 1975). Although this initially appeared to support the role of carbamoylation in toxicity other sugar containing nitrosoureas such as GANU have been found to have high carbamoylating activity but low myelotoxicity (Panasci et al.1977, Heal et al. 1979a) (Fig. 15). Hilton et al. (1978) calculated the peak isocyanate concentration formed during BCNU or CCNU decomposition in cell culture medium and showed that no L1210 toxicity occurred at these concentrations.

The weight of evidence indicates that isocyanate production is not important to the toxicity or the mode of action of the nitrosoureas in vivo. As always there are contrary reports, such as that by Gibson and Hickman (1982) who by cross resistance studies employing the bioassay showed that the isocyanates generated from BCNU and CCNU had some measure of selective toxicity to TLX5 tumour cells. They postulated that isocyanate production could be particularly important to the cytotoxicity of the nitrosoureas expressed in TLX5 lymphoma, a cell line naturally resistant to 2-chloroethylamine type alkylating agents (Connors and Hare 1975). It was also suggested that molecules that release a chloroethyl isocyanate but not a

haloalkyldiazo species could be an active antitumour agents (Gibson and Hickman 1982). Mitozolomide, in theory, could meet this criteria of isocyanate producer and this belief, in part, lead to the screening of mitozolomide against murine tumours.

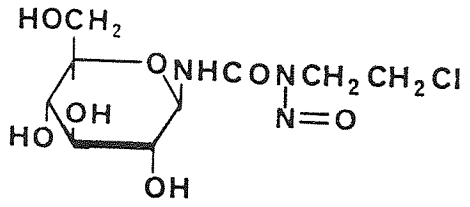
FIG. 15 The structures of chlorozotocin and GANU.



CHLOROZOTOCIN

1-(2-chloroethyl)-2-D-glycopyranosyl-1-nitrosourea

GANU



2-(2-chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosourea

A.5.2 Mitozolomide as an alkylating agent.

A.5.2.1. Production of MCTIC.

The 2-chloroethyl substituent of mitozolomide not only allows the supposition of 2-chloroethyl isocyanate generation but also the potential of alkylation through the intermediacy of 5-[3-(2-chloroethyl)-triazen-1-yl]-imidazole -4-carboxamide, (MCTIC) (Fig. 16).

MCTIC is the active species generated from 5-[3,3 bis-(2-chloroethyl)-triazen-1-yl]-imidazole-4-carboxamide, BCTIC (Shealy et al. 1975)(Fig. 17). BCTIC was synthezised by Shealy as part of the synthetic program generated by the discovery of the activity of DTIC (Shealy et al 1968). The in vivo activation of BCTIC to MCTIC via oxidative dechloroethylation is directly analogous to the demethylation and activation of DTIC (Fig 12). When tested BCTIC was found to be the most active of the imidazotriazenes against the L1210 leukemia (Shealy et al. 1968, Shealy and Krauth 1966). Despite excellent activity against murine tumours its clinical evaluation showed it offered no advantages over DTIC (Constanza et al. 1976) and that it produced severe side-effects such as leukopenia and thrombocytopenia (Falkson et al 1972).

FIG. 16 The production of MCTIC by mitozolomide decomposition.

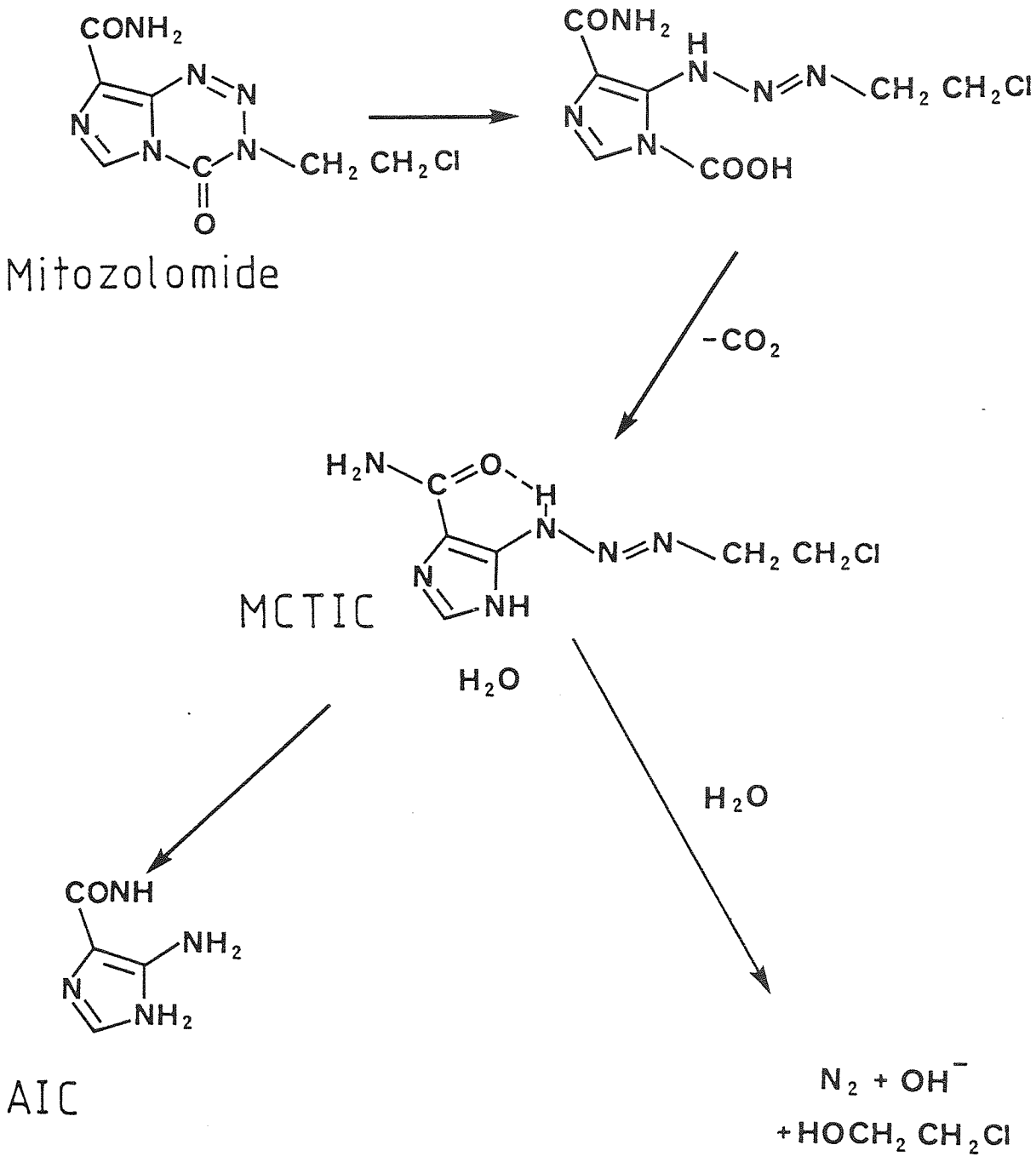
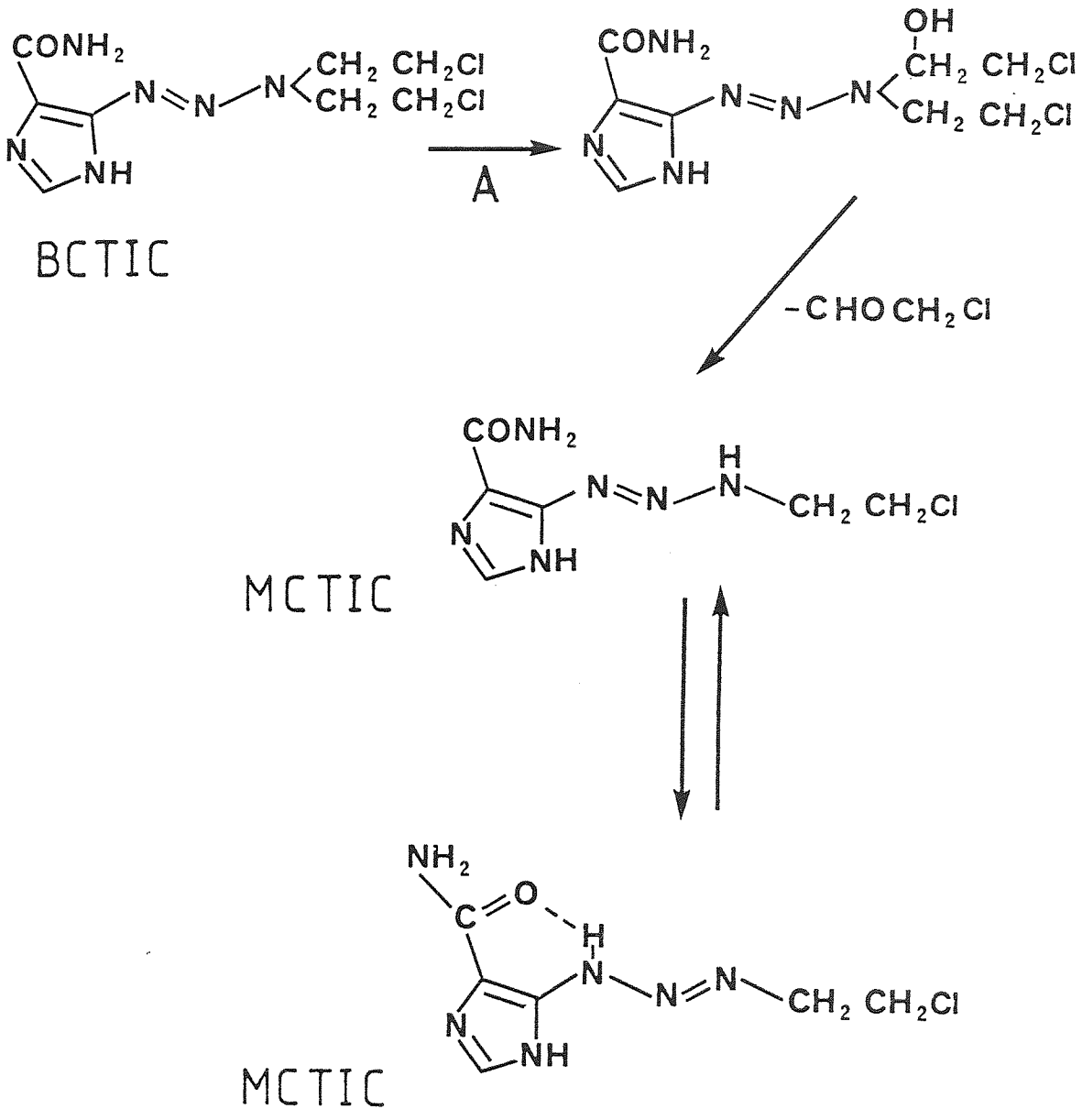


FIG. 17 The production of MCTIC by BCTIC



A = oxidative metabolism

MCTIC is a extremely unstable molecule, decomposing in water or 50% methanol to give a 70% yield of chloroethanol (Shealy et al. 1975). This suggested the production of a 2-chloroethyldiazohydroxide alkylating species in an analogous fashion to the 2-chloroethynitrosoureas, as shown in Fig. 13. There is some experimental evidence to suggest that BCTIC does act as an alkylating agent since it has been noted that L1210 resistant to BCNU is cross-resistant to BCTIC (Carter and Newman 1968), consideration of this evidence lead Shealy and co-workers to support alkylation as the major mode of action.

A.6. Alkylating agents.

A.6.1. The nitrosoureas and the mustards.

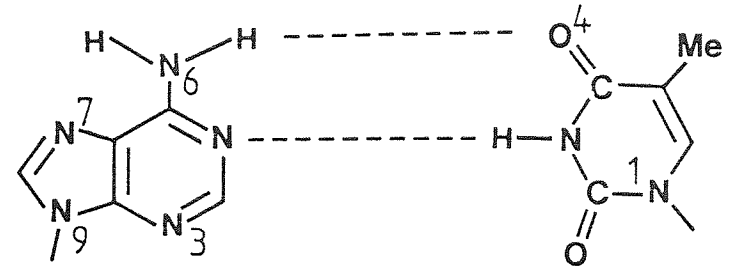
The antitumour effect of the nitrosoureas and the mustards is ascribed to the ability to alkylate and subsequently cross-link DNA (Goldacre et al. 1949, Kohn et al. 1966, Kohn 1977, Ludlum 1977). The activity of the nitrosoureas and the mustards does overlap, but there are notable cases were tumours resistant to one of the agents are not cross-resistant to the other (Audette et al. 1973). A good example is the TLX5 lymphoma. This cell line is naturally resistant to alkylating agents of the mustard type but is extremely sensitive to the nitrosoureas (Connors and Hare 1974). These observations led to the early suspicion that although nitrosoureas and mustards are capable of alkylation, the precise mechanism is different, and that biological systems were sensitive to such differences (Connors and Hare 1975).

A.6.2. Sites of alkylation on the bases of DNA.

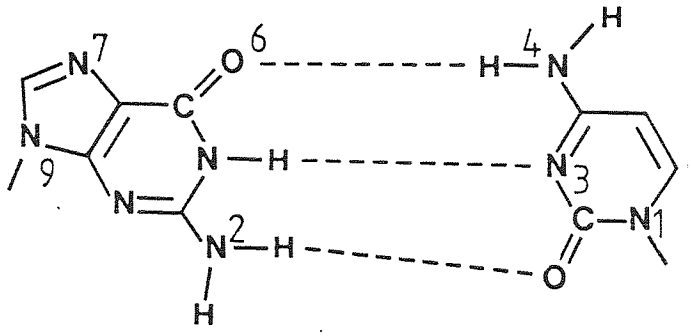
Numerous sites of alkylation of nucleic acids have now been identified (Fig. 18). The major site is the N-7 position of guanine which may account for 90% of the total alkylation (Roberts 1978). The proportion of various products formed in DNA can be explained by the rate of formation of carbonium ions and the nucleophilicity of the reaction sites in DNA. The Swain-Scott substrate constant s (Swain-Scott 1953) is a measure of the sensitivity of the alkylating agent to the nucleophile with which it reacts. Reagents of low s value are able to react more extensively with less nucleophilic centres, such as the O-atom sites of DNA, than those of high s value. Compounds which form carbonium ions readily. have low s values, reacting predominantly by S_N1 mechanisms are able to produce O-atom alkylation in DNA.

The reactions of nitrosoureas and mustards with DNA are illustrated in Fig. 19. Nitrosoureas react with a greater S_N1 character than mustards and therefore produce a measurable quantity of O^6-guanine alkylations (Tong et al. 1982a) while mustards do not.

FIG. 18 Alkylation sites on DNA bases.



ADENINE THYMINE



GUANINE CYTOSINE

FIG. 19A The reaction of nitrogen mustard with guanine

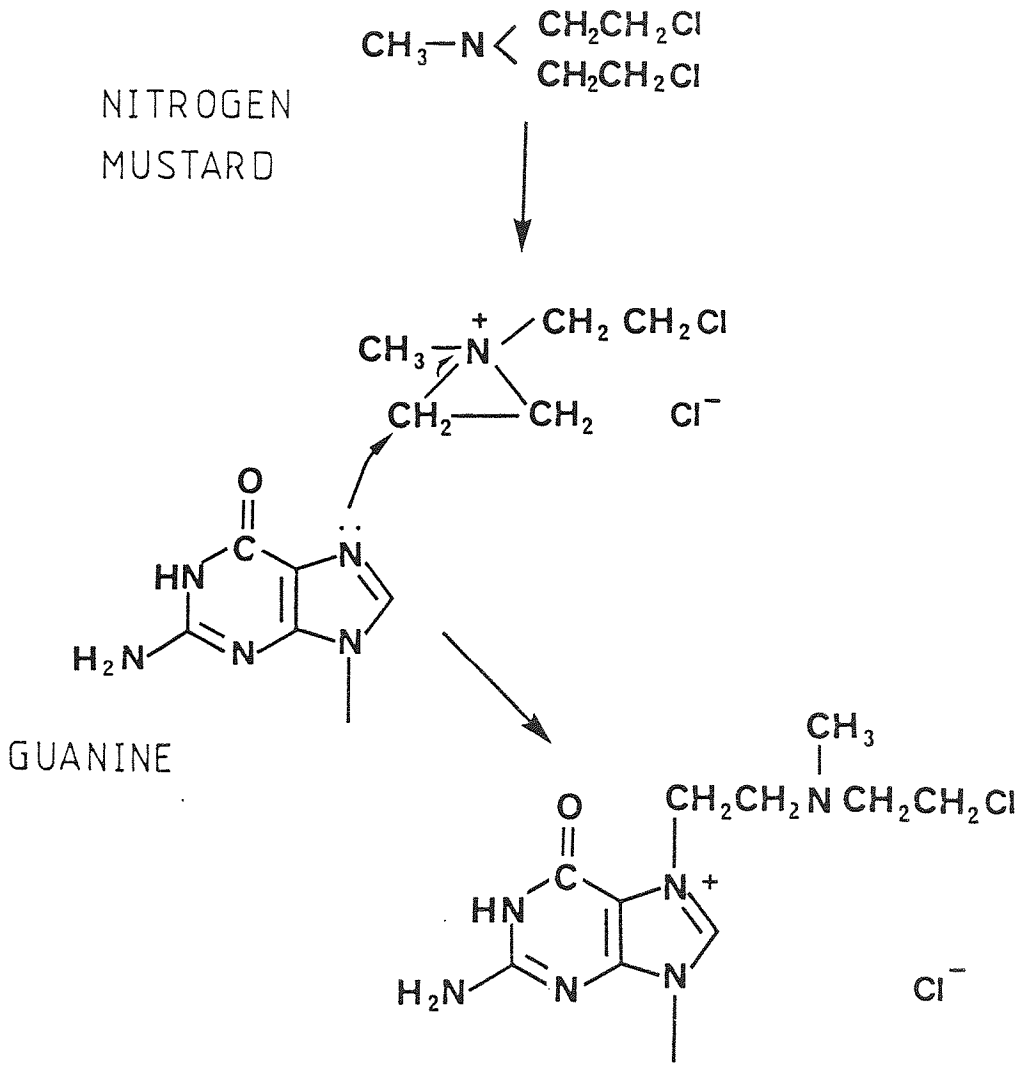
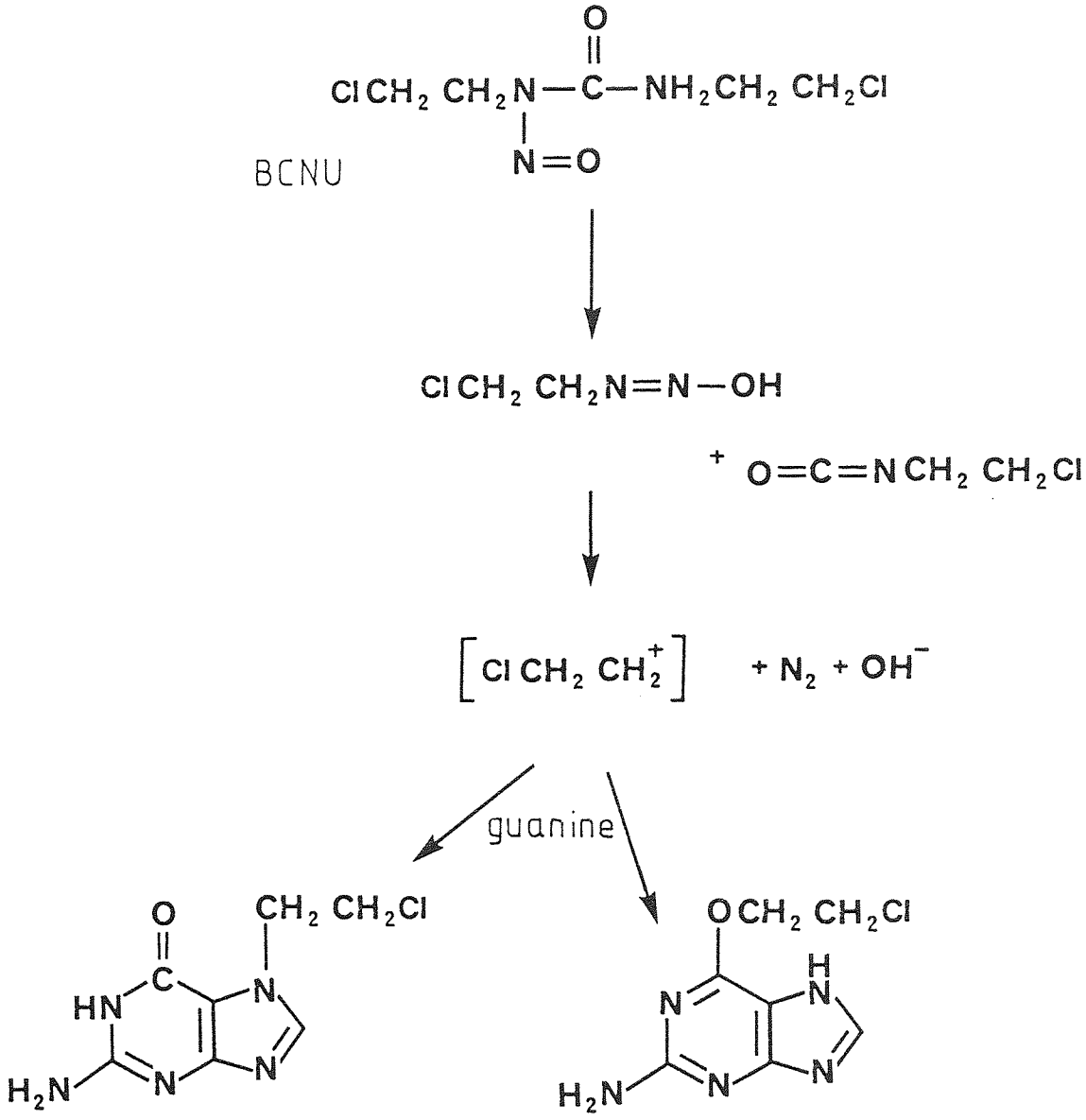


FIG. 19B The reaction of BCNU with guanine



A.6.3. The importance of base oxygen alkylations.

As can be seen in Fig. 18 the O^6-position of guanine and the O^4-position of thymine are envolved in hydrogen bonding unlike the N^7-position of guanine. Alkylation at these oxygen atoms would therefore be expected to be more disruptive of DNA function than alkylation at the N^7-position of guanine. Alkylations at O^6-guanine and O^4-thymine are pro-mutagenic lesions, leading to the misreading of the genetic code. O^6-guanine alkylation allows guanine to masquerade as adenine and so a GC-AT single point mutation occurs (Loveless and Hampton 1969, Lawley and Martin 1975, Eadie et al. 1984).

The role of O^6-alkylation in mutagenesis and carcinogenesis has been proven (Singer 1979, Rajewsky 1982, Guttenplan 1974 and Goth and Rajewsky 1974), and evidence is now accumulating that the importance of O^6-guanine adducts to cytotoxicity is totally disproportionate to the number of these lesions produced.

A.7. Methyl-DNA-methyltranferase activity.

Cells possess a repair capacity able to remove O^6-guanine and O^4-thymine alkylation, which has been termed the O^6-methyl-DNA-methyltranferase protein (Olsson and Lindahl 1980, Harris et al. 1983). Cells proficient in this type of repair are classified mer+ or mex+ while those deficient are mer- or mex-.

The mer+ phenotype presumably arose in response to the necessity to preserve the correct methylation patterns within DNA. Recently a major source of intracellular methyl groups. S-adenosylmethionine has been shown to act as a weak alkylating agent, methylating DNA by a non-enzymatic process (Lindahl and Karren 1983, Rydberg and Lindahl 1982, Barrows and Magee 1982). Such aberrant methylations produced by S-adenosylmethionine might be the natural substrate for the mer+ protein. The mer+ protein appears to possess a broad substrate specificity being able to remove methyl, ethyl, hydroxyethyl, and apparently chloroethyl adducts (Segdwick and Lindahl 1982, Pegg et al. 1984, Robins et al. 1983). Removal of chloroethyl adducts renders its activity pertinent to the cytotoxicity of chloroethylating agents.

Methyltranferase activity was first described by Samson and Cairns (1977) in E.Coli, and its presence in mammalian cells has since been demonstrated (Bogden et al. 1981, Sklar and Strauss 1981). The nature of the methyl excision repair system is unusual. The E.Coli transferase has a molecular weight of 18,000, is very heat stable and

has 4-5 cysteine groups per molecule. On transfer of the DNA adduct to an activated sulphur on a cysteine residue the protein is rendered inactive, and is therefore an example of suicide kinetics (Demple et al. 1982).

A transferase with apparently identical properties to the E.Coli protein has been found in human tissue (Demple and Karren 1983), but it is only available in small quantities and has not yet been purified to homogeneity. One major difference between the E.Coli and mammalian mer+ phenotype is the adaptive response of the E.Coli repair system on exposure to sub-lethal concentrations of MNNG and MNU (Cairns et al 1981), which is not observed in mammalian cells (Foote and Mitra 1984).

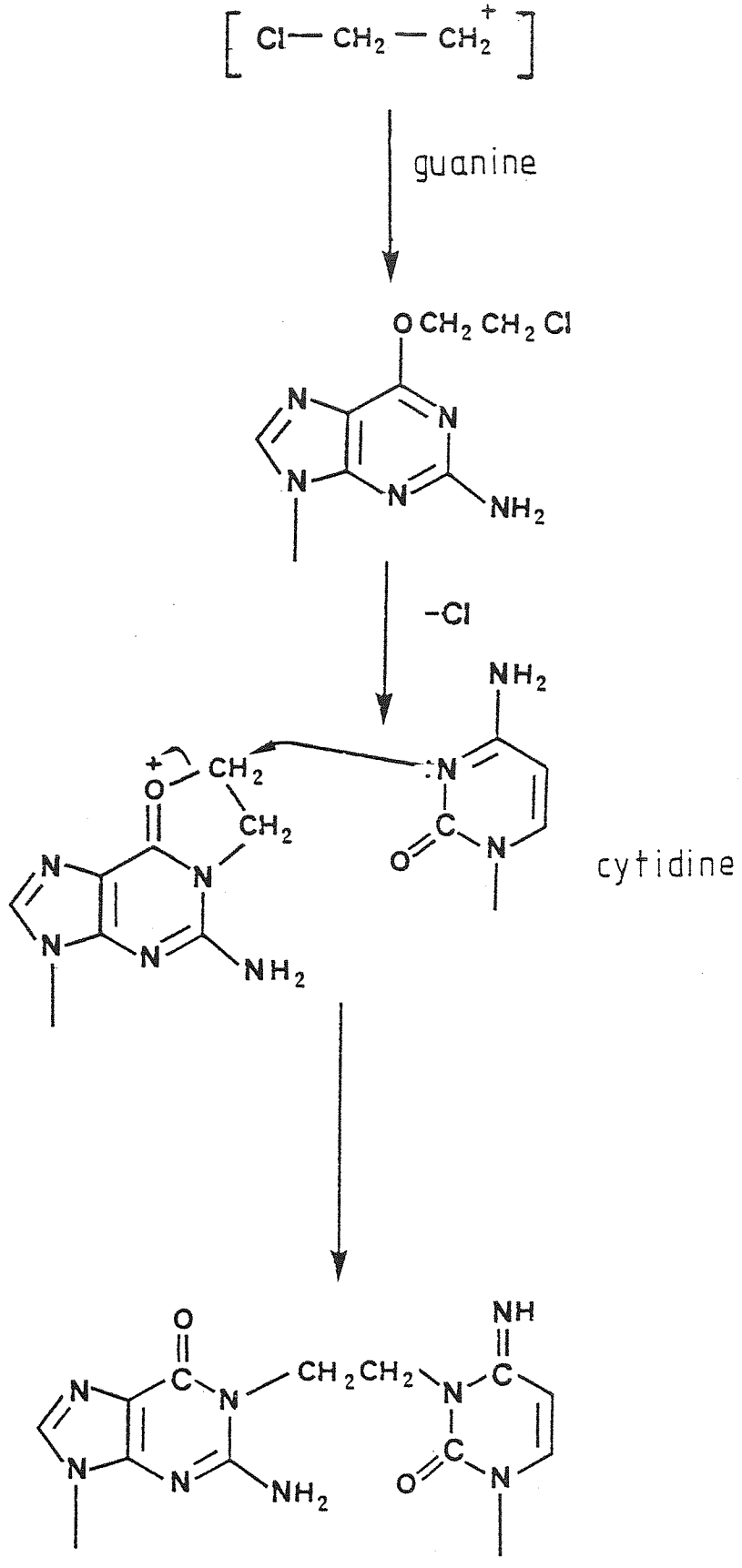
The relevance of the mer phenotype to nitrosourea cytotoxicity was demonstrated by Erickson et al. (1980b) who showed that mer+ cell lines were resistant to nitrosoureas. This indicated the importance of the relativly few O^6 alkylations produced by nitrosoureas to the mechanism of cytotoxicity.

Although it is known that nitrosoureas cross-link DNA the actual nature of the cross-link remains elusive, at present diguanyl adducts and 1-[N^3-deoxycytidyl]-2-[N^1-deoxyguanosyl] ethane residues have been identified in nitrosourea treated DNA (Tong et al. 1982b, Kohn 1977). The resistance of mer+ cell lines to nitrosoureas led to the suggestion that the crucial cytotoxic reaction of the nitrosoureas might be initial alkylation at the O^6-position

of guanine followed by a rearrangement to produce the N^3-cytidyl-N^1-quanosyl cross-link, Fig. 2Ø (Tonq et al 1982b, Robins et al. 1983, Scudierio et al. 1984). The initial attack was envisaqed as rapid while the rearrangment was expected to occur more slowly over a number of hours. Such an hypothesis was supported by previous kinetic data on the formation of cross-links in DNA (Kohn 1977, Lown et al. 1978). In the interval before cross-link formation the O^6-guanine adduct would be vulnerable to repair by the mer protein hence the resistance of mer+ cell-lines to compounds whose main cytotoxic reaction is dependent on the production of O^6-quanine alkylation.

The resistance of mer+ cell-lines to nitrosoureas in vitro may be removed by the administration of a priming dose of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) prior to the addition of the nitrosourea (Zlotoqorski and Erickson 1983). MNNG produces many O^6-guanine adducts saturating the repair capacity. If nitrosourea treatment occurs before protein synthesis can regenerate the pool of mer protein the mer+ line will then display an equivalent sensitivity to that of the mer- cell-line. The observation underlines the importance of the lesion repaired by this system to the cytotoxicity of the nitrosoureas.

FIG 20 Rearrangement of O^6-chloroethylguanine to give a N^1-guanine-N^3-cytidine cross-link

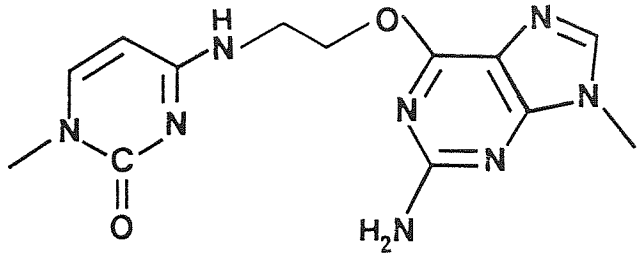


Much of the experimental evidence supporting the importance of O^6-alkylation may also be applied to a similar hypothesis of O^4-thymine alkylation leading to the formation of cross-links. The O^4-thymine position is similarly involved in hydrogen bonding and the methyltransferase protein may also operate at this position (Robins et al. 1983), although there is conflicting evidence (Muller and Rajewsky 1983).

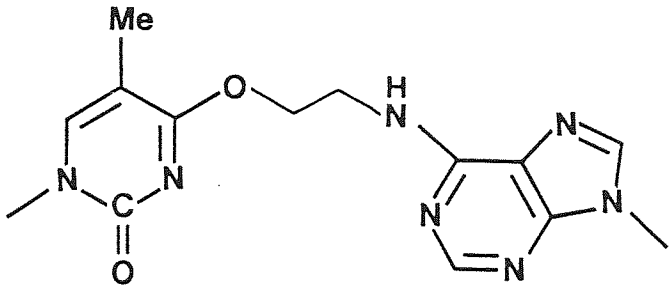
Cross-links other than the N^3-cytosine-N^1-guanine link are also possible (Fig. 21), the O^6-guanine and N^4-cytosine link is one suggested by Kohn (1977). The diguanyl link is presumed to be an intra rather than an interstrand cross-link and of little relevance to the cytotoxicity of the nitrosoureas. While the case for the N^3-cytosine-N^1-guanine link remains in the least speculative the importance of other cross-links should not be overlooked.

The fact that nitrosoureas do react by different mechanisms producing some different DNA products could explain the general biological differences between these two types of alkylators. The characterisation of the mer phenotype accounts for the specific differences of sensitivity of mer+ cells to the mustards and the nitrosoureas.

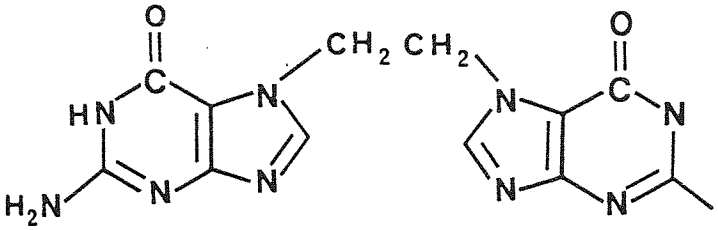
FIG. 21 Possible 2-carbon DNA cross-links



O^6-Guanine to N^4-Cytosine



O^4-Thymine to N^6-Adenine



Diguanyl

A.8.Mechanistic implications of the spectrum of activity of mitozolomide to its mode of action.

The initial demonstration of mitozolomide activity was against the TLX5S lymphoma, a cell line which is naturally resistant to alkylating agents of the 2-chloroethylamine type (ie. mustards) and sensitive to the nitrosoureas. Mitozolomide was active against the cyclophosphamide resistant L1210 leukemia , inactive against L1210 resistant to BCNU and inactive against the TLXRT lymphoma (which had induced resistant to triazenes and was cross resistant to the nitrosoureas BCNU and CCNU) (table 4). Mitozolomide is active against a broad range of tumours, TLX5, L1210, P388 leukeamia, M5076 sarcoma, B16 melanoma, mouse colon 38 cell line and the Lewis lung carcinoma (Hickman et al. 1982), an activity spectrum which resembles that of the nitrosoureas (Schabel 1976).

Experimental evidence therefore reveals a link between the mechanism of action of the nitrosoureas, mitozolomide and possibly the triazenes. Both the production of a chloroethyl isocyanate and/or the chloroethyl alkylating species by mitozolomide could provide the basis for the apparent similarity to the nitrosoureas. If mitozolomide were to generate MCTIC, alkylation of DNA could occur in a directly analogous fashion to that produced by the haloethylnitrosoureas. MCTIC provides a self-evident link to the chemistry of the triazenes.

It is particulary interesting that a number of

analogues of mitozolomide which possess a chloroethyl group are inactive.

Table 4. Activity of Mitozolomide against murine tumour lines resistant to antitumor agents.

Expt. No.	Tumour	Screening Centre	Inoculum		Treatment			Dose (mg/kg/day)	Mean[b] or median[c] life span (T/Cx 100%)[d]	Long term survivors[e] (Day)
			Route	Cell No.	Route	Days of injection	Vehicle[a]			
1	L1210	B	I.P.	10^5	I.P.	1	I	40	237	-
								20	>388	10/10 (30)
								10	>360	8/10 (30)
								5	128	-
2	L1210/BCNU	B	I.P.	10^5	I.P.	1	I	40	167	-
								20	143	-
								10	114	-
								5	107	-
3	L1210	B	I.P.	10^5	I.P.	1-4	I	40	>433	10/10 (30)
								20	>433	10/10 (30)
								10	>415	9/10 (30)
								5	142	-
4	L1210/BCNU	B	I.P.	10^5	I.P.	1-4	I	40	162	-
								20	139	-
								10	114	-
								5	110	-
5	L1210/CY	B	I.P.	10^5	P.O.	1-5	II	20	>300	10/10 (30)
								20	>285	7/10 (30)
								5	211	-

Table 4 continued ...

6	L1210	C	I.P.	10^5	I.P.	1-9	I	50	137	-
								25	>745	6/6 (60)
								25	>745	6/6 (60)
								6.25	184	-
7	LL	C	I.V.	$2x10^5$	I.P.	1-9	III	50	61	-
								25	>298	7/10 (60)
								12.5	>159	2/10 (60)
								6.25	>120	1/10 (60)
								3.12	103	
8	C26	B	I.P.	1/10 diln.	P.O.	7	II	40	>203	10/10 (68)
								20	>163	4/10 (68)

[a] - injection vehicles were as follows: I - 10% DMSO 90% Arachis oil
II - Suspension in carboxymethyl cellulose
III - Suspension in Tween 80

[b] - T/C x 100% values calculated from median life span of test (T) and control (C) animals in Experiments 1,2,6 and 7.

[c] - T/C x 100% values calculated from mean life span of test (T) and control (C) animals in Experiments 3-5 and 8.

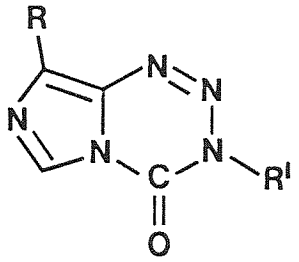
Positive controls were employed in some experiments.

Experiment 1. (BCNU; 40 mg/kg; T/C >600; 5/5 long term survivors).
Experiment 4. (BCNU; 40 mg/kg; T/C = 166; 0/5 long term survivors).
Experiment 7. (Cyclophosphamide; 90 mg/kg; T/C >253; 9/10 long term survivors).
Experiment 8. (MeCCNU; 40 mg/kg; T/C >170; 7/10 long term survivors).

A.9. The analogues of mitozolomide.

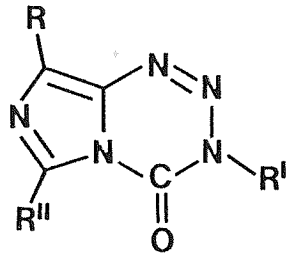
A number of analogues of mitozolomide have been synthesised and tested (table 5 and 6). Some observations concerning the structure activity relationships may be made. Generally substitution at the C_8 position on the imidazole ring is less important to antitumour activity than that at the 3-(2-chloroethyl) side-arm. Certain substitutions at carbon 8 do destroy activity such as the replacement of the carbamoyl by a carboxyl group. Activity is strictly confined to either a methyl or chloroethyl group at position 3. The loss of activity by homologues of greater carbon number than the chloroethyl is common in alkylating agents, as is the fact that methyl compounds are less active and less toxic than compounds bearing chloroethyl groups. The structure activity relationships obtained therefore point to the chloroethyl side-arm as the major element which determines possession of antitumour activity. The imidazo substituents could influence activity by producing electronic effects over the entire molecule so affecting the reactivity of the chloroethyl group.

TABLE 5 Analogues of mitozolomide.



DRUG	R	R'	activity
MITOZOLOMIDE	$CONH_2$	CH_2CH_2Cl	ACTIVE
METHAZOLOSTONE	$CONH_2$	Me	ACTIVE
ETHAZOLOSTONE	$CONH_2$	CH_2CH_3	INACTIVE
ANALOGUE 1	CO_2H	CH_2CH_2Cl	INACTIVE
ANALOGUE 2	CN	CH_2CH_2Cl	INACTIVE
ANALOGUE 3	CO_2Et	CH_2CH_2Cl	INACTIVE
ANALOGUE 4	SO_2NH_2	CH_2CH_2Cl	ACTIVE
ANALOGUE 5	SO_2NHMe	CH_2CH_2Cl	ACTIVE
ANALOGUE 6	SO_2NMe_2	CH_2CH_2Cl	ACTIVE

TABLE 6 Structure activity relationship of imidazotetrazinones against TLX5S lymphoma (Q01DX01).



(i) 5 out of 5 cures < 1 dose level			(ii) T/C (%) 125 - 200			(iii) T/C (%) < 125		
R	R'	R''	R	R'	R''	R	R'	R''
$CONH_2$	CH_2CH_2Cl	H	CO_2H	CH_2CH_2Cl	H	$CONH_2$	$CH_2CH_2CH_2Cl$	H
$CONH_2$	CH_2CH_2Cl	Me	SO_2Me	CH_2CH_2Cl	H	$CONH_2$	CH_2CH_3	H
SO_2NH_2	CH_2CH_2Cl	H	$CONH_2$	Me	H	CN	CH_2CH_2Cl	H
SO_2NHMe	CH_2CH_2Cl	H	CN	Me	H	CO_2Et	CH_2CH_2Cl	H
SO_2NMe_2	CH_2CH_2Cl	H	SO_2Me	Me	H	$CONHC_6H_5$	CH_2CH_2Cl	H
ACTIVE						INACTIVE		

B. MATERIALS

B. MATERIALS.

B.1. Biological materials.

B.1.1. Cells lines used in this study.

The TLX5S, sensitive to nitrosoureas and the TLX5RT, with induced resistance to dimethyltriazene were all obtained from the Institute of Cancer Research, London (Audette et al 1973). The Lewis lung carcinoma (3LL), was that used at the Mario Negri Research Centre, Milan, Italy. The human leukemic cell-line, K562 was obtained from Dr M.Tisdale of the University of Aston in Birmingham. GM0892A human lymphoblastoma cells and the Burkitt lymphoma derived Raji cell-line were obtained from Mill Hill laboratories, London, courtesy of Dr. P.Karran and were classified in those laboratories as O^6-guanine methyltransferase proficient or deficient respectively (Harris et al. 1983).

B.1.2. Animals.

CBA/CA male and female mice (18-22g) were supplied by Bantim and Kingman (Hull). Animals were kept for at least a week before they were used for the experiments and were fed on a 41B modified breeding diet (Pilsbury, Birmingham) with water ad libitum.

C57Bl/6 male mice (18-22g) were supplied by Charles-River, Italy.

B.2. Chemicals and investigational compounds

B.2.1. Purchased.

Chloroethyl isocyanate, DNP, sodium flouride, sodium cyanide, and oligomycin were obtained from Sigma Chemical Co., Poole, Dorset.

Dow Corning silicon oil was purchased from Asda's (Birmingham).

B.2.2. Gifts.

BCNU was obtained as a gift from the drug synthesis and chemistry branch, National Cancer Institute, Bethesda, Maryland, U.S.A., Nitrogen Mustard was a gift of the Boots company, Nottingham, U.K., Methotrexate was a gift of the Lederle Company, Gosport. Chlorambucil was synthesized at the Chester Beatty Research Institute, London.

B.2.3. Synthesized.

Mitozolomide, methazolastone, ethazolastone were synthesized in this department by Prof. M.F.G.Stevens, G.Baig and Dr.M.Threadgill. MCTIC was synthesized by G.Baig using the method of Shealy et al. (1975).

B.3. Radiochemicals.

[6-^{14}C-imidazo]-Mitozolomide 3.41 x 10^{-3} Cimmol-1 (14.08 μCi/mg) was provided by May and Baker Ltd., Dagenham. 5-(methyl-^{3}H)thymidine (5 CimMol^{-1}), (5-^{3}H)uridine (27

Cimmol-^{1}), (2,5,8-^{3}H)adenosine (40 Cimmol-1), (^{3}H)- water (0.09 Cimmol-1), (1,2-^{3}H) polyethyleneglycol (27Cimmol-^{1}) were all obtained from Amersham International Ltd.

B.4. Scintillation fluids.

Fiso-fluor m.p.c. was obtained from Fisons plc. Loughborough, England. PCS scintillation fluid was purchased from Hopkins and Williams, Romford, England. PPO/PPOP scintillation fluid was prepared in the laboratory by the dissolution of PPO (4g/l) and PPOP (lg/l) obtained from Fisons plc. Loughborough, England in scintillation grade toluene obtained from Hopkins and Williams Ltd. Romford, England.

B.5. Media.

R.P.M.I. 1640 with 25mMol Hepes, Newborn calf serum, fetal calf serum, Penicillin (5000 units/ml) were all purchased from Gibco, Paisley, Scotland.

B.6. Enzymes.

Glutathione reductase, γ-glutamyl transpeptidase and α-chymotrypsin were all purchased from Sigma Chemical Co., Dorset.

B.7. Buffers.

All chemicals were obtained from BDH Ltd., Poole, Dorset and of reagent grade unless otherwise stated.

B.7.1. Sorensons Buffer (Glutathione reductase assay buffer).

Sodium dihydrogen phosphate (39ml of Ø.2M) and disodium hydrogen phosphate (61ml of Ø.2M) were made up to 2ØØml with distilled water giving a final solution of Ø.1M phosphate. The pH was adjusted to pH 7.Ø.

B.7.2. α-Chymotrypsin assay buffer.

A solution of 1Ø.55g of calcium chloride dihydrate in 25Øml of Ø.2M tris(hydroxy-methyl)aminomethane (Sigma Chemical Co. Ltd., Poole, Dorset) was adjusted to pH 7.8 with hydrochloric acid and diluted to 1 litre. To this solution was added 432ml of methanol, (BDH spectrophotometer grade), making the final buffer 25.6% methanol (w/w) and Ø.5M Calcium (Hummel 1959).

B.7.3. γ-Glutamyl transpeptidase.

Tris-(hydroxymethyl)aminomethane (5Øml of Ø.2M) and hydrochloric acid was diluted with distilled water to 2ØØml final volume and the pH adjusted to 8.6.

B.7.4. Walpoles buffer.

Sodium acetate trihydrate (166ml of a solution of

2.722g /200ml) was added to acetic acid (6.005g/100ml of distilled water). The acetic acid was obtained from Fisons, Loughborough, England and was of hplc grade. Diethylamine (0.73g/l) was added to the above solution and the whole was adjusted to a final pH of 4.2.

B.7.5. Buffer for the separation of DNA bases by hplc.

Phosphate buffer (25mM KH_2PO_4) and diethylamine (0.5M) adjusted to pH 3.5.

B.7.6. Phosphate buffered saline (P.B.S.).

The final solution contained, sodium chloride (150mM), potassium dihydrogen phosphate (4.3mM) and dipotassium hydrogen phosphate (0.7mM).

B.7.7. Flow-cytofluorimetry staining solution.

Propidium iodide obtained from Calbiochem Behring Co. U.S.A. was dissolved in a 0.1% w/v solution of sodium citrate to a final concentration of 50 g/ml. To this solution Nonidet P40 (Sigma Chemical Co. Ltd., Poole, Dorset) was added (30 μl/l).

B.7.8. Erythrocyte cell lysis buffer.

Ammonium chloride (7.5g/l) was added to a solution of tris-HCl (0.016M) and the pH adjusted to 7.2. (Boyle 1968).

B.8. Hplc standards.

3-(2-hydroxyethyl)-1,2,3-benzotriazin-4-one was synthesized by Dr M Threadgill. Adenine and guanine were purchased from Sigma Chemical Co. Ltd., Poole, Dorset. AIC was obtained from BDH CHemicals Ltd., Poole, Dorset.

B.9. Equipment.

Tissue culture flasks 80cm^3, 25 cm^3 and twenty-four multi-well dishes (133 x 88mM) were purchased from Nunc, Gibco Ltd., Paisley, Scotland.

C. METHODS

C. METHODS.

C.1. Cells used in this study.

C.1.1 TLX5S Lymphoma.

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

C.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 sub-curative dose of BCNU (5mg/kg), also administered i.p. Tumour cells which survived this treatment were harvested and transplanted back into mice which were subsequently treated with an increased concentration of BCNU. This procedure was repeated until the survival of mice treated with the maximum tolerated concentration of BCNU (40mg/Kg) was no greater than that of the untreated control mice.

C.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 sub-curative concentration of dimethyl triazene (12.5mg/kg), also administered i.p. Tumour cells which survived this treatment were harvested and transplanted back into mice where they were subsequently exposed to a higher level of dimethyltriazene. This procedure was repeated until the survival of mice treated with the maximum tolerated level of the dimethyltriazene (50mg/kg) was no greater than that of the untreated control mice.

C.1.4. Lewis lung carcinoma (3LL).

The Lewis lung carcinoma was discovered by Dr. Margaret R. Lewis of the Wistar Institute in 1951. This tumour originated spontaneously as a carcinoma of the lung of a C57Bl/6 mouse (Mayo 1972).

C.1.5. GM0892A.

The GM0892A cell-line is a human lymphoblastoma cell-line derived from normal individuals (Harris et al. 1983). These cells were classified as O^6-Guanine-DNA-methyltransferase deficient. that is mer- (or mex-).

C.1.6. Raji.

Raji is a Burkitt lymphoma derived cell-line and were classified as O^6-Guanine-DNA-methyltransferase proficent, that is mer+ (or mex+), (Harris et al. 1983).

C.1.7. K562.

The K562 human leukemia cell-line was obtained from Dr.M.Tisdale of the University of Aston in Birmingham.

C.2. In vivo tumour maintainence.

C.2.1. Routine passage of TLX5 cell-lines.

The TLX5 lymphoma cell-lines were maintained by weekly passage under asceptic conditions into male CBA/CA mice (18-22g). Ascitic fluid (Ø.1ml) was withdrawn from the peritoneal cavity of a donor mouse and diluted with 1Øml of sterile isotonic saline . The cell suspension (Ø.1ml) of approximately $2x1Ø^5$ cells was injected i.p. into recipient mice.

C.2.2. Routine passage of 3LL cell-line.

The 3LL carcinoma cell-line was maintained by passage every 15 days under asceptic conditions into C57Bl/6 male mice (2Ø+/-2g). A single cell suspension was prepared from the tumour of the donor mouse, from which $1Ø^5$ cells were injected i.m. into the recipient mouse.

C.3. In vitro tissue-culture techniques.

C.3.1. Extraction of TLX5 mouse ascites cells.

Ascitic fluid was withdrawn from the peritoneal cavity of CBA/CA mice bearing a routine passage of TLX5 cell lymphoma and was mixed with sterile saline (37^oC). If necessary, saline (5ml) was syringed into the peritoneal

cavity, removed and added to the above cell solution. The cells were sedimented on a labofuge bench centrifuge at approximately 500g and washed with erythrocyte lysis buffer (B.7.8), to remove red cell contamination. The cells were then recentrifuged and resuspended in R.P.M.I. 1640 media.

For short term use cells were suspended in R.P.M.I. 1640 media supplemented with 10% horse serum, but maintainence in tissue culture required R.P.M.I. 1640 media supplemented with 20% fetal calf serum initially, which after two weeks could be replaced by 10% newborn calf serum in R.P.M.I media.

C.3.2. Maintainence of <u>in vitro</u> cell-lines.

Stock cultures were maintained in exponential phase at a density of $0.05-3x10^6$ cells/ml under an atmosphere of 10% CO_2 in air. R.P.M.I. 1640 media supplemented with 10% newborn calf serum was used as the culture medium unless otherwise stated.

C.3.3. <u>In vitro</u> growth inhibition studies.

Logarithymically growing cells were plated out at $5x10^4$ cells/ml and drug solutions added as appropiate. After a 72 hr incubation the cells were counted using a Coulter-counter and the percentage inhibition of growth determined.

C.3.4. Preparation of *in vitro* 3LL cells from *in vivo* samples.

3LL tumours growing i.m. were removed weighed and washed in a Petri dish with phosphate-buffered saline (PBS), after which fragments were minced with scissors and the vegetative and necrotic areas separated. Vegetative cells were treated as in the method of Starace et al. (1982) to produce a single cell suspension. The suspension was forced through a 18GX x 1 1/2 needle and resuspended in R.P.M.I. 1640 media. The cell solution (1ml of 0.5×10^5 cells/ml) was plated out into sterile multiwell dishes. Cells were maintained at 37°C under an atmosphere of 10% CO_2 in air.

C.4. Enzyme assays.

C.4.1. Glutathione reductase assay.

Glutathione reductase activity in cell extracts was determined spectrophotometrically at 25°C by following the oxidation of NADPH at 340nm. The assay mixture contained 0.2m KCl, 1mM EDTA, cell extract and 1mM oxidized glutathione in 0.1M phosphate, pH 7.0. The reaction was initiated by the addition of NADPH to a final concentration of 0.1mM and followed in a Beckman DU 7 spectrophotometer.

To prepare the cell extract, cells were sedimented on a labofuge centrifuge at approximately 500g and resuspended in a small volume of the assay buffer (200-500 μl). The cell suspension was then sonicated at 10kcs for

10s using a MSC sonic oscillator.

C.4.2. γ-Glutamyl transpeptidase assay.

γ-Glutamyl transpeptidase activity was determined by the colorimetric method of Jacobs (1971). The assay system consisted of γ-glutamyl-p-nitroanilide (3.2 μmole), glycylglylcine (22.0 μmole), magnesium chloride (11.0 μmole) and γ-glutamyl transpeptidase enzyme in tris-HCl buffer, pH 8.6 in a total volume of 3ml. After a 45min incubation at 37°C the reaction was stopped by the addition of 5.0ml of 0.0075N NaOH. The liberated p-nitroaniline was determined by measuring the absorbance at 410 nm in a Beckmann DU 7 spectrophotometer.

C.4.3. Chymotrypsin assay.

α - Chymotrypsin activity was determined by the method of Hummel (1959). Benzoyl-L-tyrosine ethylester (BTEE) was dissolved in the chymotrypsin assay buffer (B. 7.2.) at a concentration of 15.7mg/100ml. Exactly 3ml of the BTEE substrate solution was placed in a quartz cuvette with a 1cm light path, the reaction was initiated by the addition of 150 μl of a solution of enzyme in water and the reaction followed by the increase in absorbance at 256 nm.

C.4.4. Protein determination.

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

C.5. Incorporation of radioactive precursors into acid-insoluble material

C.5.1. The incorporation of radioactive thymidine and uridine.

For precursor incorporation studies mouse ascites cells were used at a concentration of 1.0×10^6 cells/ml and tissue culture cells at 0.5×10^6 cells/ml. Cells were incubated with drugs for various periods of time as indicated in the figure legends.

After the drug/cell incubation pulse labelling was carried out for a 1hr period with the radioactive precursors (2.5 μCi/ml). Incorporation into acid-insoluble material was determined by filtering the cell suspension through a Whatman GF/C glass fibre disc with saline, followed by 10 vol of ice cold 0.2N perchloric acid and 5 vol of absolute ethanol. The discs were dried at 70°C for 1hr and the radioactivity determined in a PPO/POPOP scintillation fluid.

C.5.2. The incorporation of radioactive adenosine.

The differential incorporation of adenosine into DNA and RNA was determined by first measuring total incorporation into acid-insoluble material as above . For incorporation of radioactivity into DNA cell suspensions

were mixed in an equal volume of 1N NaOH and allowed to stand overnight. The alkali insoluble material was washed onto a GF/C disc and washed with 2Ø vol of saline, and 5 vol of absolute ethanol. The discs were then processed and counted as above. The incorporation of the label into RNA was taken as the difference between total acid-insoluble material and alkali-insoluble material (Mandel et al. 1974).

C.5.3. Measurement of the thymidine pool size.

Tissue culture cells (Ø.5 x $1Ø^6$ cells/ml) were incubated with drug as indicated in the figure legends. They were then pulse labelled with thymidine (2.5 μCi/ml) for 1hr and 1Øml portions sedimented in a Labofuge bench centrifuge for 5 min at 5ØØg. The cell pellet so obtained was rapidly washed with ice-cold saline and recentrifuged. The cells were lysed with Ø.5M perchloric acid and the supernatant nuetralized with 5M KOH. The radioactivity of a small aliquot was determined in PCS scintillation fluid.

C.6. Transport studies.

C.6.1. Uptake of mitozolomide

TLXS mouse ascites cells were removed in saline and erythrocyte contamination was destroyed by washing with red cell lysis buffer, see C.3.1., (Boyle 1968). The cells were resuspended in R.P.M.I 1640 tissue culture medium at a concentration of $2x10^7$ cells/ml and equilibrated for 10 min in a shaking water bath at 37°C, or in an ice-water bath at 4°C, under an atmosphere of 10% CO_2 in air. Uptake was initiated by the addition of mitozolomide (14.08 μCi/ml) in DMSO. At specified time-points samples (200 μl in triplicate) were removed to an eppendorf tube which contained 100 μl of a silicon oil: corn oil (10:3) mixture and 50 μl of 98% formic acid. After centrifugation (900g) for 1min the tubes were frozen in liquid nitrogen, and cut at the oil/acid boundary. Each portion was placed in a scintillation vial, allowed to thaw and mixed with fiso-fluor scintillation fluid. The vials were counted in a Beckman scintillation counter.

The oil layer contained radioactivity from the extracellular solution and the acid layer contained the intracellular radioactivity. To establish the cell volume a 1ml sample of a cell suspension was treated with polyethyleneglycol and a further sample with $[H^3]$-H_2O. Once equilibrium had been established the samples were treated as for the experimental tubes.

C.6.2. Cell-medium distribution ratio

Cells were prepared as for transport studies and treated with a range of drug concentrations. Once equilibrium had been acheived (15 min), 200 μl portions were processed in triplicate as for transport studies. Cell volume was determined as above.

C.6.3. Effect of metabolic inhibitors on the uptake of mitozolomide.

TLX5 mouse ascities cells ($2x10^7$ cells/ml) were suspended in R.P.M.I. 1640 medium in a shaking water bath at 37°C. The cells were treated with one of the following, DNP (1mM), NaCN (1mM), NaF (20mM), or oligomycin (0.1mM). Following a 30 min incubation period uptake was initiated by the addition of mitozolomide (14.08 μCi/ml) in DMSO and the determination of the equilibrium value of mitozolomide concentration proceeded as detailed in C.6.1. The uptake was followed for 30 min, which was sufficent for equlibrium to be achieved. The uptake of mitozolomide was investigated at 4°C as well as 37°C.

C.6.4. The effect of AIC on the uptake of mitozolomide.

TLX5S mouse ascites cells ($2x10^7$ cells/ml) were equilibrated at 4°C. AIC and radioactive mitozolomide were added to the cells in a molar ratio of 1:1 and the initial uptake of mitozolomide determined as detailed (see C.6.1.).

C.7. The fate of the [6-^{14}C]imidazo-mitozolomide label.

C.7.1. Incorporation of [6-^{14}C]imidazo-mitozolomide into acid precipitable material.

TLX5S mouse lymphoma cells were ruspended in R.P.M.I. 1640 medium containing 10% fetal calf serum at a concentration of 2x10^6 cells/ml in the presence of [6-^{14}C]-mitozolomide (14.08 μCi/ml) at 37^{o}C under an atmosphere of 5% CO_2 in air. At appropiate times samples (1ml) were removed, sedimented by centrifugation, washed with saline and lysed with 0.2N $HClO_4$ (500 μl). The acid-insoluble material, after centrifugation, was taken up in fiso-fluor scintillation fluid and the radioactivity determined in a scintillation spectrometer. The ability of cells to synthesize DNA was monitored following the incorporation of 5-(methyl-^{3}H)-thymidine into acid insoluble material as previously described, C.5.1.

C.7.2. Breakdown of [6-^{14}C]imidazo-mitozolomide as measured by hplc.

The decomposition of [6-^{14}C]-mitozolomide and the production of [6-^{14}C]-AIC in R.P.M.I. 1640 media at 37^{o}C was determined by the hplc method of Slack et al. (1983). Separation was acheived by eluting the column with 5% methanol in Walpoles acetate buffer (B.7.4.), these conditions were maintained for 2.5 min at which point the methanol content was increased linearly to 35% over 2 min. The final conditions were held for 7 mins. The flow-rate was 2 ml/min throughout. When a radioactivity detector was used

in series with a U.V. detector the above protocol was slightly modified. The initial condition of 5% methanol was held for 5 min then increased linearly to 35% over 2 min, the final conditions were held for a further 10 min. The flow-rate was maintained at 1 ml/min. The elutant from the column was collected in 200 μl aliquots, mixed with fiso-flour scintillant and counted in a Beckmann scintillation counter.

C.7.3. Incorporation of the [6-^{14}C]imidazo-mitozolomide label into adenine and guanine bases.

TLX5S mouse lymphoma cells at a concentration of $2x10^6$ cells/ml (50ml), were incubated in R.P.M.I. 1640 medium with mitozolomide 0.14 μCi/ml for 5hr. The cells were sedimented by centrifugation, washed with saline and lysed with 0.2N $HClO_4$ (2ml). The acid-insoluble material so obtained was washed twice with ice-cold distilled water and lyophilized. The lyophilized material was heated to 80°C in 1N HCl (1ml) for 1hr. The resulting acid hydrolysate was seperated by reverse phase chromatography on a 5μM C^{18} column eluted with 3-10% acetonitrile in phosphate buffer (B.7.5.) over 40 min at a flow rate of 0.5 ml/min. Adenine and guanine were used as hplc standards. The method used was based on that of Tong et al. (1982a).

The elutant was monitored with a Waters LC spectro-photometer and a coincidence ESI Nuclear analyser (Rotheroe and Mitchell, Nuclear division, Ruislip, Middlesex). The elutant was collected in small aliquots and dissolved in

fiso-flour scintillation fluid for scintillation detection.

C.8. Flow-cytometry.

Flow-cytometry provides a rapid means of investigating agents which preturb the cell-cycle. The method quantifies the DNA content of individual cells producing a DNA histogram which shows the number of cells with a particular DNA content. Measurement is based on staining cells with a fluorescent dye that binds quantitatively to DNA, in this case propidium iodide. When a sample of stained cells is introduced into the flow-cytometer they are manipulated so that a flow of single cells passes a laser light source, this excites the dye to fluoresce and the light so formed is measured giving the DNA content for each cell.

Since the position of a cell in the cell-cycle, whether G_1,S or G_2/M is identified by its DNA content the DNA histograms produced also detail the number of cells in each stage of the cycle. An unperturbed population of cells will cycle at an constant median rate so that its DNA histogram remains essentially unchanged, reflecting the fact that there is the same number of cells in each stage of the cell-cycle at any one time. If a sample of cells are treated with an agent that interferes with the cell-cycle prior to flow-cytometric evaluation the number of cells in each cell cycle stage is altered, either due to a complete block at one point, or to the lengthening of the time it takes fora cell to progress through a particular stage, DNA synthesis

for example. Such changes in distribution of a cell through the cell-cycle are reflected in the DNA histograms produced.

C.8.1. The preparation of cell samples for flow-cytometic evaluation.

The method used to prepare *in vivo* tumour samples was reported by Erba et al. (1983). 3LL tumours growing in C57Bl/6 mice were removed, weighed, and washed in a Petri dish with phosphate buffered saline (PBS). The tumour fragments were then minced with scissors to seperate the vegetative and necrotic part of the tumour.

The vegetative part of the tumour was used to prepare a single cell suspension for analysis by flow-cytometry using the method of Starace et al. (1982). Tumour fragments were forced through a 18 Gx$1^{1}/2$ needle and resuspended in Hank's solution maintained at 4°C. The cells were then stained with propidium iodide (P.I.) by adding P.I. solution (3ml), (see section B.7.6.), to 200 µl-300 µl of cell suspension. The cell suspension was store at 4°C for 20-30 min before analysis. The suitability of the preparation, the specificity of staining, and the abscence of cell aggregates were checked by fluorescent microscopy before the assay was preformed. Leukocytes taken from a C57Bl/6 mouse were used to standardize the flow-cytometer.

Cytofluorometric analysis was preformed using a 30L cytofluorograph. The fluorescence pulses were detected in a spectral range between 580 and 750 nm (to exclude the

overlapping region of excitation and emission spectra of unbound P.I.) and integrated. To calculate the percentage of cells in cell-cycle phases the method of Krishan and Frei (1976), was used.

The co-efficent of variation (CV) of the leukocyte standard was between 1.5% and 2.5%, while the Go-G_1 phase of 3LL cells was between 4-6% CV. Each cytofluorographic assay was preformed on $20-40x10^3$ cells.

C.8.2. The effect of mitozolomide on the cell-cycle traverse of 3LL cells <u>in vivo</u>.

Mice bearing 3LL tumours were injected i.v. in the tail vein with mitozolomide dissolved in DMSO and saline (1:10). At appropiate times mice were sacrificed, the tumours removed and cell suspensions prepared for flow-cytometry as described above. At each time-point 4 control mice and 4 druq treated mice were sacrificed. Evaluation of the anti-tumour effect of mitozolomide was carried out on day 21 after tumour implantation. Tumour weights and the number of metastases were recorded and compared with controls.

C.8.3. The effect of mitozolomide and MCTIC on the cell-cycle traverse of 3LL cells *in vitro*.

Logarithymically growing cultures of 3LL cells were prepared as described previously, (C.3.4.), and drug solutions of mitozolomide and MCTIC added to a final concentration of Ø.4 μM, 4.Ø μM and 4Ø.Ø μM. Samples were taken for flow-cytometric evaluation at 8, 12 and 24hr. At 24hr fresh media was supplied and the cells allowed to recover for 24hr, at which time a further sample was removed for flow-cytometric evaluation.

C.8.3.1. The effect of mitozolomide and MCTIC on the uptake of radioactive thymidine by 3LL cells *in vitro*.

In vitro cultures of 3LL cells were prepared as described (C.3.4.). After plating for 24hr 3LL cellr were treated with the drugs under investigation. One set of cells were resupplied with fresh media after 1hr and at 24hr all cells were supplied with radioactive thymidine (1.Ø μCi/ml). Labelling was allowed to proceed for 1hr at which time the level of radioactivity incorporated into cells was determined.

C.8.3.2. Growth inhibition of mitozolomide and MCTIC against 3LL cells *in vitro*.

A logarithymically growing culture of 3LL cells was prepared as described in (C.3.4.). Cells were exposed to

drug for 1hr and 24hr, the cells were counted in a Coulter counter at 24hr to determine the inhibition of growth.

C.9. Determination of the chemical half-lives of mitozolomide and selected analogues.

The half-lives of mitozolomide and a number of its analogues were determined in phosphate buffer (Ø.2M) at pH 6.Ø, 7.Ø, 7.5, and 8.Ø. Solutions of drug were made up to give an absorbance of approximately 1.Ø. The solutions were maintained in a Beckmann DU 7 spectrophotometer at 37°C and monitored at pre-set time intervals. The decrease in absorbance with time was measured at the max of the compound under study. The half-lives were calculated using a plot of the log. concentration of the drug versus time.

D. RESULTS

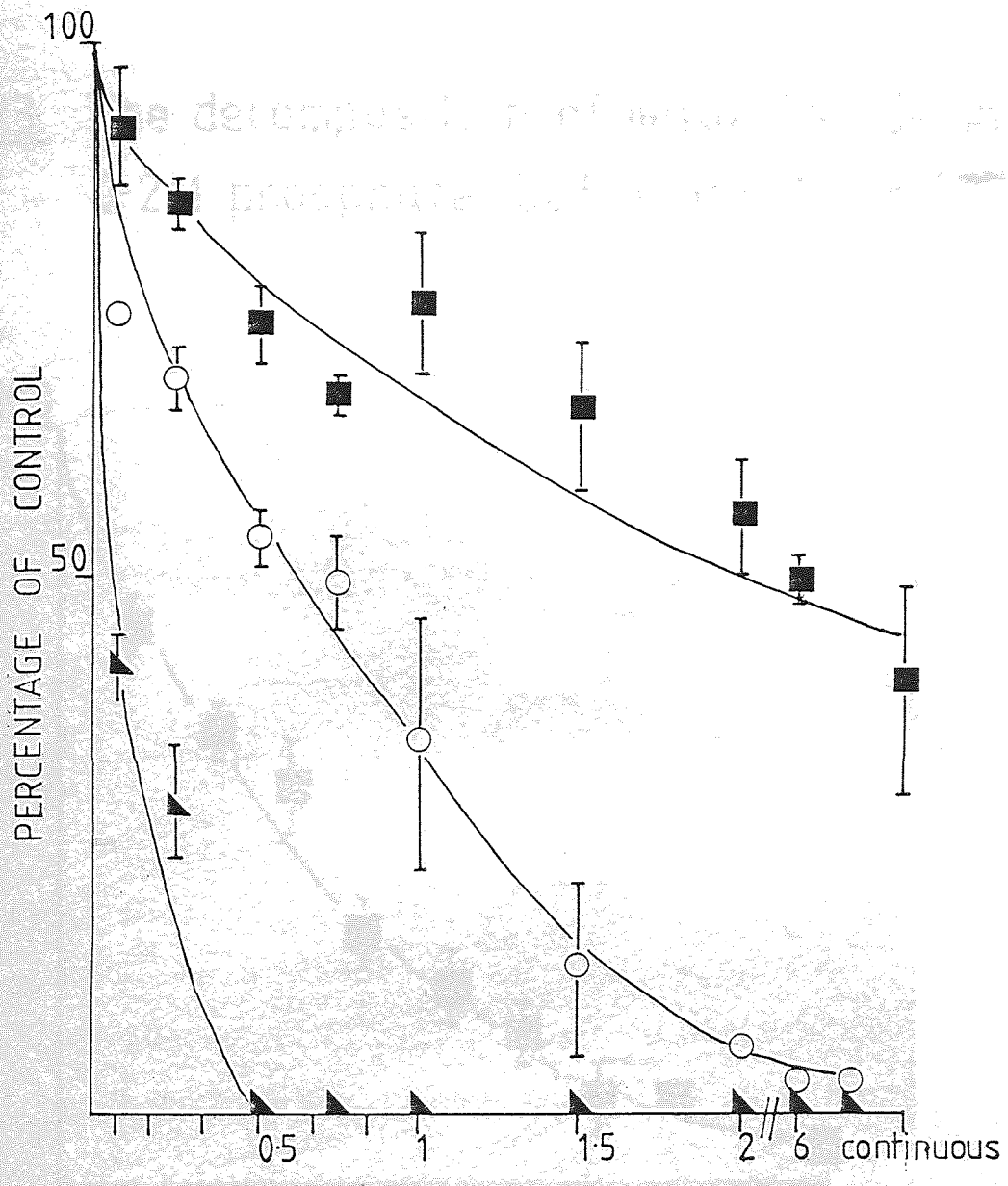
D. RESULTS.

D.1. Time-course of mitozolomide cytotoxicity *in vitro*

Logarithmically growing cells K562 cells ($0.5x10^5$ cells/ml) were treated with various concentrations of mitozolomide, samples were taken at appropiate times and the media removed by centrifugation. After washing with sterile saline, fresh media was resupplied. The cells were plated out ($0.5x10^5$ cells/ml) and incubated for three days before counting.

The percentage inhibition of growth against time of exposure to mitozolomide is shown (Fig 22 and 23). The time of exposure to drug required to produce a given cytotoxic effect is inversely proportional to the drug concentration, 50% inhibition of growth is achieved by approximately a 5min, 40min, and 120min exposure to 125 μM, 50 μM, and 5 μM mitozolomide concentrations respectively. This time-course of cytotoxicity may be compared with the plot of disappearance of drug in phospate buffer (0.2M), both incubations being at pH 7.5. The degree of cytotoxicity is proportional to the extent of mitozolomide decomposition.

FIG. 22 The time-course of cell death of K562 cells on exposure to mitozolomide in R.P.M.I. media at p.H 7·5



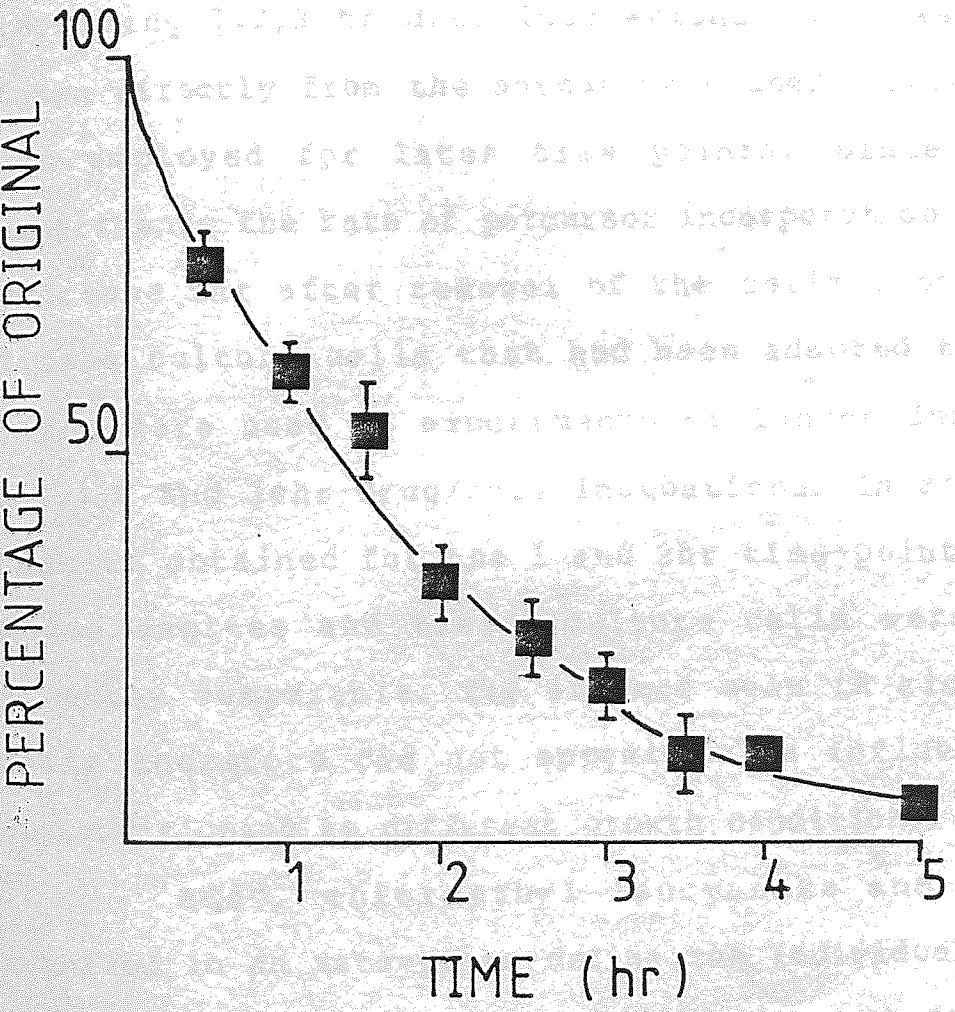
ID_{50} for mitozolomide against K562 cells = 5·0 μM

■ = 5·0 μM mitozolomide (50% growth inhibition)

○ = 50 μM mitozolomide (99% growth inhibition)

◣ = 125 μM mitozolomide (>99% growth inhibition)

FIG.23. The decomposition of mitozolomide in 0·2M phosphate buffer (pH 7·5) at 37°C



D.2. Precursor incorporation experiments.

Reactive species often damage DNA and RNA with cytotoxic consequences. Using TLX5S cells the effects of equitoxic concentrations of mitozolomide, MCTIC, BCNU and chloroethyl isocyanate on the incorporation of thymidine into DNA, uridine into RNA and of adenosine into DNA and RNA were determined over a 24hr period. For early time-points, involving 1,2,3 hr drug incubations mouse ascites cells taken directly from the animal were used. These cells were not employed for later time points, since in control experiments the rate of percursor incorporation was found to decrease 5hr after removal of the cells from the mouse. Tissue culture cells that had been adapted to growth *in vitro* were used in experiments of longer duration, i.e. 1,2,4,8 and 24hr drug/cell incubations. In all cases the results obtained for the 1 and 2hr time-points for which mouse ascites and tissue-culture cells were used were directly comparable. The effects seen in tissue-culture cells therefore did not appear to be influenced by any preconditioning to different growth conditions.

BCNU, chloroethyl isocyanate and MCTIC were examined in an attempt to define the individual effects of alkylation and carbamoylation on DNA and RNA and with reference to the potential metabonates of mitozolomide. BCNU carbamoylates via the chloroethyl isocyanate which it is suggested mitozolomide might also produce. MCTIC another possible metabonate of mitozolomide should alkylate DNA via

the production of the chlorodiazo species in an analogous fashion to BCNU.

D.2.1. Inhibition of thymidine incorporation into acid-insoluble material.

In Fig 24 the effects of a 1,3,8 and 24hr drug/cell exposure on 5-(methyl-^{3}H) thymidine incorporation into DNA are given. Most remarkable is the rapid and extensive inhibition produced by BCNU. At the 1hr time-point a 1Ø M concentration of BCNU causes greater than 6Ø% inhibition of thymidine incorporation. Chloroethyl isocyanate produces no effect on thymidine incorporation while the effects of mitozolomide and MCTIC are very similar, inhibition being first discernible at 3hr and increases through 8hr to 24hr. The only difference between the effect of MCTIC and mitozolomide occurs at 24hr when MCTIC produces a greater inhibition than mitozolomide.

Fig 24: Inhibition of thymidine incorporation into acid insoluble material.

○ = Mitozolomide

■ = MCTIC

▲ = BCNU

△ = 2-Chloroethyl isocyanate

Concentrations given refer to mitozolomide. The concentrations of all other agents are equitoxic to those of mitozolomide. The various LD_{50}'s of these compounds to the TLX5S cells as determined by three day growth inhibition studies are as follows:-

Mitozolomide = 4.0 +/- 1.0 μM

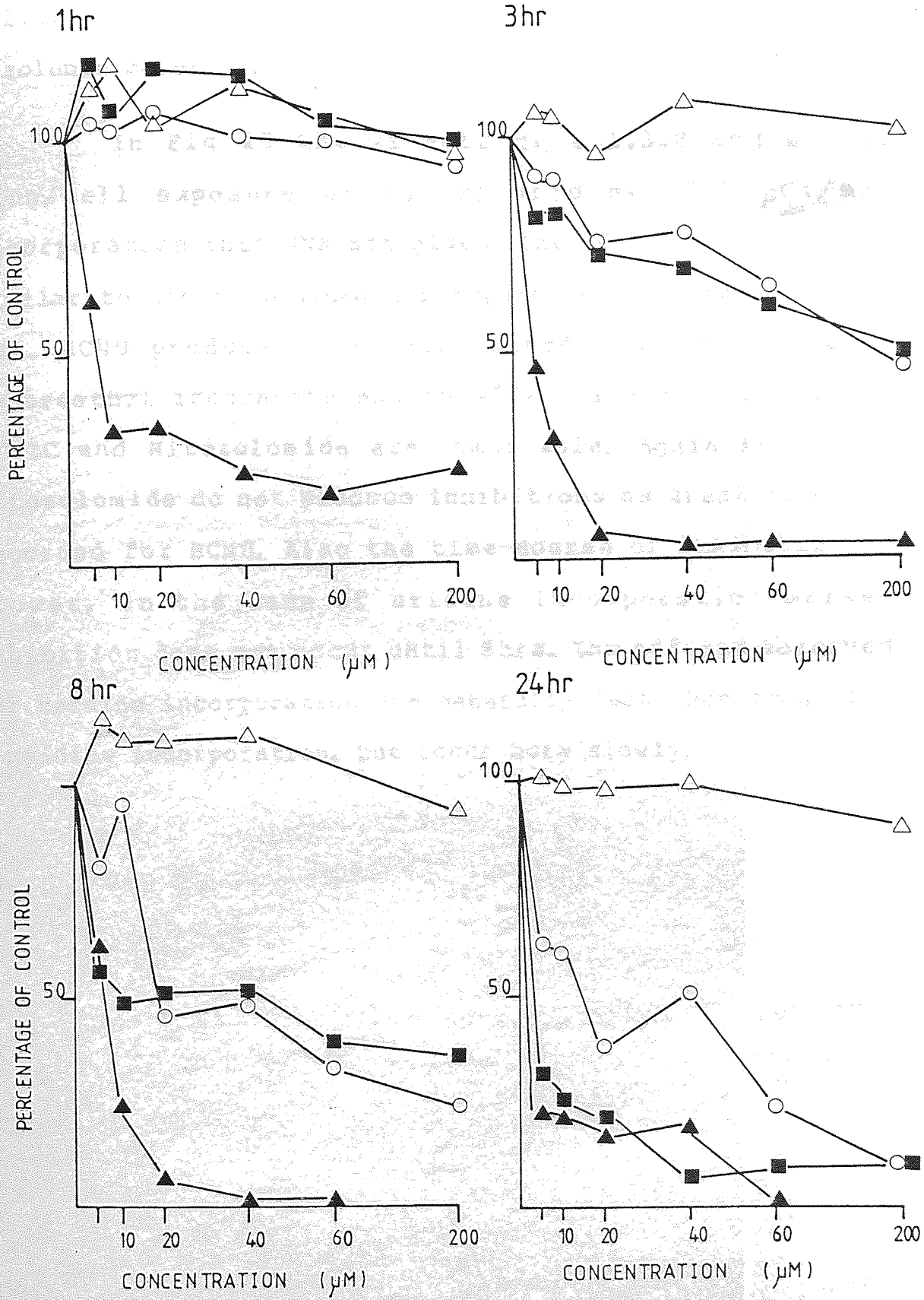
MCTIC = 4.0 +/- 1.0 μM

BCNU = 1.0 +/- 2.0 μM

2-Chloroethyl isocyanate = 35 +/- 5.0 μM

All points are the average of at least 3 separate experiments. Times given are those of drug/cell incubation prior to 1 hour incubation with radioactive 5-(methyl-^{3}H) thymidine. The 1 and 3 hour time-points were carried out using TLX5S mouse ascites cells while the 8 and 24 hour incubations TLX5S tissue-culture cells.

FIG. 24 Inhibition of thymidine incorporation into acid insoluble material



D.2.2. Inhibition of uridine incorporation into acid insoluble material.

In Fig 25 the effects of a 1,3,8 and a 24hr drug/cell exposure on (5-^{3}H)-uridine (2.5 μCi/ml) incorporation into RNA are given. The overall results are similar to those obtained for thymidine incorporation into DNA. BCNU produces the most marked and rapid effect, chloroethyl isocyanate has no effect and the effects of MCTIC and Mitozolomide are comparable. Again MCTIC and mitozolomide do not produce inhibitions as great as those recorded for BCNU. Also the time-course of inhibition is slower, in the case of uridine incorporation marked inhibition does not occur until 8hrs. The effects observed for uridine incorporation are generally less than those for thymidine incorporation, but occur more slowly.

Fig 25: The inhibition of uridine incorporation into acid insoluble material.

○ = Mitozolomide

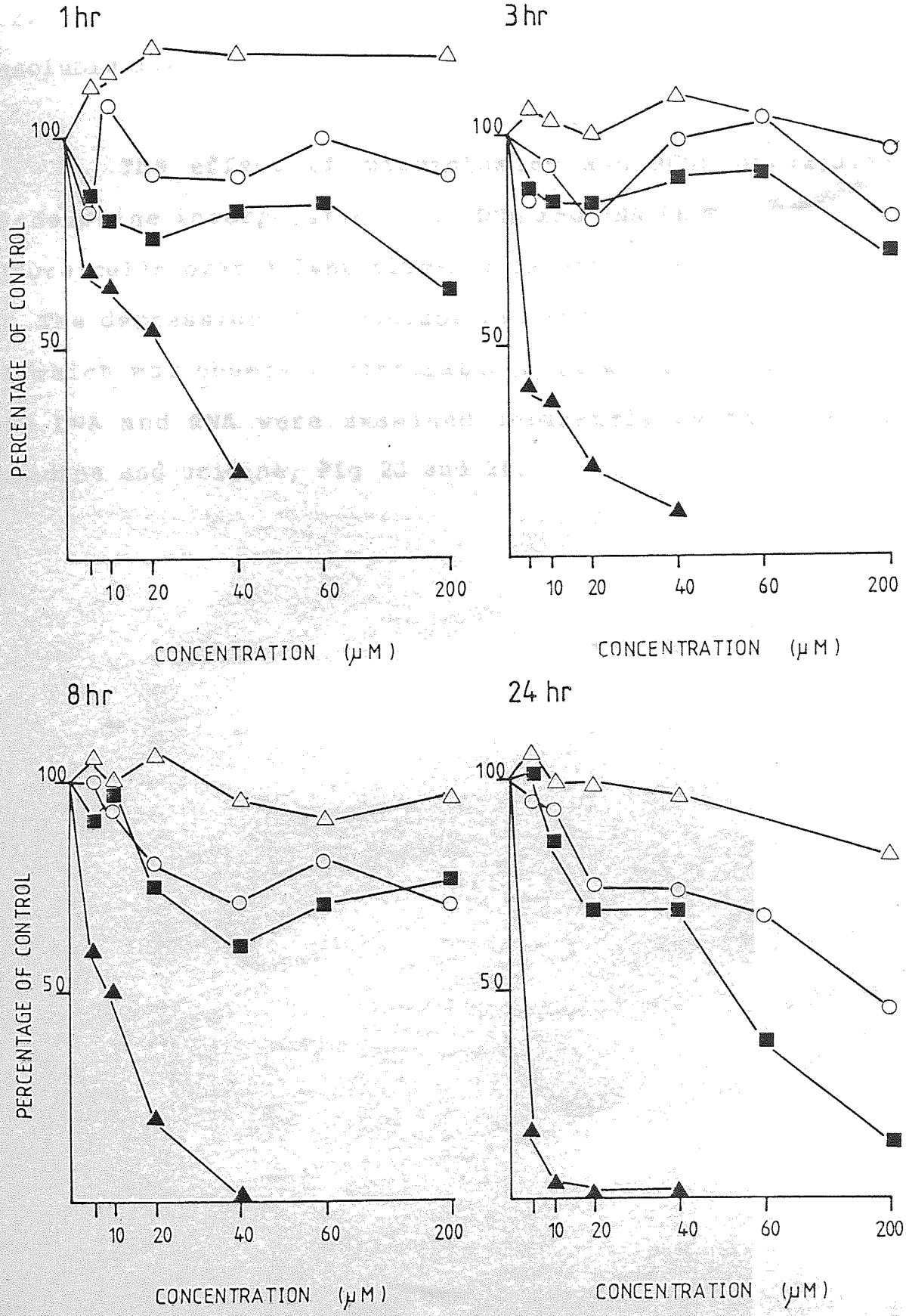
■ = MCTIC

▲ = BCNU

△ = 2-Chloroethyl isocyanate

Concentrations given refer to Mitozolomide, the concentrations of all other agents are equitoxic to those of mitozolomide. All points are the average of at least 3 separate experiments. Times given are those of drug/cell incubations prior to 1 hr incubation with radioactive (5-^{3}H)uridine. The 1 and 3 hr time-points were carried out using TLX5S mouse ascites cells, while the 8 and 24hr incubations employed TLX5S tissue-culture cells.

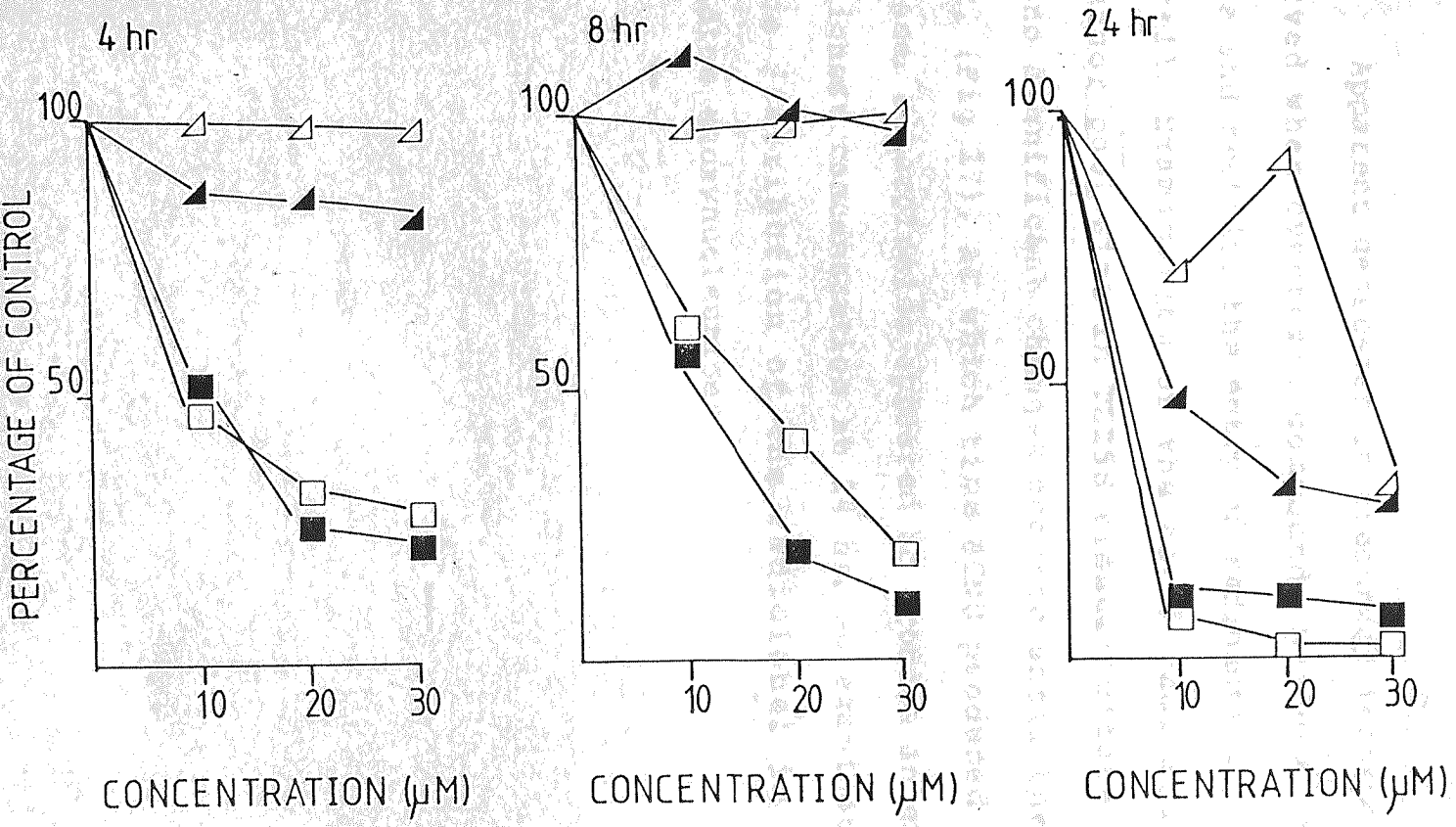
FIG. 25 Inhibition of uridine incorporation into acid insoluble material



D.2.3. Inhibition of adenosine incorporation into acid insoluble material.

The effect of mitozolomide and BCNU on (2,5,8-^{3}H)adenosine incorporation into DNA and RNA in TLX5S tissue culture cells over a 24hr time-course was investigated, (Fig 26). The depression of precursor incorporation into DNA and RNA which was observed correlated well with that revealed when DNA and RNA were examined separately by the use of thymidine and uridine, Fig 23 and 24.

FIG. 26 Inhibition of adenosine incorporation into acid insoluble material



△,◢ = Mitozolomide

□,■ = BCNU

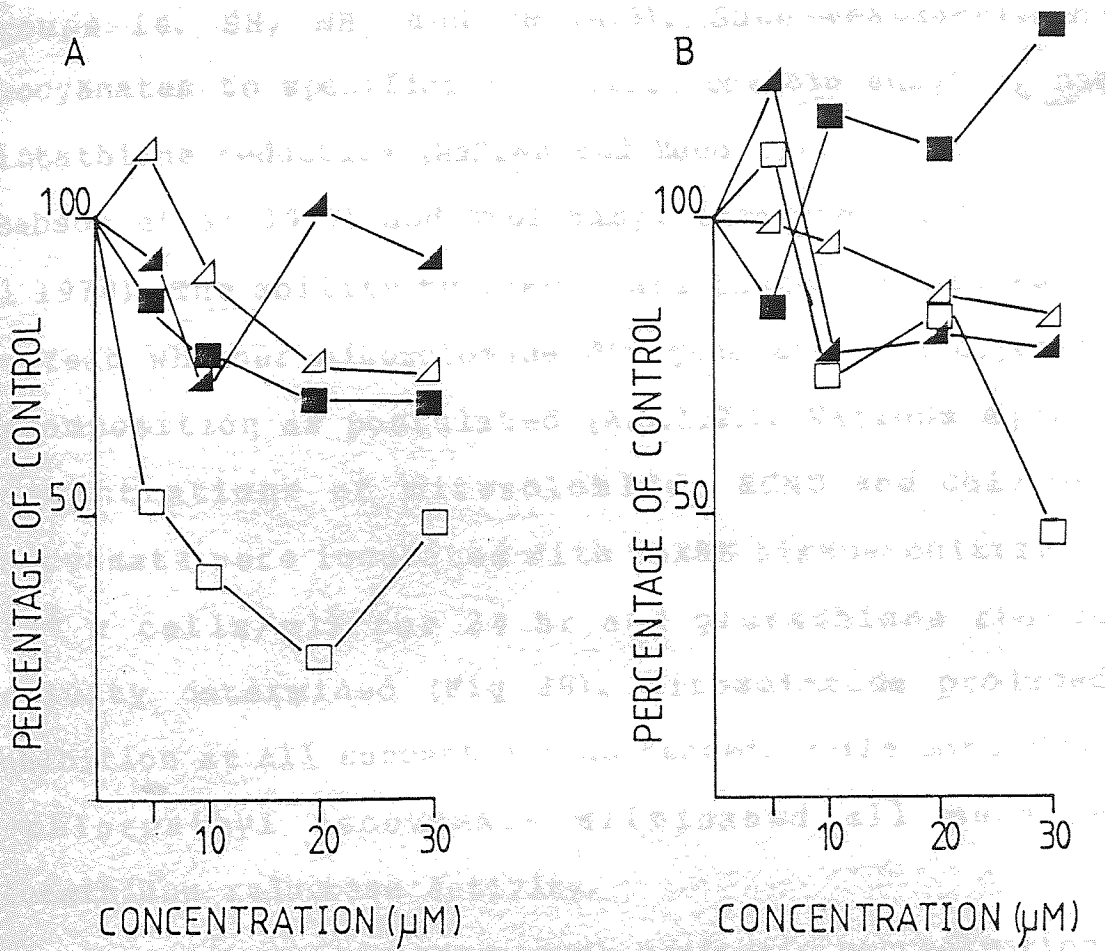
Closed symbols = effect on DNA

Open symbols = effect on RNA

D.2.4. The effect of mitozolomide and BCNU on the thymidine pool size of TLX5S tissue-culture cells.

Apparent decreases in precursor incorporation may be observed when cytotoxic compounds produce cell membrane damage and restrict the entry of radioactive precursors into the cell. Examination of the total radioactivity of the precursor pool size in TLX5S tissue-culture cells showed that no significant changes occured until the 24 hr time-point, (Fig 27), at which time BCNU produced the greatest decrease. Mitozolomide appeared to produce an effect only at the highest concentration at 24 hr. No examination was made of the distribution of the radiolabel in the various thymidine deoxynucleotides.

FIG. 27 Effect of mitozolomide and BCNU on the total pool size of 5(methyl ^{3}H) thymidine and metabolites in TLX5S cells



A = BCNU

B = Mitozolomide

The times of drug / cell incubations are :-

◢ = 2 hr ◿ = 4 hr

■ = 6 hr □ = 24 hr

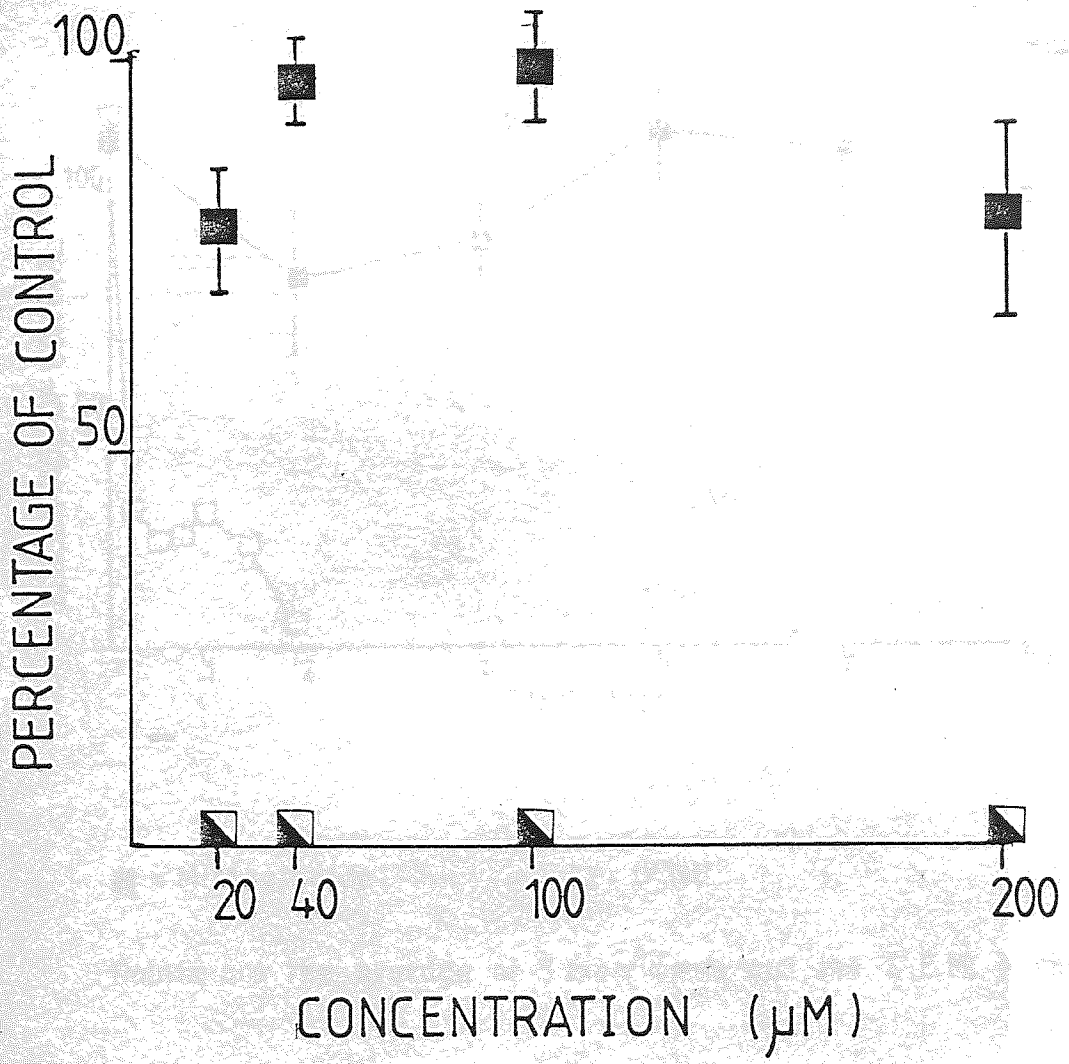
D.3. Production of an isocyanate.

Isocyanates are capable of reacting with biological groups ie. SH, NH_2 and OH (A.5). Such reactions enable isocyanates to specifically inhibit certain enzymes, namely glutathione reductase (Babson and Reed 1978), α-chymotrypsin (Babson et al 1977) and γ-glutamyl transpeptidase (Lakai et al 1978). The ability to inactivate these enzymes was used to test whether mitozolomide did generate an isocyanate on decomposition as postulated (A.5.1.2.). Various equimolar concentrations of mitozolomide, BCNU and chloroethyl isocyanate were incubated with TLX5S tissue-culture cells (10^6 x cells/ml) for 24 hr and glutathione reductase activity determined (Fig 28). Mitozolomide produced no inhibition at all concentrations tested, while both BCNU and 2-chloroethyl isocyanate eliminated all measurable glutathione reductase activity.

In a similar experiment equitoxic concentrations of mitozolomide and BCNU were incubated with TLX5S tissue culture cells. At various time-points cell samples were taken (20ml), washed with saline and sonicated in glutathione reductase buffer (B.7.1.). The supernatant obtained on centrifugation was assayed for glutathione reductase activity (Fig 29). Mitozolomide caused no reduction in the enzyme activity even after a 24 hr exposure time while BCNU produced almost total inhibition at 2 hr and marked inhibition was observed within a few minutes.

Incubation of purified chymotrypsin (Fig 29) and glutamyl transpeptidase (Fig 3Ø) with different equimolar concentrations of BCNU and mitozolomide again demonstrated the total lack of effect of mitozolomide compared to the almost complete inhibition produced by BCNU.

FIG. 28 Effect of mitozolomide, BCNU, and 2-chloroethyl isocyanate on glutathione reductase activity in TLX5S cells.

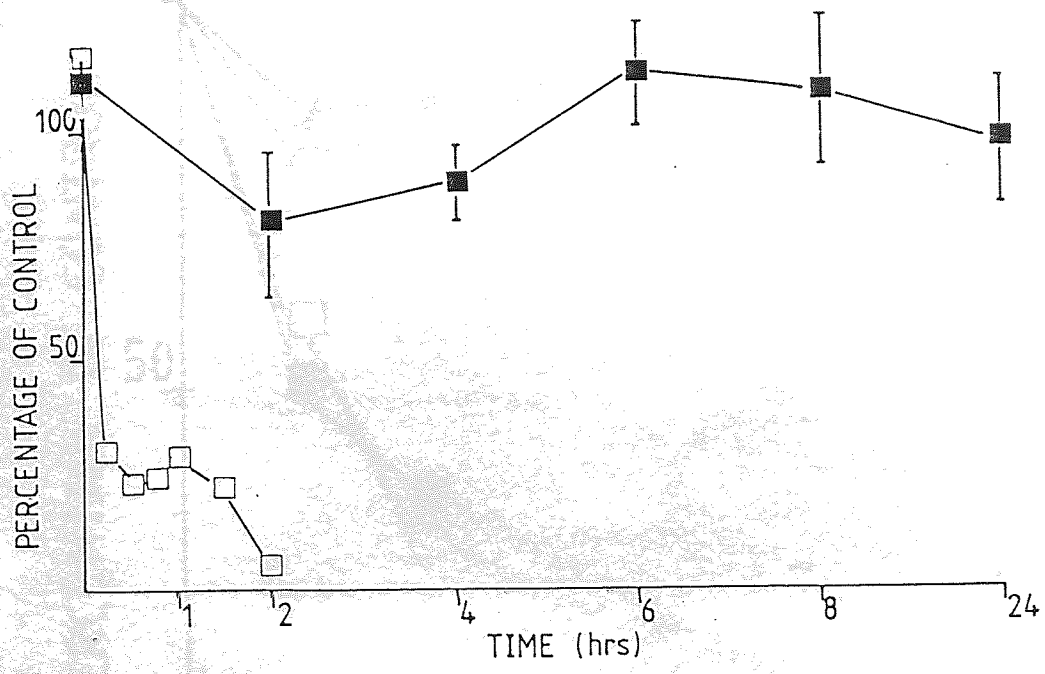


■ = Mitozolomide ◣ = 2-Chloroethyl isocyanate

□ = BCNU

Results are the average of 3 experiments and the S.E.M is given

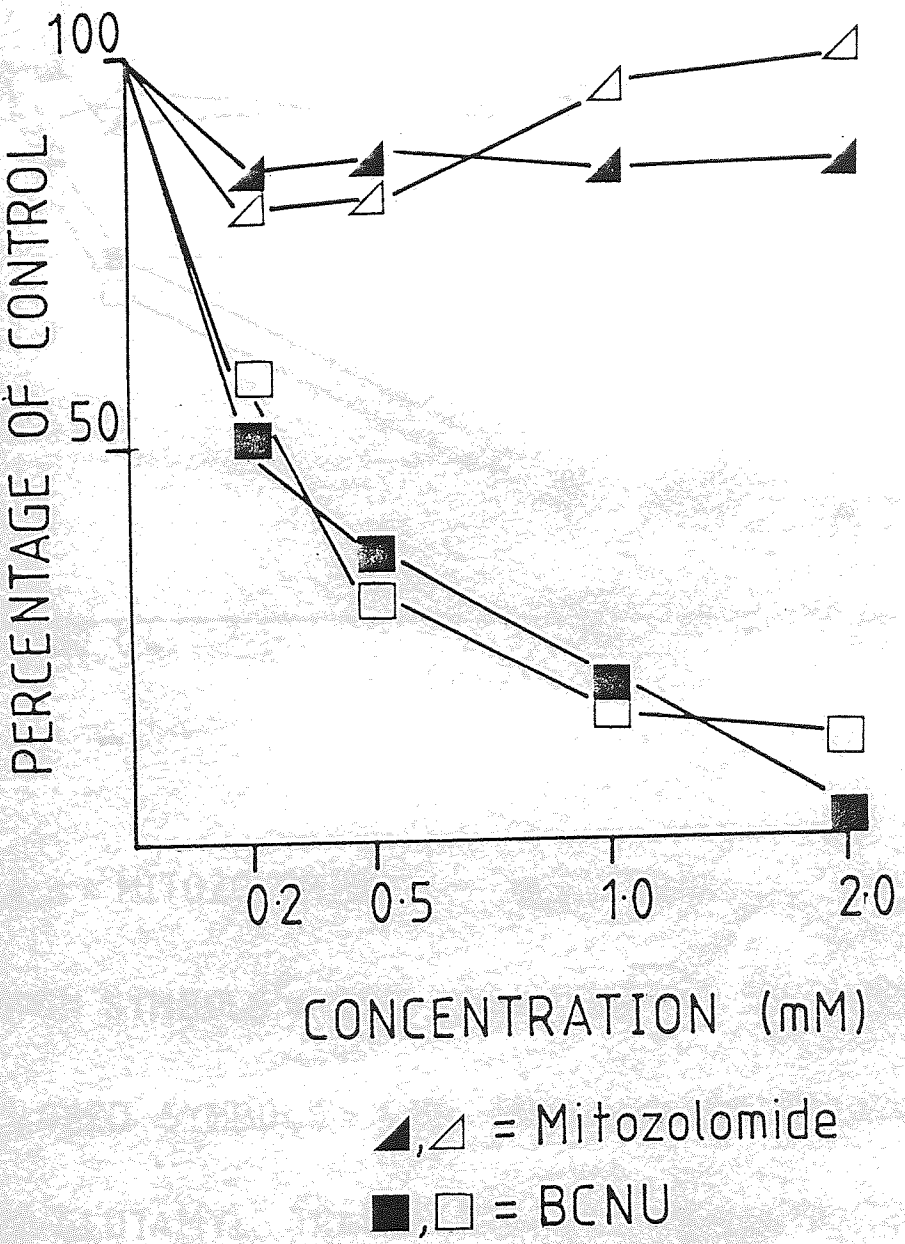
FIG. 29. The time-course of the inhibition of glutathione reductase activity in TLX5S tissue culture cells by mitozolomide and BCNU



■ = Mitozolomide □ = BCNU

Points are the average of 3 experiments and the S.E.M. is shown.

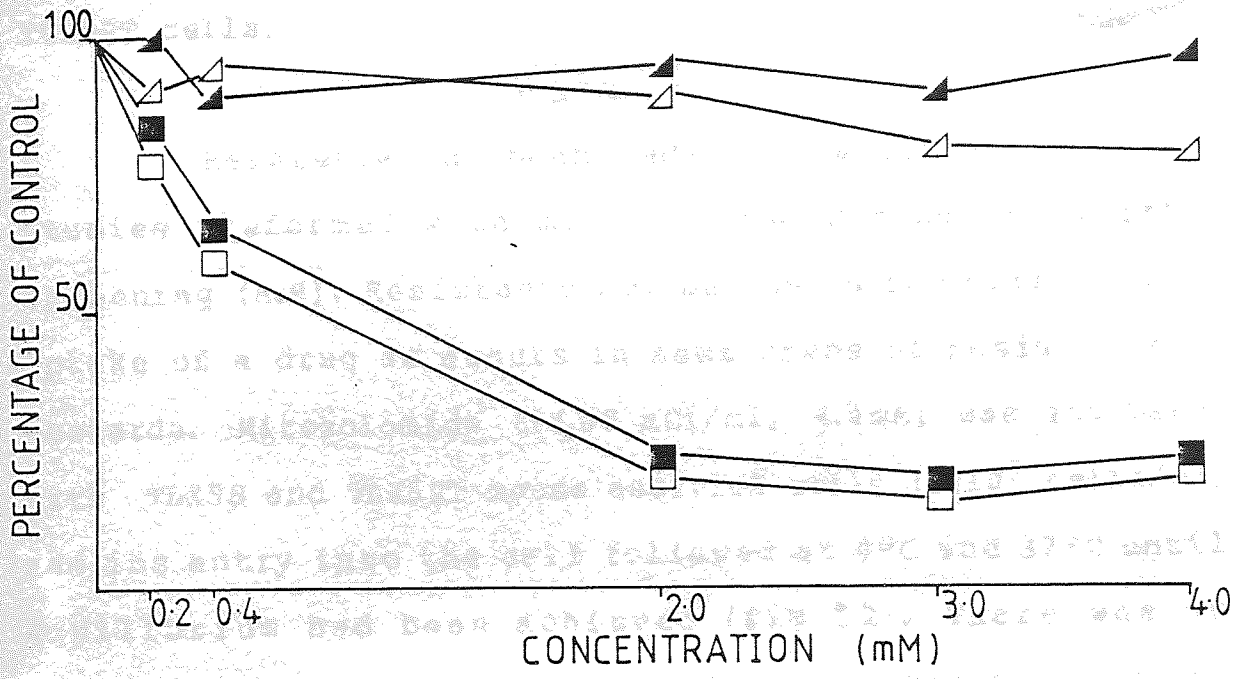
FIG. 30 Effect of equimolar concentrations of mitozolomide and BCNU on chymotrypsin activity



Open symbols = 2 hr Closed symbols = 4 hr

Chymotrypsin concentration = 0·57 mg/ml.

FIG. 31 Effect of equimolar concentrations of mitozolomide and BCNU on γ-glutamyl transpeptidase activity.



◢,◿ = MITOZOLOMIDE ■,□ = BCNU

OPEN SYMBOLS = 2HR DRUG/ENZYME INCUBATION

CLOSED SYMBOLS = 4 HR DRUG/ENZYME INCUBATION

γ-GLUTAMYL TRANSPEPTIDASE CONCENTRATION = 10 mg/ml

D.4. Uptake and incorporation of [^{14}C]imidazo-mitozolomide into TLX5S cells.

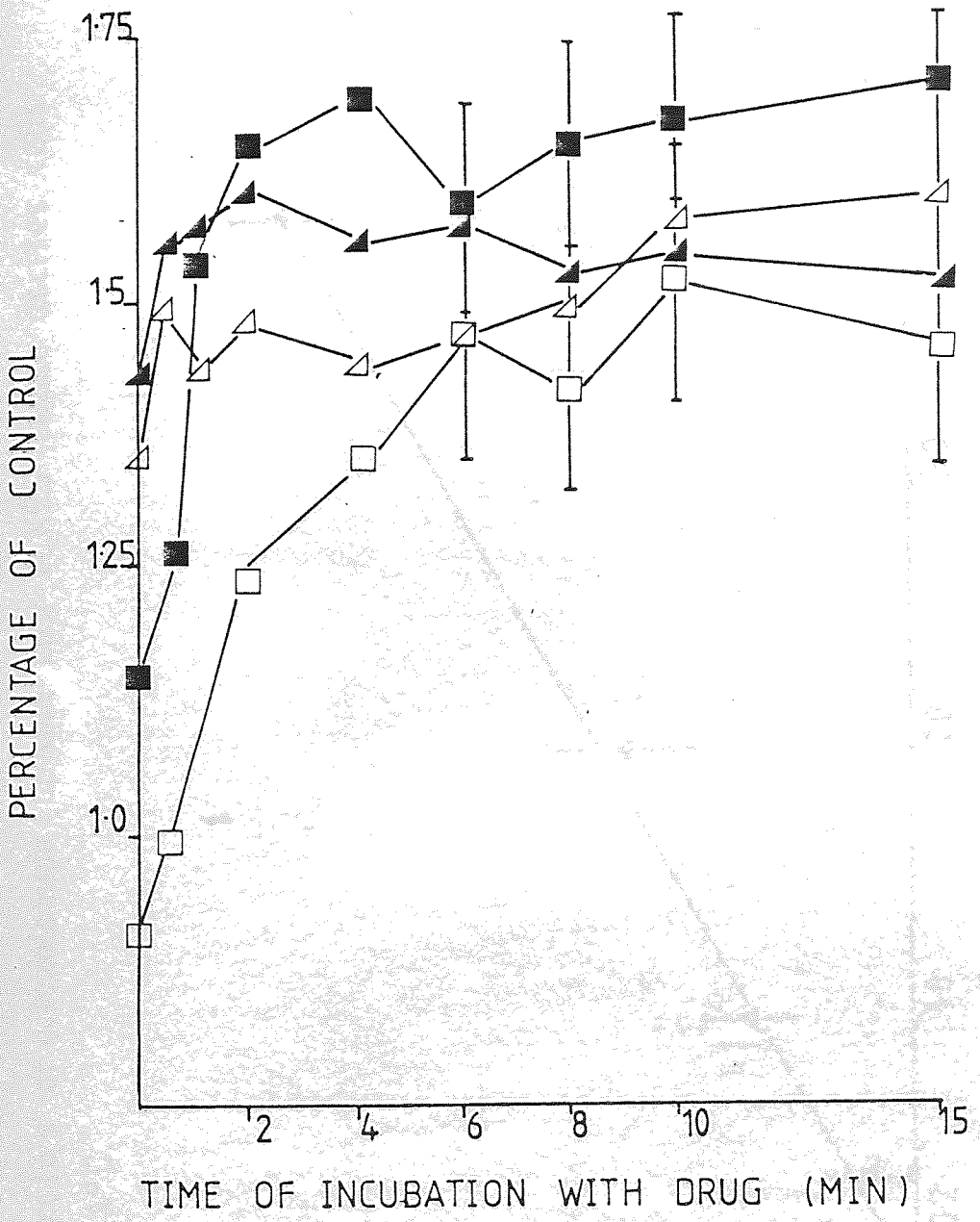
D.4.1. Uptake of [^{14}C]-imidazo mitozolomide into TLX5S and TLX5RT cells.

Reference has been made to the cross-resistance studies preformed with mitozolomide during its initial screening (A.8). Resistance may be due to the differential uptake of a drug as occurs in some cases of resistance to mustards. Mitozolomide (14.Ø8 μCi/ml, 4.4mM) was incubated with TLX5S and TLX5RT mouse ascities cells (2x1Ø^7 cells/ml) and its entry into the cell followed at 4^{o}C and 37^{o}C until equilibrium had been achieved (Fig 32). There was no significant difference between the equilibrium values of mitozolomide obtained in the sensitive or resistant cell line.

The uptake profiles for the different concentrations of mitozolomide were obtained. The rate of uptake versus concentration was directly proportional (Fig 33) and showed no signs of saturation. The cell:medium distribution ratio achieved by mitozolomide using TLX5S cells at 37^{o}C , 4^{o}C and with cells that had been preincubated with the metabolic inhibitor DNP (1mM) for 3Ømin at 37^{o}C was only slightly greater than 1.Ø (Fig 34). There was therefore no enhanced concentration of mitozolomide within cells. Incubation of TLX5S cells with a

variety of metabolic inhibitors DNP (1mM), NaCN (1mM), NaF (20mM) and oligomycin (0.1mM) for 30mins prior to mitozolomide addition did not produce any significant effects on drug uptake (Table 7).

FIG. 32 Uptake of [C^{14}-imidazo]-mitozolomide into TLX5S and TLX5RT mouse ascites cells at 4 and 37°C.

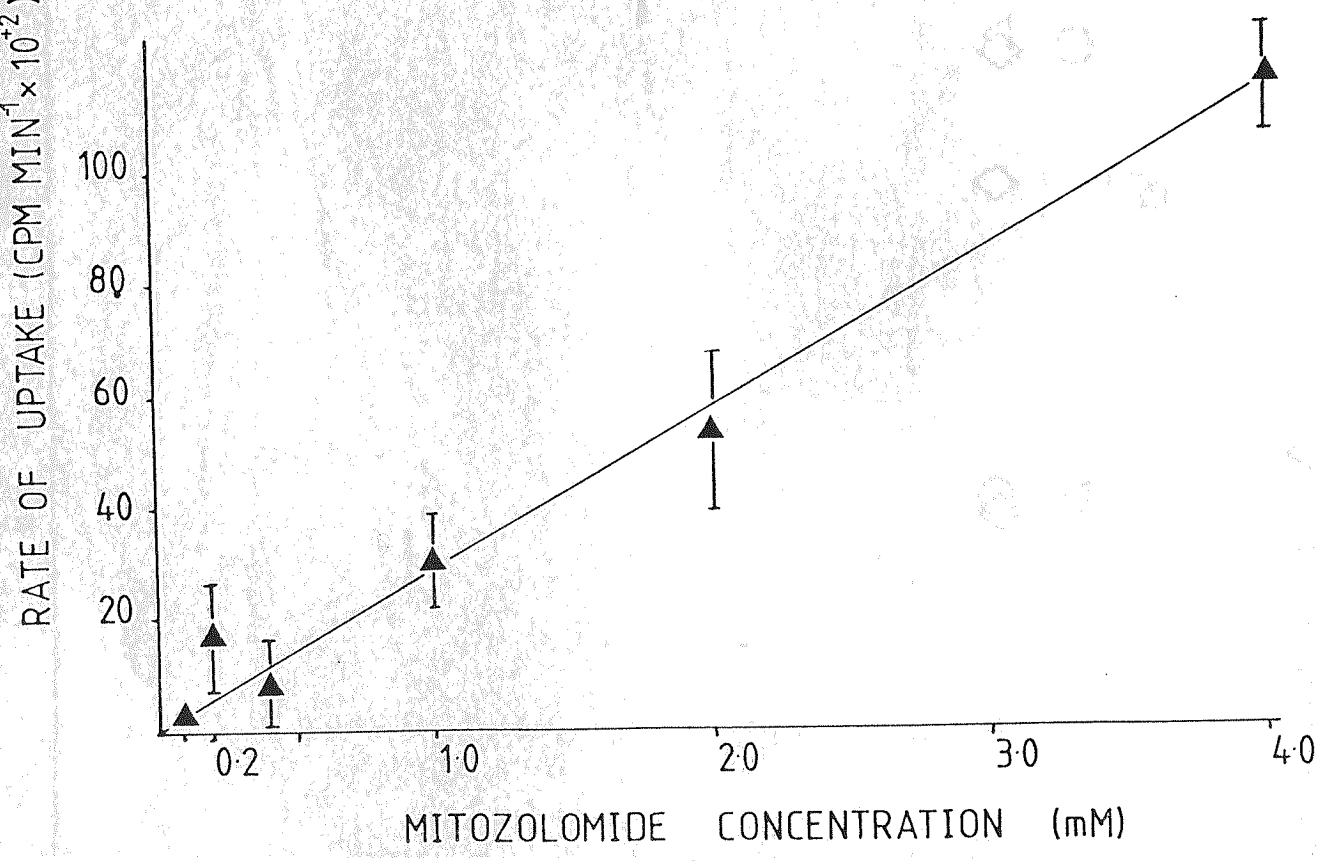


◿ = TLX5S CELLS AT 37°C □ = TLX5S CELLS AT 4°C

◢ = TLX5RT CELLS AT 37°C ■ = TLX5RT CELLS AT 4°C

ERROR BARS SHOWN REFER TO TLX5S AND TLX5RT (□, ■ respectively) AT 4°C. ALL POINTS ARE THE AVERAGE OF 3 EXPERIMENTS.

FIG 33 Kinectics of mitozolomide uptake into TLX5S mouse ascites cells at 4°C.



EACH POINT IS THE AVERAGE OF 3 EXPERIMENTS. S.E.M. IS GIVEN

FIG. 34 Cell/Medium distribution of mitozolomide

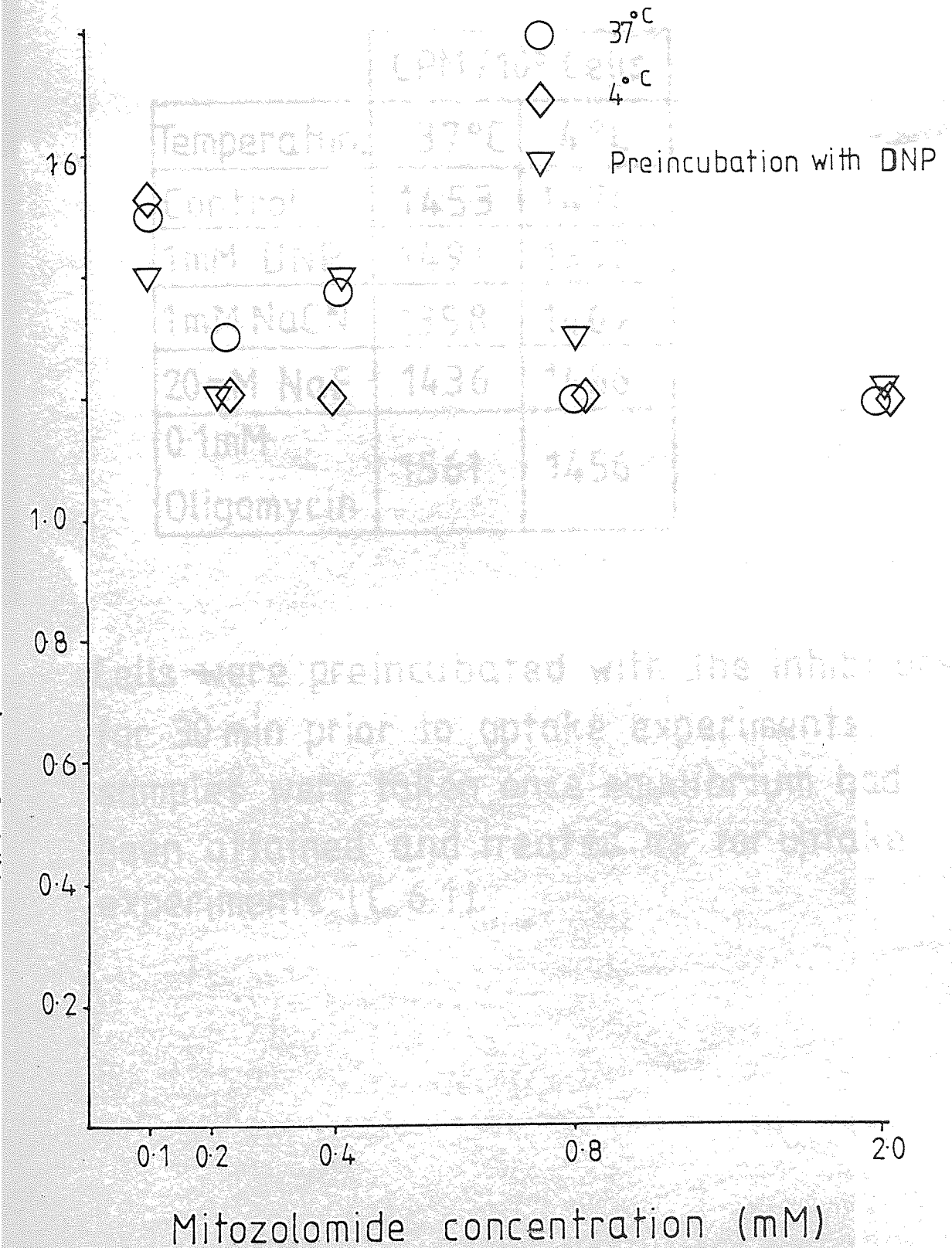


TABLE 7 Effect of metabolic inhibitors on the uptake of mitozolomide

	CPM / 10^6 Cells	
Temperature	37°C	4°C
Control	1453	1474
1mM DNP	1491	1552
1mM NaCN	1358	1402
20mM NaF	1436	1456
0·1mM Oligomycin	1561	1456

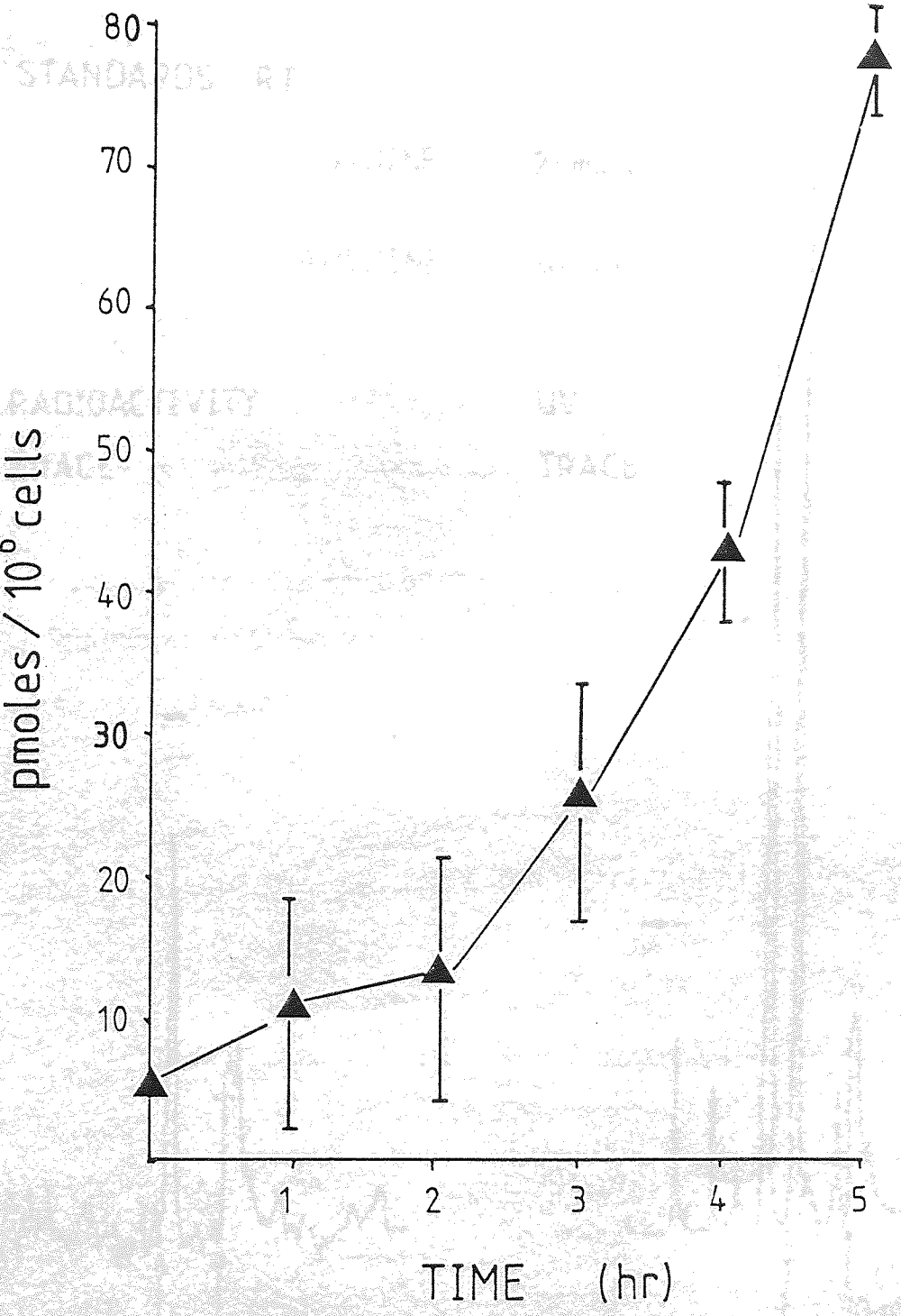
Cells were preincubated with the inhibitors for 30 min prior to uptake experiments, samples were taken once equilibrium had been attained and treated as for uptake experiments. (C.6.1).

D.4.2 Incorporation of [^{14}C]imidazo-mitozolomide into the acid insoluble material of TLX5S mouse ascites cells.

On incubation of [6-^{14}C]imidazo- mitozolomide (14.Ø8 μCi/ml) with TLX5S cells radioactivity was found to become associated with the acid precipitatable material over a 5 hour time-course (Fig 35). There was an initial lag period of approximately 2 hr where incorporation of the [^{14}C]imidazo label was low, after which the rate of incorporation increased until at the 5 hr time-point there were 8Ø.Ø pmoles of radioactive material present/1Ø^{6} cells.

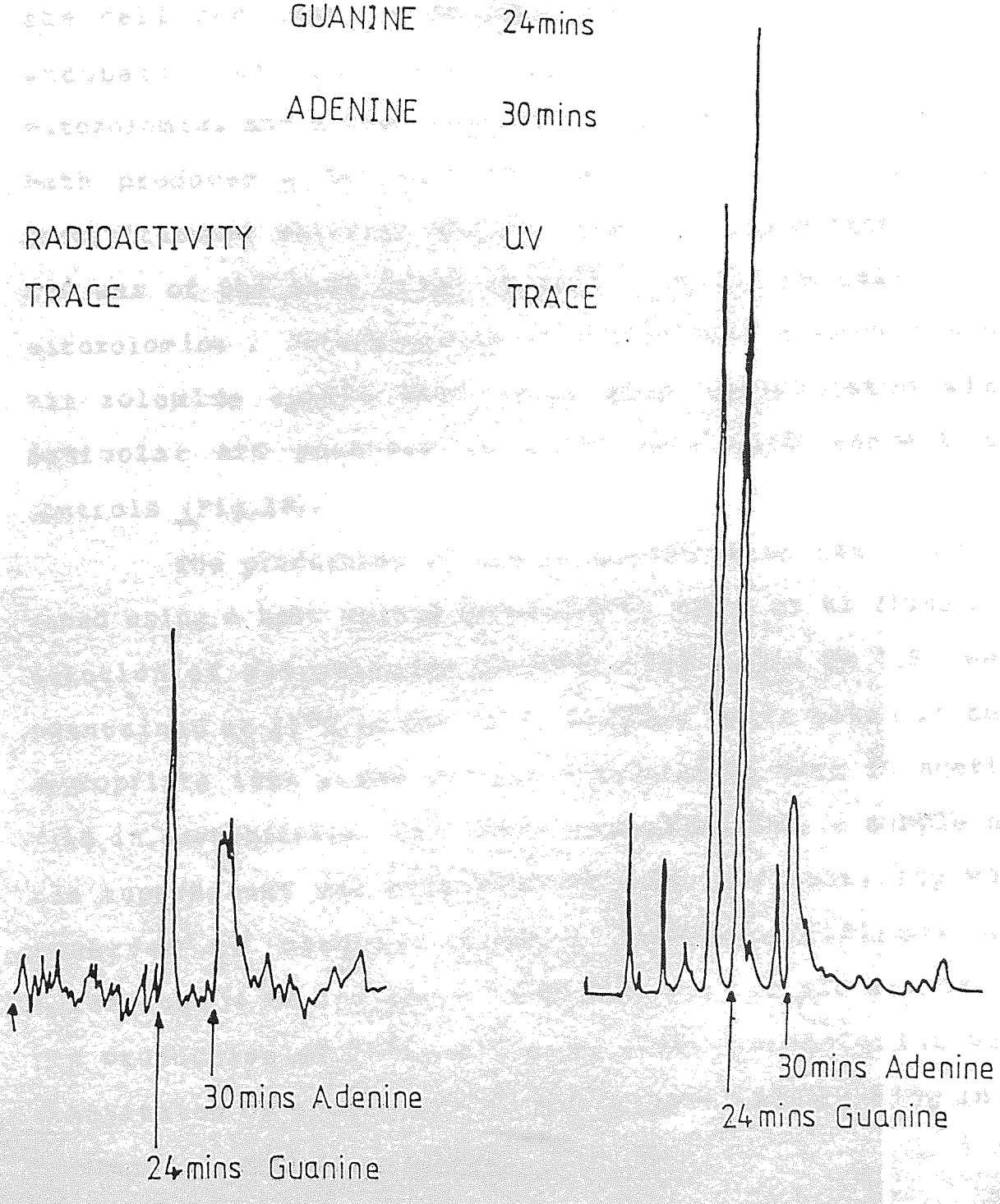
Samples of the acid precipitable material labelled with [^{14}C]imidazo-mitozolomide were prepared by the incubation of labelled mitozolomide (Ø.141μCi/ml) with approximately 5Øml of TLX5S mouse ascites cells (2x1Ø^{6}cells/ml) for 5hr. After acid hydrolysis of the precipitate the hydrolysate was separated by hplc using a protocol designed to analyse the bases of DNA. Peaks of radinactivity were found which corresponded to U.V peaks produced by adenine and guanine (Fig.36). The hplc elutant was collected, mixed with scintillant and counted. The radioactivity present in the peaks coincided with adenine and guanine standards and accounted for all the radioactivity present in the hydrolysate. Any precipitate left after acid hydrolysis was washed and when counted found to be unlabelled

FIG. 35. Incorporation of the [C^{14}-imidazo]-mitozolomide label into the acid-precipitable material of TLX5S mouse ascites cells.



Points are the average of 3 experiments. SEM is given.

FIG. 36 Hplc traces showing coincidence of C^{14}-imidazo]-mitozolomide derived radioactivity with adenine and guanine standards.



D.4.3. Production of [^{14}C]AIC from [^{14}C]imidazo-mitozolomide

Labelling of nucleic acids could occur through the breakdown of mitozolomide to MCTIC and consequent production of [^{14}C]-AIC (A.5.2.1.). [^{14}C]-AIC might then be salvaged by the cell and used in de novo purine biosynthesis. Co-incubation of TLX5S mouse ascites cells with [^{14}C]-mitozolomide and either non-radioactive mitozolomide or AIC both produced a decrease in the labelling of the acid precipitatable material (Fig.37). The inhibition produced by AIC was of the same order of that produced by unlabelled mitozolomide . Determination of the initial time-course of mitozolomide uptake into cells when co-incubated with equimolar AIC produced no difference with respect to controls (Fig.38).

The production of AIC by mitozolomide was investigated using a hplc method developed by Slack et al (1983). A solution of mitozolomide in RPMI 1640 media pH 7.5 was maintained at 37^{o}C in the dark. Samples were taken at the appropriate imes , the protein precipitated with 5% acetic acid in acetonitrile, and, after centrifugation, a sample of the supernatant was separated by hplc. Radioactivity was observed to progress from a peak identifiable as mitozolomide to one which co-eluted with an AIC standard. The production of [^{14}C]-AIC from [^{14}C]-mitozolomide was quantitated by collection of the elutant and counting in a Beckman scintillation counter (Fig. 39).

FIG. 37 Inhibition of the uptake of [C^{14}-imidazo]-mitozolomide label by non-labelled mitozolomide and AIC.

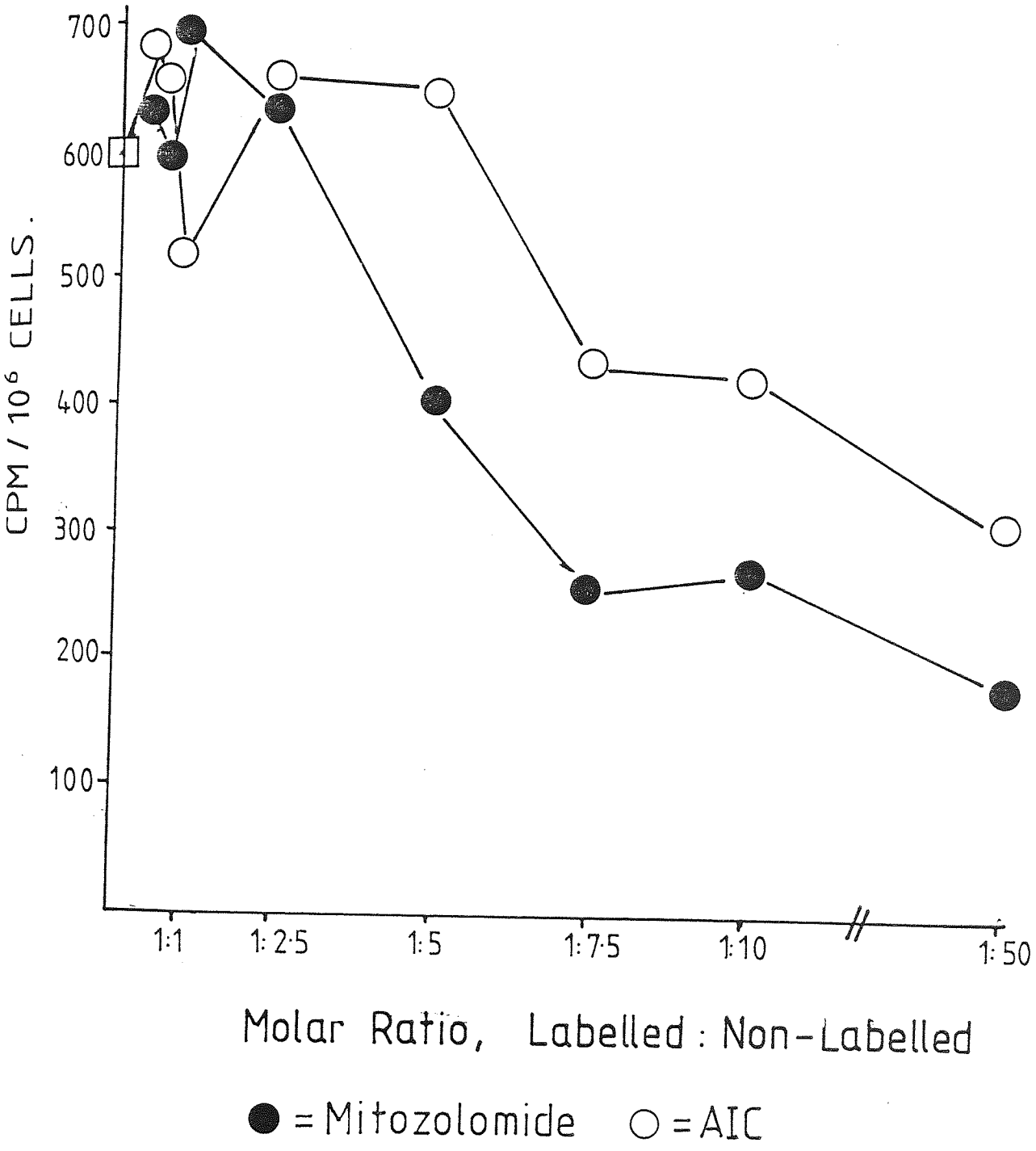
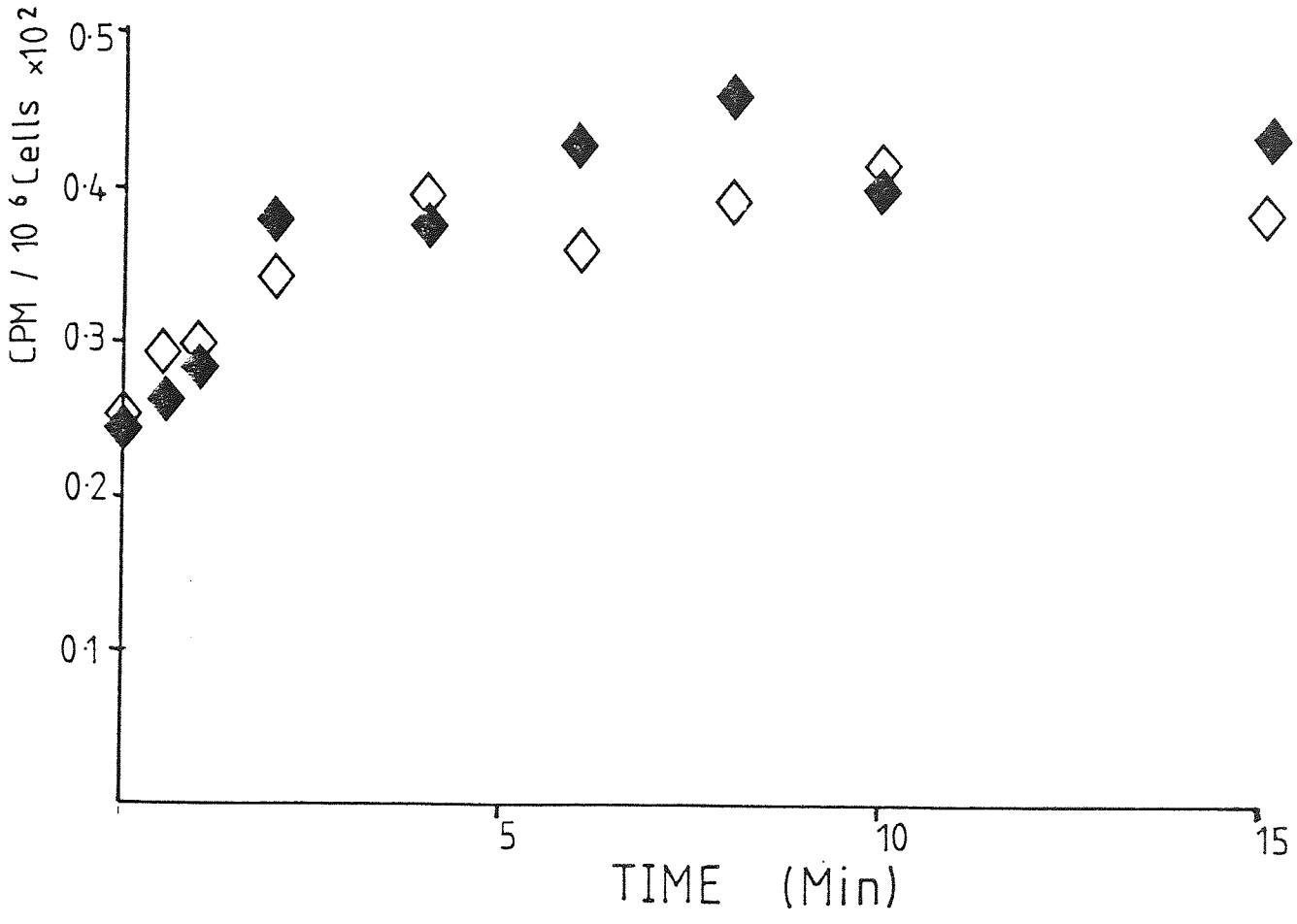


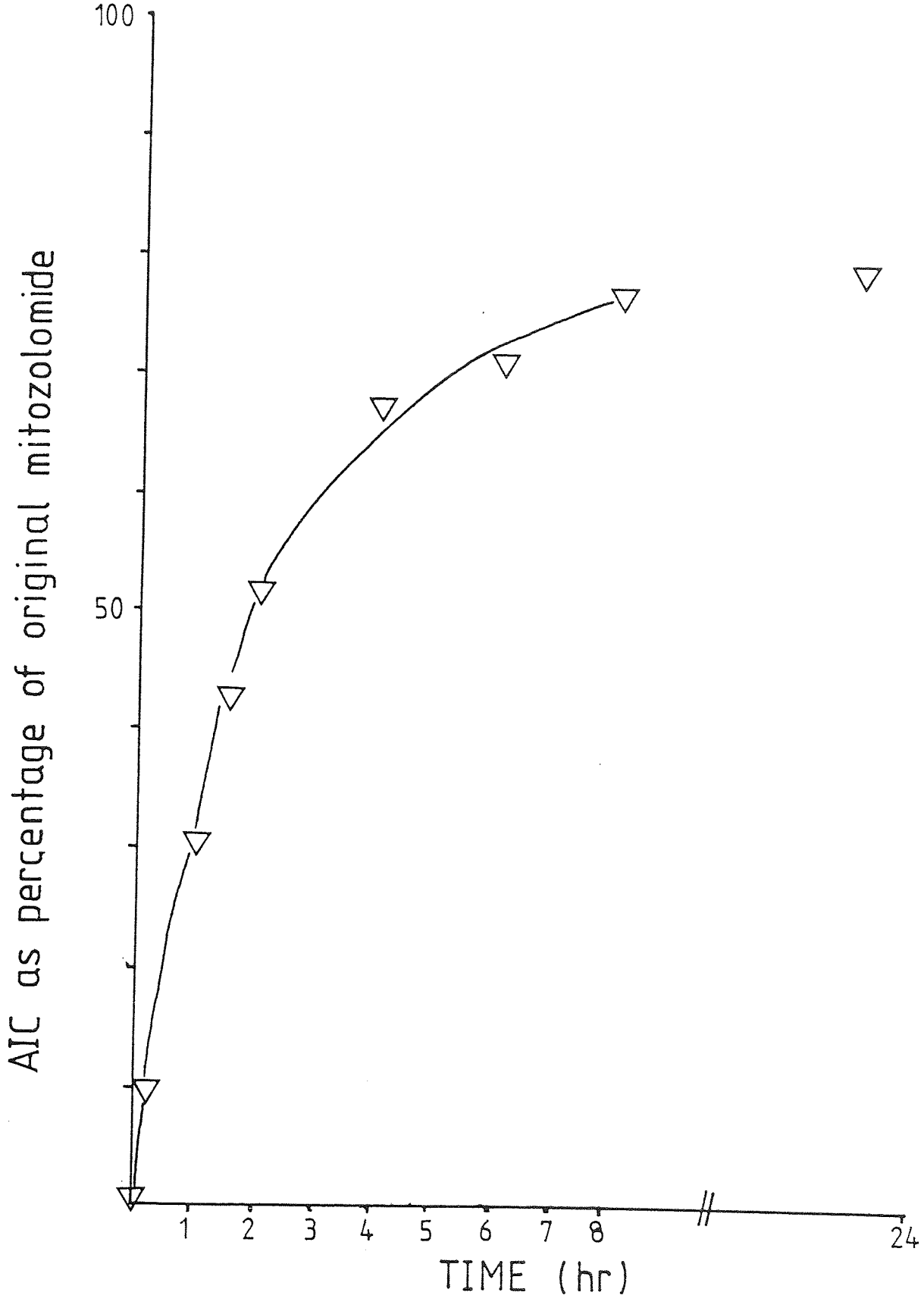
FIG. 38 Effect of AIC on the initial uptake of mitozolomide.



◆ = Control

◇ = AIC

FIG. 39 PRODUCTION OF AIC AS DETERMINED BY RADIOACTIVITY



D.5 Flow-cytometry

D.5.1. The effect of mitozolomide on the cell-cycling of 3LL cells *in vivo*

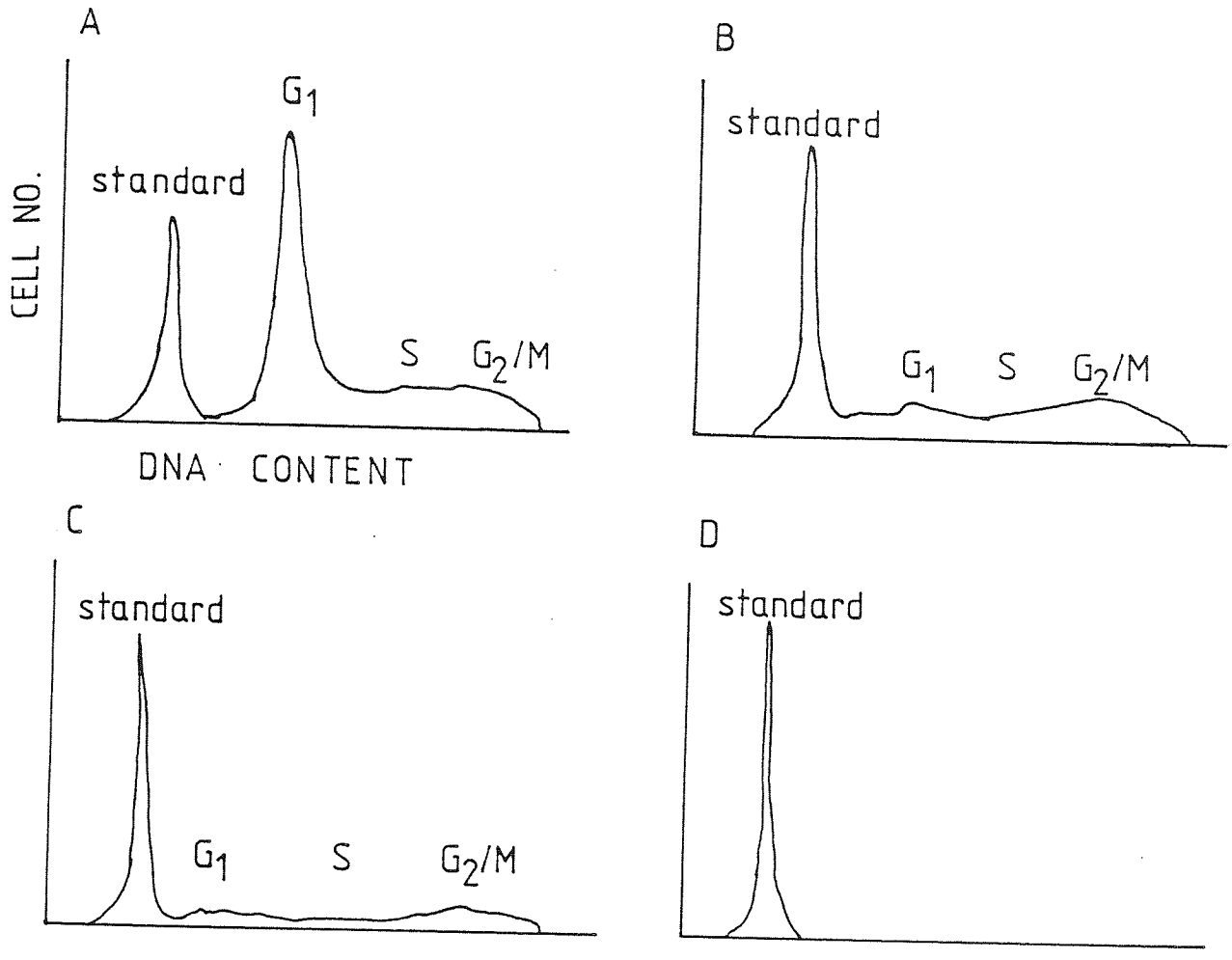
C57Bl/6 mice bearing 10 day old Lewis lung carcinoma (10^5 cells/0.1ml/mouse) i.m. were treated with mitozolomide (20mg/kg). Mice were sacrificed at 24, 48 and 96hr post treatment (4 mice/time-point) and the tumour mass removed. The vegetative part of the tumour was dissected out and prepared as a single cell suspension for propidium iodide staining and eventual processing by a flow-cytometer. The DNA histograms so obtained relate to the cell-cycle as previously explained (C.8).

In Fig 40 the potent antitumour effect of mitozolomide is very clearly illustrated. At 24 hr the G_1 population had disappeared and there was an increase in the G_2/M population. At 48hr those tumour cells still evident were predominantly in the G_2/M stage of the cell-cycle and by 96hr no tumour cells could be detected. These results are represented as percentages of the total cell population (Fig 41) which clearly demonstrates the G_2/M block at both 24 and especially 48hr.

The antitumour effect of mitozolomide illustrated in Figs 40 and 41 was confirmed by the autopsy results obtained from treated and control mice at 21 days post mitozolomide treatment (Table 8). All tumour parameters were significantly reduced in the treated animals compared to the

controls, the average tumour weight was 2.7g less in the treated animals, only 1 animal in 8 of the treated group had metastases and even then only one very small secondary tumour site was found (Ø.58g). Of the control animals 8/9 suffered multiple metastases of average weight 3.85 +/- 1.13g.

FIG. 40 DNA histograms obtained on treatment of Lewis Lung carcinoma *in vivo* with mitozolomide.



A = CONTROL
B = 24 hr post-mitozolomide treatment
C = 48 hr post-mitozolomide treatment
D = 96 hr post-mitozolomide treatment

Four mice were sacrificed at each time-point, the flow cytomatograms reproduced are typical examples of the results obtained. The control given was produced at 24hr; control mice were sacrificed at each time-point but no significant differences were observed.

Fig 41: Change in the cell-cycle distribution of Lewis lung carcinoma when treated with Mitozolomide *in vivo*

The data obtained from the flow cytomatograms as presented in Fig 40 is expressed as the percentage of the total number of cells in each cell cycle. The points are the average results obtained from four mice per time-point.

△ = Control

▲ = treated

A = G_1 cells

B = Early S stage

C = Late S stage

D = G_2/M cells

FIG. 41 Change in the cell-cycle distribution of Lewis Lung carcinoma when treated with mitozolomide in vivo

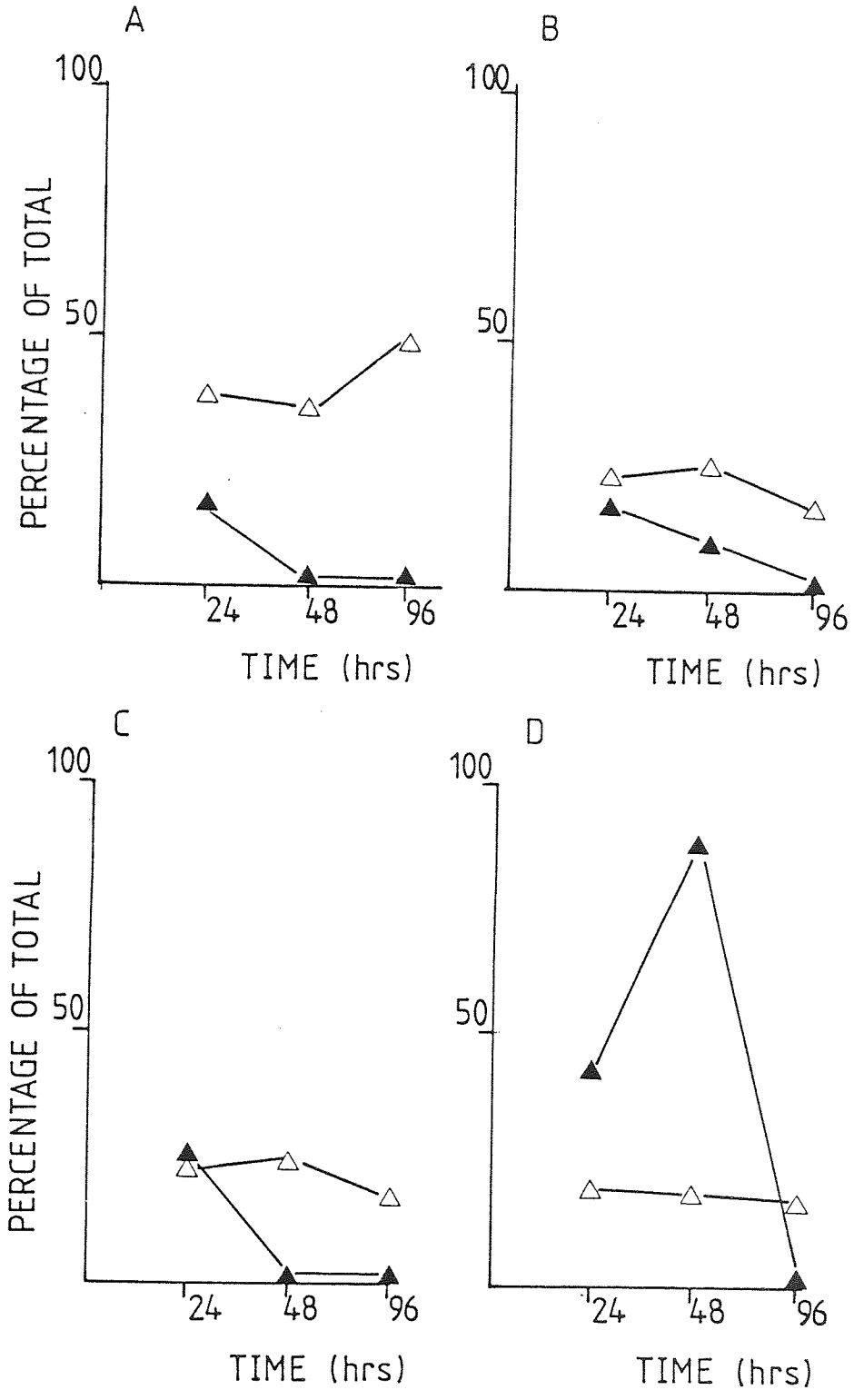


TABLE 8 Animal autopsy data for treatment of Lewis lung carcinoma *in vivo* with mitozolomide

	animals (g)	tumour (g)	animal with metastases	METASTASES number/ mouse	METASTASES weight (g)
CONTROL	23·8±0·5	7·29±0·3	8/9	8·0± 2	38·5±11·3
MITOZOLOMIDE	22·0±0·4	4·56±0·6	1/8	1	0·52

C_{57} 3l/6 mice bearing 21 days old 3LL (10^5 cells/0·1ml/mouse) i.m.
Mitozolomide 20 mg/kg i.p on day +10 after tumour transplantation

D.5.2. The effect of mitozolomide and MCTIC on the cell cycling of 3LL cells *in vitro*

In order to clearly define the cell-cycle perturbations produced by mitozolomide and to compare the effects with those of MCTIC a flow-cytometric examination of Lewis lung cells treated with mitozolomide and MCTIC *in vitro* was carried out. As the perturbations observed in DNA histograms are often concentration dependent (Hill et al 1981), 0.4, 4.0 and 40.0 μM drug concentrations were used. Cells were sampled by flow-cytometry after 8,12 and 24hr exposure to drug containing media and after drug removal by the replacement of media (24hr recovery time-point),(Fig.42).

No effect was seen until the 24hr time-point at which time the G_1 peak diminished and there was a clear enhancement of the G_2 peak in both MCTIC and mitozolomide treated cells (40 μM). After 24hr recovery the effect was accentuated, both sets of drug treated cells showed a loss of the G_1 population, enhancement of the late S population and a peak of G_2/M cells (40 M). No effect was recorded for the 0.4 M concentration throughout the experiment. The first effect produced by the 4.0 μM concentration was observed at the 24hr recovery time-point, a small G_2/M block being evident. Unlike the higher drug concentration of 40 μM the G_1 peak persisted

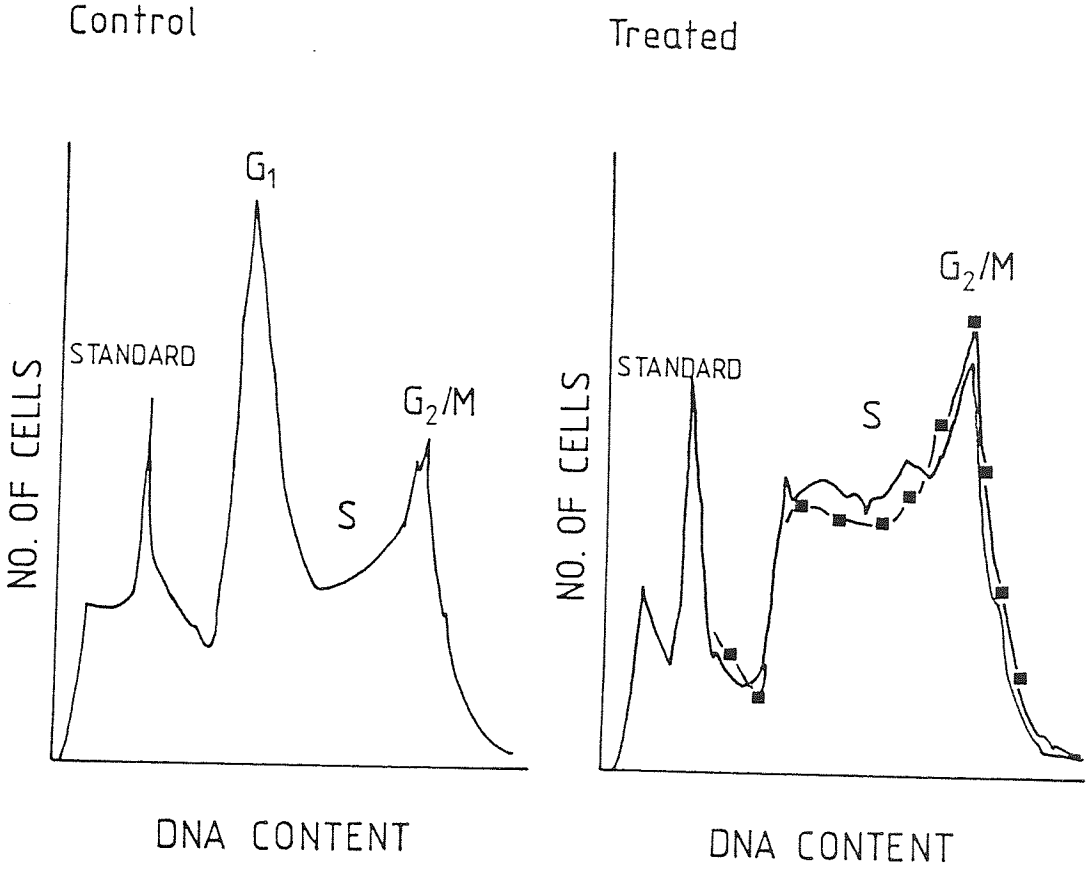
The cytotoxicity and effect on thymidine

incorporation of mitozolomide and MCTIC was determined for Lewis lung cells growing *in vitro* using two drug/cell exposure times of 1 and 24hr. For cytotoxicity experiments 3LL cells were taken from tumours growing *in vivo* , plated out and allowed to grow for 24hr, in order that logarithmic growth might be obtained. Drug solutions were added and the media resupplied after washing with sterile saline inorder to remove any remaining drug at 1 and 24hr. The cells were left for 2 days and counted (Fig 43a).

Logarythimically growing cultures of Lewis lung cells were prepared in an analogous fashion for the thymidine incorporation experiments as for those used for cytotoxicity evaluation. Drug containing media was removed at 1 and 24hr. At 24hr both sets of cells were incubated with radioactive thymidine for 1hr before the incorporated radioactivity was determined (Fig 43b). In both cytotoxicity and precursor incorporation experiments an increase of effect was observed for cells treated with mitozolomide for 24hr over those treated for only 1hr. Longer time exposures of MCTIC produced no significant difference in either cytotoxicity or precursor incorporation when compared to the 1hr exposure.

FIG. 42 Effect of mitozolomide and MCTIC on the in vitro cell-cycle progression of Lewis Lung carcinoma.

(I) 24 hr drug/cell incubation

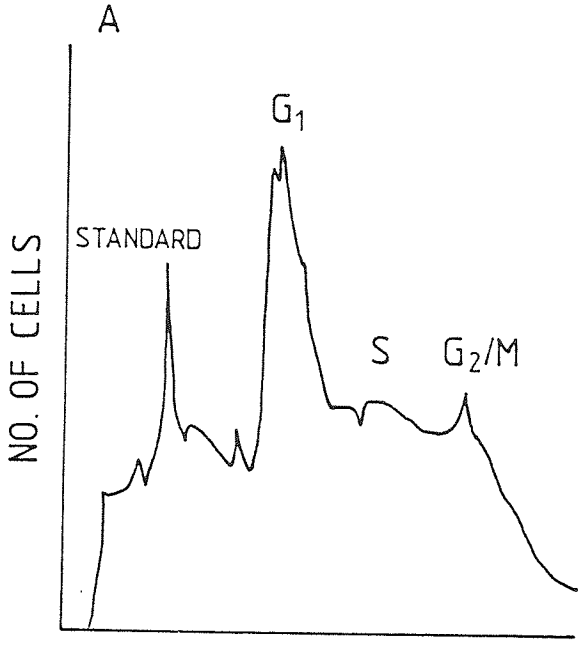


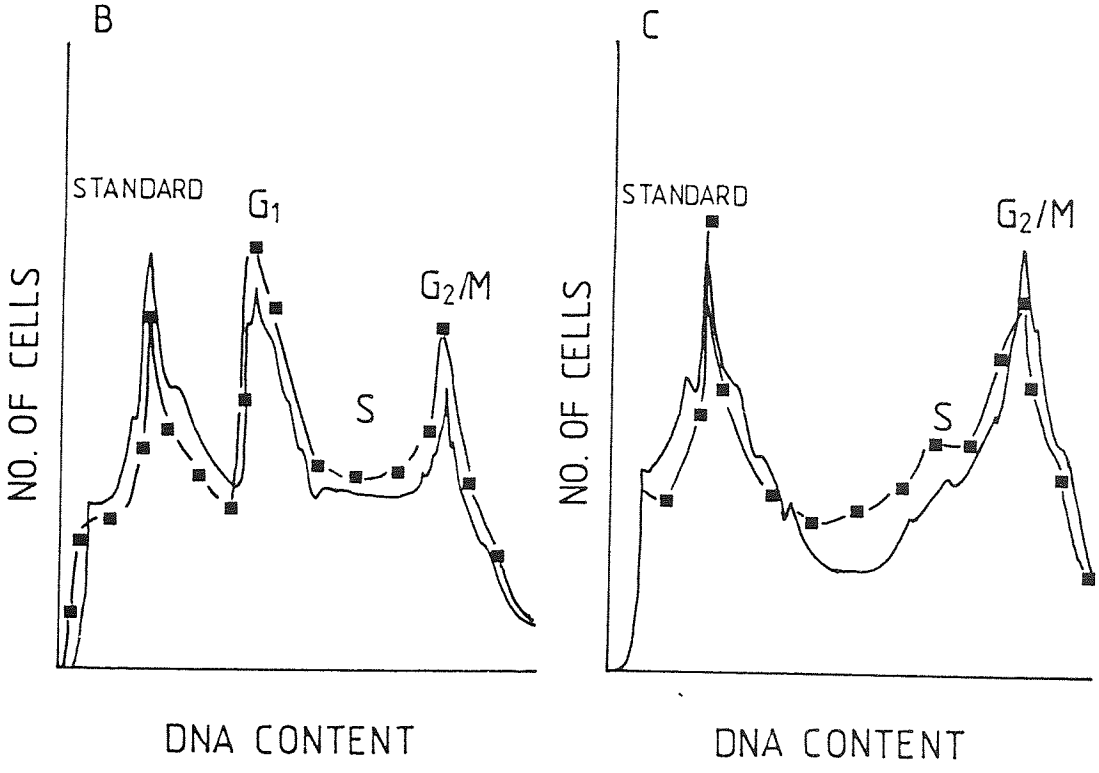
——— = 40 μM MCTIC

–■–■– = 40 μM Mitozolomide

FIG. 42 Continued

(II) 24 hr recovery

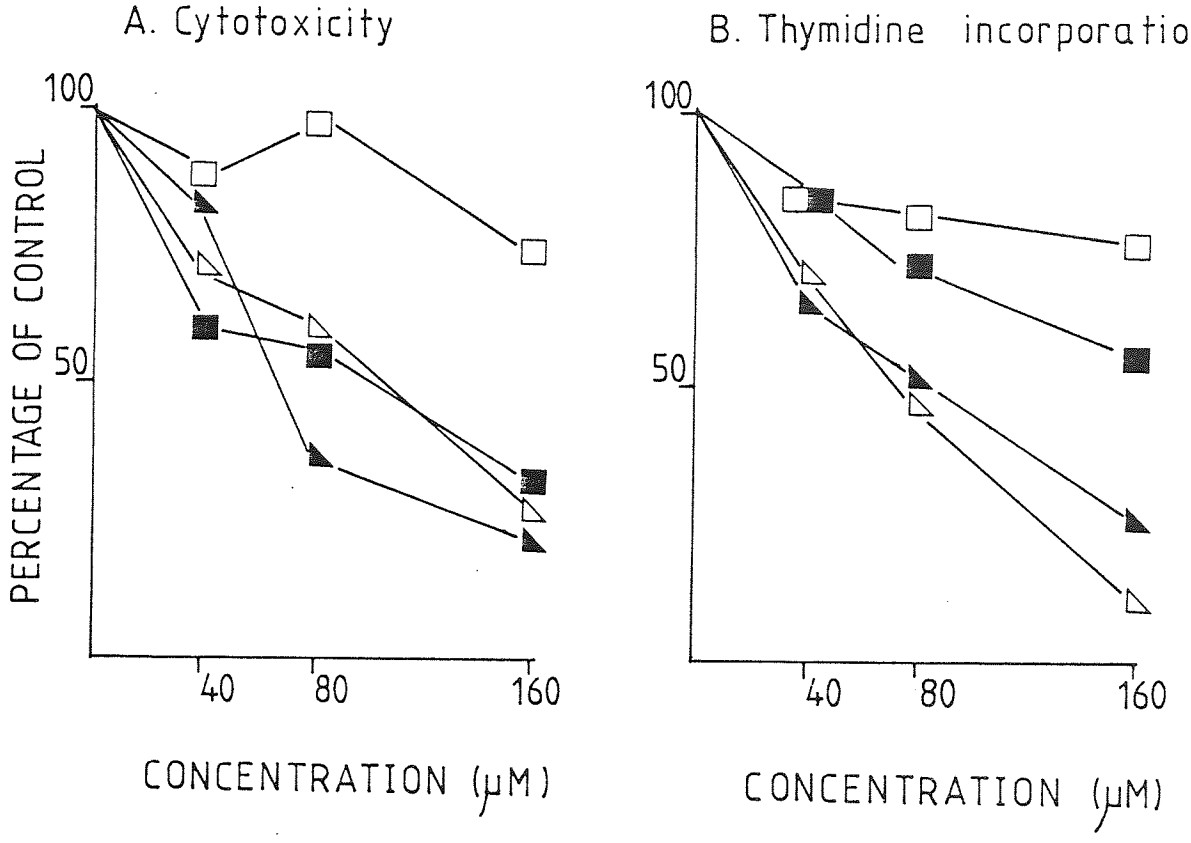




——— = MCTIC —■—■— = Mitozolomide

A = Control B = 4·0 μM of drug C = 40 μM of drug

FIG. 43 Effect of mitozolomide and MCTIC on cell growth and thymidine incorporation of *in vitro* Lewis Lung cells



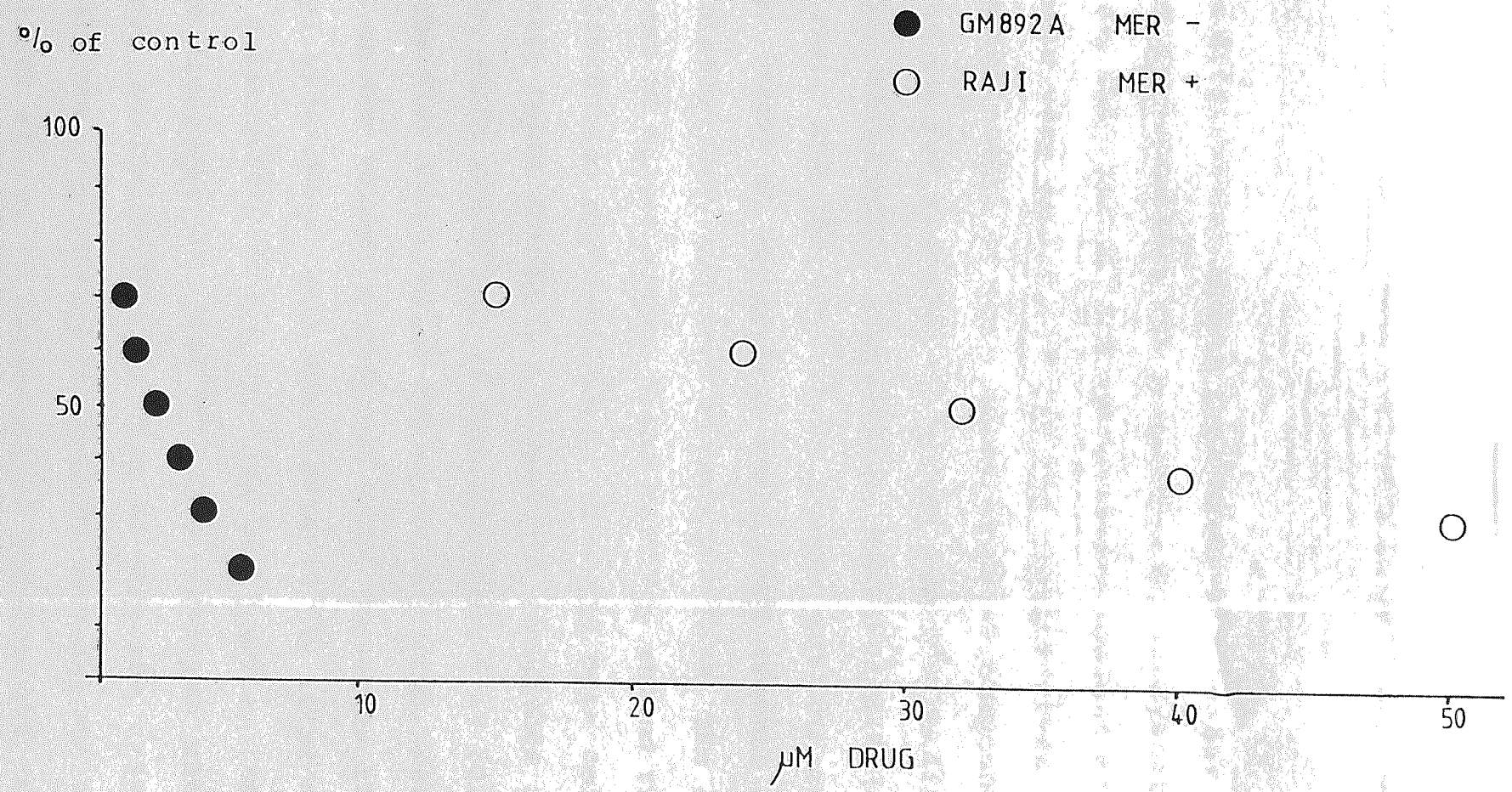
□ = Mitozolomide 1hr drug/cell exposure
■ = Mitozolomide 24hr drug/cell exposure
◺ = MCTIC 1hr drug/cell exposure
◣ = MCTIC 24hr drug/cell exposure

D.6. Investigation of the cytotoxicity of mitozolomide and analoques against mer- and mer+ cell-lines.

D.6.1. Differential growth inhibition of mitozolomide to mer+ and mer- cell-lines.

Mer+ and mer- cells ($\emptyset.5x1\emptyset^4$ cells/ml) were treated with various concentrations of mitozolomide and allowed to grow for 72hr at which time cell number was determined. Fig 44 illustrates the resistance of the Raji cell-line (mer+) to the toxic effects of mitozolomide when compared to the GMO892A. The mer- cell line is approximately ten times more sensitive to mitozolomide than the mer+ cell line.

FIG.44 GROWTH INHIBITION OF MER+ AND MER- CELLS BY MITOZOLOMIDE



D.6.2 Differential growth inhibition of selected imidazotetrazinones and control compounds to Mer+ and Mer- cell lines.

The differential growth inhibition of a number of analoques of mitozolomide, the 2-chloroethyl isocyanate, BCNU, mustine, chlorambucil and methotrexate against mer+ and mer- cells were determined and the results given in Table 9. The resistance of mer + cells to those compounds not believed to act via alkylation at the O^6-guanine position, ie. chloroethyl isocyanate, methotrexate (an antimetabolite), and the alkylating agents mustine and chlorambucil, (which do not produce appreciable amounts of O^6-quanine alkylation), was approximately 2-fold. The 3-methyl analogue of mitozolomide, methazolastone, showed the greatest differential cytotoxicity to mer+ and mer- cell lines. The mer+ cells displayed a comparable resistance to mitozolomide, BCNU, and MCTIC, of approximately 6-1Ø fold resistance. The five analogues of mitozolomide tested, were all modified on the imidazole ring and still possessed the 2-chloroethyl side-chain. Only one displayed any level of differential cytotoxicity to the mer+ and mer - cells. that is the ester (analogue 3). This drug was inactive when tested against murine models *in vivo* as was the cyano analogue, while the sulphonamide derivatives were all active *in vivo* (A.8) but showed no differential cytotoxicity to mer- cell lines *in vitro*.

TABLE 9 Effect of various cytotoxics on the growth of Mer + and Mer - cells

	GROWTH INHIBITION						
	MER+			MER-			
	$ID_{50}\mu M$	n	SD	$ID_{50}\mu M$	n	SD	x R
METHAZOLASTONE	140	3	20	10	3	7	14
MITOZOLOMIDE	20	5	7	2	3	0·5	10
MCTIC	26	5	12	3	2	0·5	10
BCNU	17	3	3	3	4	0·5	6
2-CHLOROETHYL ISOCYANATE	77	3	30	50	2	15	1·5
ANALOGUE 2	90	2	10	59	2	8	1·5
ANALOGUE 3	29	4	2	5	4	1	5
ANALOGUE 4	18	4	5	7	3	1	3
ANALOGUE 5	20	5	5	12	2	1·5	2·5
ANALOGUE 6	16	4	4	7	2	0·1	2
NITROGEN MUSTARD	0·2	2	0·5	0·1	3	0·04	2
CHLORAMBUCIL	1	2	0·5	0·6	2	0·3	2
METHOTREXATE	13	1		10	2	0·6	1

FOR THE STRUCTURES OF MITOZOLOMIDE ANALOGUES SEE TABLE 5

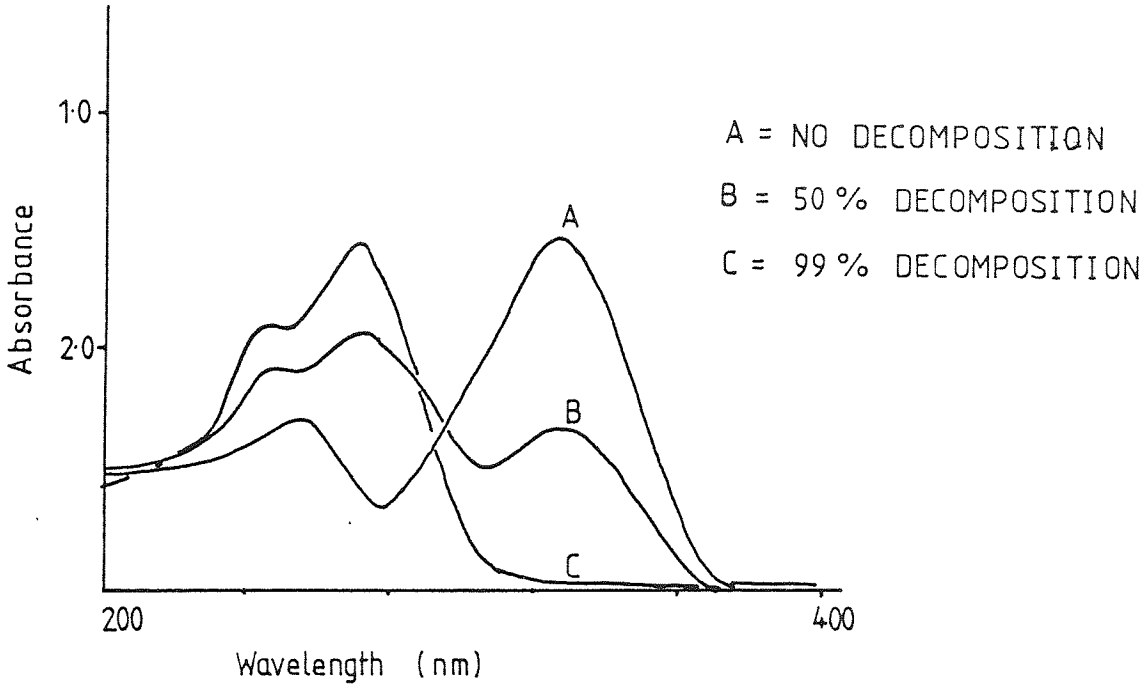
$xR = Mer+\ ID_{50} / Mer-\ ID_{50}$

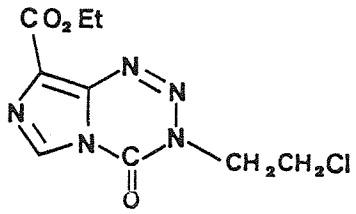
D.7 Determination of the half-lives of Mitozolomide, MCTIC and selected analoques of mitozolomide.

Chemical half-lives were determined in order to investigate whether any correlation existed between this parameter and anti-tumour activity. Half-lives were measured using a Beckman Du-7 spectrophotometer which monitored the absorbance at the λ-max of the compound under observation and maintained the temperature constant at 37°C. A series of spectra obtained using analogue 3 at Ø, 5Ø, and 99% decomposition are presented in Fig 45. The plot of log. concentration versus time for analogue 3 is also given, (Fig 46). Such plots were obtained for all the compounds under investigation and the half-lives and rate constants of decomposition (K) subsequently calculated, (Table 1Ø).

The decomposition of mitozolomide and its analogues showed a marked dependence on pH, all the compounds being more stable in acid rather than alkaline conditions, (Table 1Ø), Fig 47. Analoque 6 showed the same trend, while MCTIC was most stable in the pH range 7.Ø-7.5.

FIG. 45 Breakdown of analogue 3 in phosphate buffer, pH 7·5 and 37°C





ANALOGUE 3

FIG. 46 Breakdown of analogue 3 in phosphate buffer:-

Log. concentration v Time

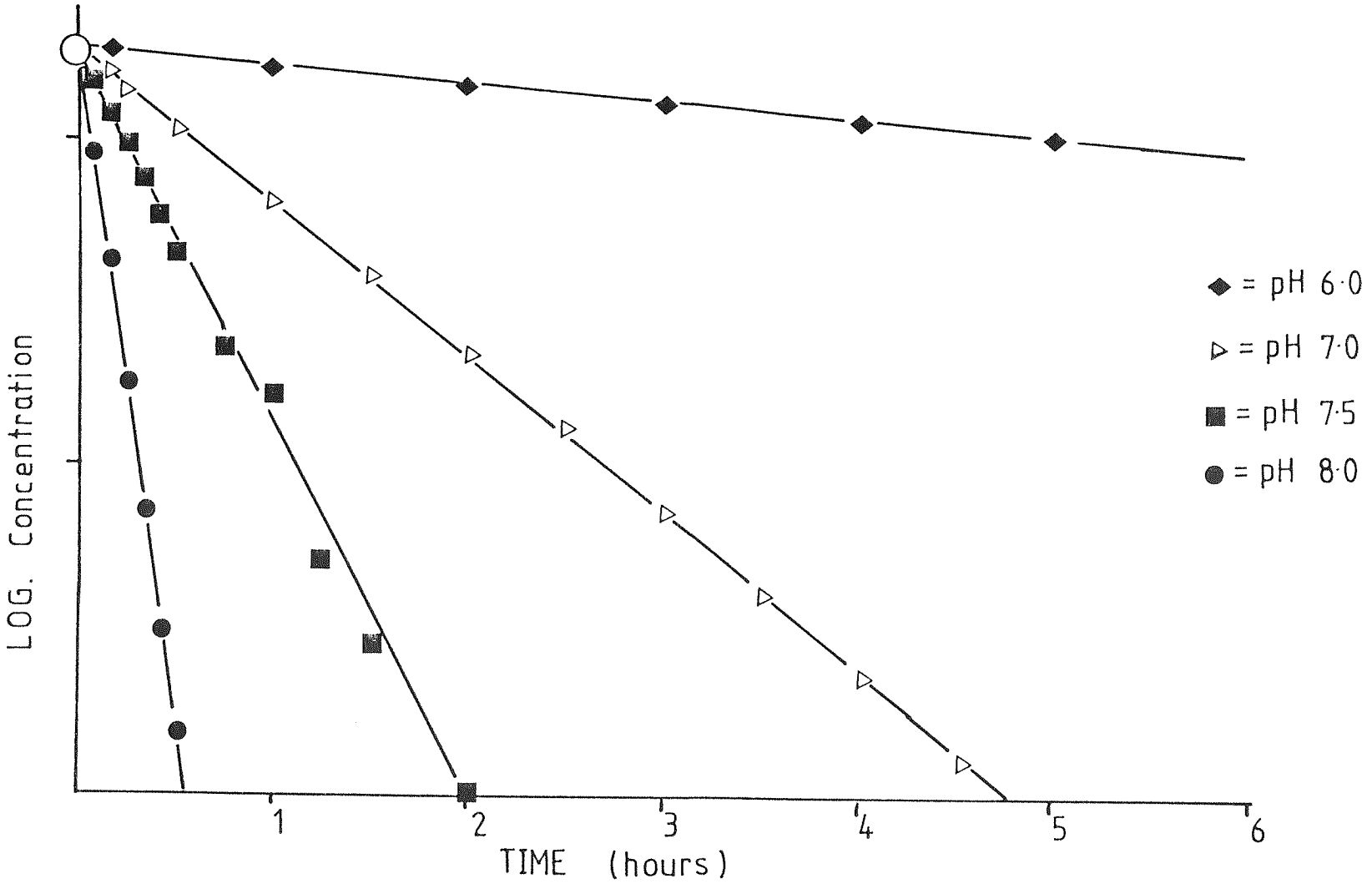


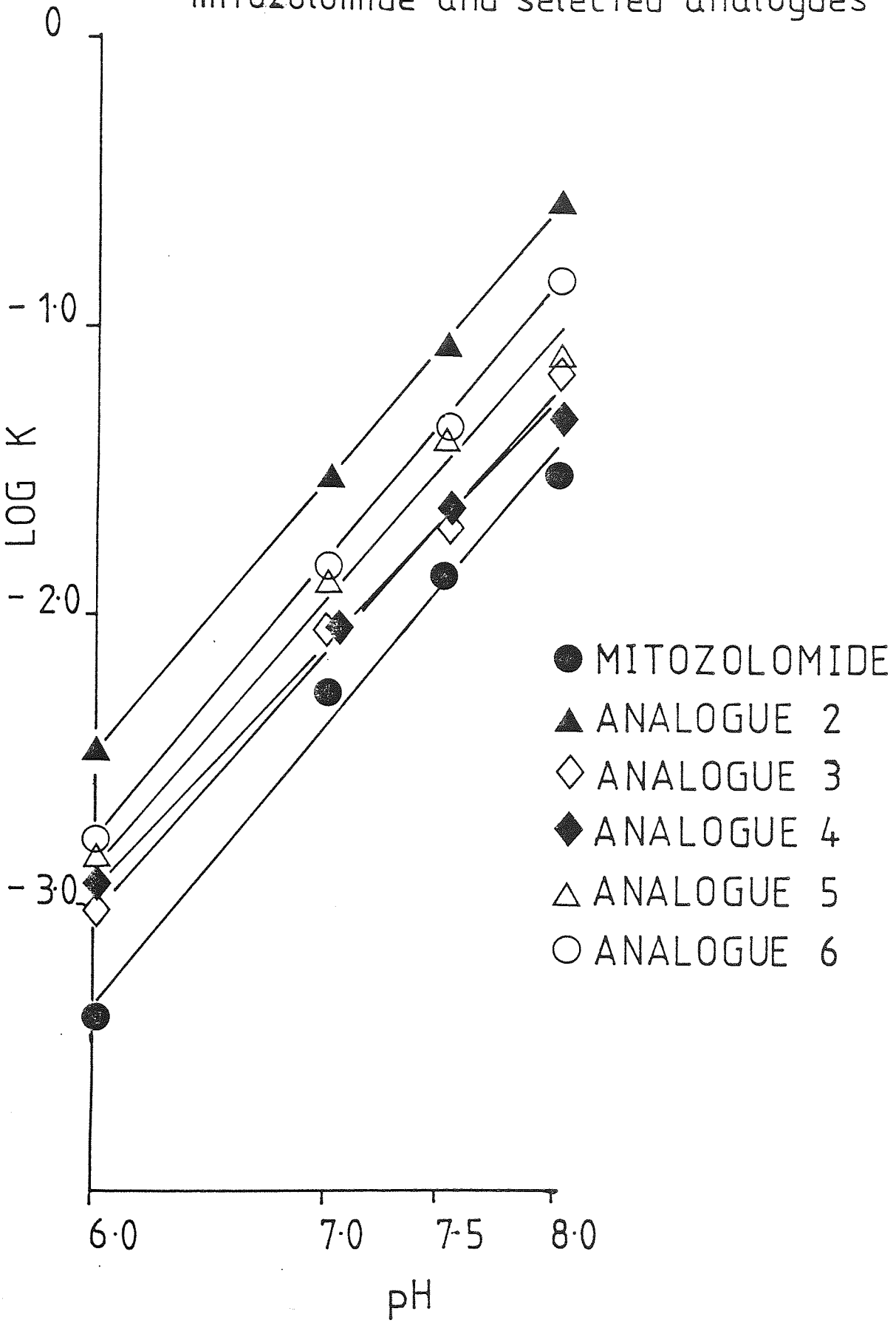
TABLE 10 Half-lives of mitozolomide and selected analogues in phosphate buffer at 37°C

	pH 6·0			pH 7·0			pH 7·5			pH 8·0		
	$t_{1/2}$ min	S.E.M	K min^{-1}	$t_{1/2}$ min	S.E.M	K min^{-1}	$t_{1/2}$ min	S.E.M	K min^{-1}	$t_{1/2}$ min	S.E.M	K min^{-1}
MITOZOLOMIDE	1531	37	0·00045	125	17·8	0·0055	54	5·0	0·0128	22	6·0	0·0315
ANALOGUE 2	209	3·0	0·0033	23	4·0	0·03	8	0·6	0·0866	2·5	0·5	0·2772
ANALOGUE 3	736	27	0·00094	74	2·0	0·0094	31	0·5	0·022	9·6	0·5	0·072
ANALOGUE 4‡	537	–	0·0012	76	–	0·0091	36	–	0·0193	16	–	0·043
ANALOGUE 5	452	20	0·0015	54	8·0	0·0128	19	1	0·0036	9	0·6	0·077
ANALOGUE 6*	426	11	0·0016	47	6·0	0·0147	16	1·5	0·043	10	1	0·0693
MCTIC	1·5	–	0·462	5·5	–	0·126	5·5	–	0·126	4·5	–	0·154

Values obtained from at least 3 experiments, except :- * only 2 experiments

‡ only 1 experiments

FIG. 47 Plot of Log K versus pH for mitozolomide and selected analogues



E. DISCUSSION

E.DISCUSSION:

Mitozolomide represents a new group of cytotoxic agents with high potency against a range of experimental tumours. This study attempted to determine the chemical species responsible for cytotoxicity and if possible to discover the mechanism by which antitumour effect is exerted.

As discussed in section A.5 the candidates for the role of antitumour species are 2-chloroethyl isocyanate and Diazo-IC produced by one pathway of decomposition, and MCTIC by an alternative pathway. Mitozolomide possesses a novel chemical structure, the imidazotetrazinone ring system, and therefore the possibility also existed that this unique molecule could exert its cytotoxic effect per se, without resort to the previously mentioned metabonates. It was envisaged that mitozolomide might inhibit specific enzymes crucial to the function of a tumour cell. A combination of any of the above hypothesis and as yet undiscovered mechanism of action might also eventually be found to explain the actions of mitozolomide.

Preliminary stability studies had shown a rapid breakdown of mitozolomide in aqueous conditions at pH 7.4 ($t^1/2$ 98 mins) (Stevens et al 1984). The time-course of mitozolomide toxicity to K562 cells *in vitro* was investigated (Fig 22) and when compared to a decomposition curve of mitozolomide obtained under the same conditions (Fig 23), it was found that the expressed cytotoxicity was dependant on the total amount of drug that had decomposed.

Presumably an active species is generated on decomposition which binds irreversible to a cellular receptor, since cytotoxicity is still expressed on drug removal. Over short time periods the decomposition of high concentrations of mitozolomide will generate large quantities of active species, the production of equivalent amounts of cytotoxic species by low concentrations will take longer. The time-course of toxicity is consequently longer for low initial concentrations of mitozolomide than for high concentrations.

The highest concentration of mitozolomide used (125.0 μM) produced approximately 60% inhibition of cell growth within 5 mins, the reaction of the cytotoxic species appears therefore to occur extremely rapidly. The rate limiting step of the initial cytotoxic event is the generation of the reactive moiety.

The experiments of Gibson (1982) suggested that the antitumour activity of mitozolomide to tumours *in vivo* did not require metabolism. Consideration of these early experiments also led to the conclusion that cytotoxicity was exerted by a metabonate. Therefore initial attention was focussed on the identity of the reactive moiety/moieties produced by mitozolomide and particularly as to which of these species had biologically important effects.

Many reactive agents exert their effects through the production of cytotoxic lesions in DNA and RNA. A measure of the gross damage to DNA caused by such agents may be obtained from precursor incorporation experiments, for

example the uptake of radioactive thymidine into DNA nd uridine into RNA.

The results of precursor incorporation experiments are particularly susceptible to the influence of drug effects unrelated to the direct damage of nucleic acids. Any change in the radioactive pool size of the precursor can lead to distortions in the results obtained from precursor incorporation experiments (Bianchi et al. 1983, Russell and Partick 1980). When examined BCNU and mitozolomide were found to have no early effect on the total pool size of thymidine and its metabolites (Fig 27). The first significant effects were not produced until the 24hr time-point. Therefore results obtained from precursor incorporation experiments ,up to this time, were not considered to be merely reflections of changes in radioactive pool sizes.

The effects of mitozolomide, BCNU, MCTIC and 2-chloroethyl isocyanate on thymidine and uridine incorporation into DNA and RNA respectively, over a 24hr time-period were investigated (Fig 24 and 25).

A number of conclusions may be drawn from these experiments (Fig 24 and 25). Firstly the isocyanate does not effect precursor incorporation into DNA and RNA, even at the 24hr time-point. Isocyanates are known to react at a number of sites concerned with DNA structure and function, therefore an effect might be expected. Particularly isocyanates may carbamoylate histone proteins (Woolley et al. 1976, Pinskey et al. 1979 and Sudhaker et al. 1979.).

Although most work in this field relates to methyl isocyanate which is derived from methyl nitrosourea (MNU) it is reasonable to assume that 2-chloroethyl isocyanate would produce similar carbamoylation reactions. It is possible that 2-chloroethyl isocyanate on carbamoylation of histone proteins could subsequently chloroethylate DNA thus producing a DNA-protein cross-link.

The inhibition of enzymes involved in nucleic acid synthesis has also been reported. DNA nucleotidyltranferase was found to be inhibited by BCNU and 2-chloroethyl isocyanate in cell free systems, although it is notable that in L1210 cells inhibition of DNA synthesis by BCNU could occur without inhibition of DNA nucleotidyltranferase *in vivo* (Wheeler and Bowdon 1968). Baril et al. (1975) have reported the inhibition of DNA polymerase II by alkylisocyanates produced by BCNU and CCNU. A cell free system was used to demonstrate this inhibition and high concentrations of agent were used (1mM). Such levels of isocyanate are unlikely to be qenerated in pharmacologically realistic conditions (Hilton et al. 1978).

Despite the ability of the isocyanates to react with numerous sites associated with nucleic acids, Fornace et al. (1978) found that chloroethyl isocyanate did not inhibit the U.V stimulated incorporation of thymidine into normal human fibroblasts. On consideration of this evidence the absence of any direct effect on precursor incorporation should not have been totally unexpected. Although using this

technique there appears to be no deleterious effects on DNA or RNA function, the reactions of the isocyanate may cause disruptions in the ability of cells to repair DNA damage, this aspect of isocyanate toxicity will be discussed later.

BCNU causes rapid and marked inhibition of thymidine and uridine incorporation into acid insoluble material, 10 μM producing a 70% reduction in the incorporation of thymidine at 1hr and a 40% reduction in uridine incorporation. As 2-chloroethyl isocyanate when supplied directly has no effect on precursor incorporation it is possible to assume that the inhibition produced by BCNU is predominantly due to alkylation damage. A caveat to this supposition is that the biological effects produced by supply of 2-chloroethyl isocyanate directly may not be equivalent to those produced when the isocyanate is generated intracellularly by BCNU. However as equivalent concentrations to those which failed to produce any effect on precursor incorporation did inhibit cellular enzymes (see later), in the case of BCNU, the incipient isocyanate does not appear to contribute directly to the observed depression of precursor incorporation.

Mitozolomide and MCTIC do not display similar rapid inhibitory effects to those produced by BCNU; inhibition of thymidine incorporation becomes significant only after 8hr whereas BCNU produces inhibition of both parameters within 1hr. The inhibition of uridine incorporation produced by BCNU, MCTIC and mitozolomide is less in all cases than that of thymidine. The results acheived by following adenosine

incorporation into DNA and RNA after BCNU and mitozolomide treatment are comparable to those obtained when DNA and RNA synthesis were examined separately using thymidine and uridine (Fig 26).

Mitozolomide showed most similarity of effect to that of MCTIC. It is accepted that MCTIC, a chlorodiazo species alkylates DNA. If mitozolomide does generate MCTIC, as previously suggested, then the contrast of effect of MCTIC and mitozolomide to that of BCNU, another chlorodiazo producing agent requires explaination. There are a number of plausible theories.

The heightened effect of BCNU might be due to the inhibition of DNA repair caused by isocyanate production. The inhibition of the repair of single strand breaks produced in DNA by ionizing radiation is a characteristic of strongly carbamoylating nitrosoureas, and has been the subject of interest for some time (Kann et al. 1974. 1980a and b). Specifically isocyanates have been shown to inhibit the ligase step of excision repair (Fornace et al. 1978). Fornace et al. (1978) suggested that this effect could amplify cytotoxicity, since an excision type repair mechanism is envolved in the repair of DNA damage caused by alkylation. Failure of the ligase step of this repair process would generate single strand breaks. Sariban et al. (1984) whose results also showed that carbamoylation may inhibit nucleotide excision repair supported the hypothesis that such an effect may increase the cytotoxicity of

alkylating agents which also produce isocyanates.

Heal et al.(1979a) investigated the effect of carbamoylation on the repair of nitrosourea induced DNA alkylation damage in L1210 cells and although they found no actual increase in cytotoxicity a delay in single-strand break repair was observed. Such an effect could possibly accentuate any initial alkylation damage to DNA produced by BCNU and be reflected as a further decrease in precursor incorporation.

Production of MCTIC by mitozolomide leads to the generation of AIC. AIC has been shown to stimulate [^{14}C]-guanine incorporation into the polynucleotides of sarcomas and adenocarcinomas in rodents. Beal (1976) working with Novikoff hepatoma cells in culture noted that both DNA and RNA synthesis were enhanced by the addition of AIC. Co-production of AIC and an agent capable of alkylating DNA, due to the stimulatory effect of AIC, might cause an apparent decrease in DNA damage as evaluated by precursor incorporation. Hence a proportion of the damage to nucleic acids produced by mitozolomide and MCTIC might be masked when measured using precursor incorporation.

Generation of the chloroethyldiazo species by different parent molecules might also influence the position at which it reacts with DNA. Indeed in a series of nitrosoureas, Tong et al. (1982a) found variation in the resulting alkylation adducts and suggested that this phenomenom might be responsible for the observed differences in toxicity and antitumour effect. It is possible that MCTIC

and mitozolomide produce more selective damage to DNA, so enabling the same level of cytotoxicity as BCNU, but as there is less non-specific damage to nucleic acids, accounting for the observed contrast in precursor incorporation.

The different rate at which precursor inhibition is manifested cannot be explained by differences in half-lives. BCNU which produces its effect most rapidly has a half-life of 60min (Hilton et al. 1978) and mitozolomide 98 min (Stevens et al. 1984), all values relating to aqueous conditions. Such differences in rate of effect might again be due to the differences in the spectrum of DNA and possible RNA adducts produced and hence differences in the type of damage caused.

In conclusion the precursor incorporation experiments showed a similarity between MCTIC and mitozolomide and that these agents did not produce inhibition to the same extent as BCNU.

The production of chloroethyl isocyanate and Diazo-IC, not addressed directly by the experiments discussed above, was next investigated. As mentioned previously isocyanates are capable of carbamoylation, readily reacting with biological groups ie. SH, NH_2 and OH (A.5.1.1). The ability of a compound to carbamoylate lysine *in vitro* is often used as a measure of carbamoylation capacity (Wheeler et al. 1975). The relevance of this method of measurement has been criticised by Weinkam and Lin (1982) who believed that the extent of lysine carbamoylation was often a

function of the half life of the parent compound.

There are a number of experimental observations which do shed doubt on the usefulness of the test for carbamoylation based on lysine. It has been shown that the inactivation of α-chymotrypsin by cyclohexyl isocyanate was not affected by the addition of a 70-fold excess of lysine, and ACNU [1-(4-amino-2-methylpyrinidine-5-yl)-methyl-3-(2-chloroethyl)-3-nitrosourea] which was classified as a " non-carbamoylator" had certain biological effects suggestive of carbamoylation (Tew and Wang 1982). In a similar manner to ACNU, BCNU possesses a greater carbamoylation activity in biological systems than expected (Sariban et al. 1984). Sariban et al. (1984) reported that BCNU behaves in vitro as if its carbamoylation capacity were equivalent to 1-(2-chloroethyl)-3-(cyclohexyl)-1-nitrosourea (CCNU), although its carbamoylation ability when determined using lysine was 3 times less than that of CCNU.

Reservations concerning the lysine based carbamoylation assay lead to the use of a test which exploited the ability of the isocyanates to produce site-specific inhibition of a number of enzymes. In particular isocyanates are known to inhibit glutathione reductase (Babson and Reed 1978), α-chymotrypsin (Babson et al. 1977) and γ-glutamyl transpeptidase (Laki et al. 1978). Glutathione reductase is readily measured in cells and therefore in vitro experiments as well as direct/drug enzyme incubations could be preformed. Use of three different enzymes (glutathione reductase, α-chymotrypsin, and γ-glytamyl tran-

speptidase) should also eliminate any effects due to different reaction sites in individual enzymes. A test for carbamoylation based on the inactivation of these enzymes was considered to provide a more sensitive and biologically relevant test than the carbamoylation of lysine.

When investigated mitozolomide was found to be devoid of all carbamoylating activity, producing no measurable inactivation of glutathione reductase, Fig 28 and 29. The different effects of mitozolomide from those of BCNU and 2-chloroethyl isocyanate are illustrated in Fig 28. After a 24hr incubation of TLX5 cells with BCNU or 2-chloroethyl isocyanate there was no measurable glutathione reductase activity while mitozolomide produced no change from the controls. The time-course of inhibition of glutathione reductase by BCNU is very rapid, marked inhibition being produced in 1Ø mins (Fig 29). Again no effect was observed on mitozolomide addition. Chymotyrpsin and γ-glutamyl transpeptidase were also inhibited by BCNU and not by mitozolomide (Fig 31 and 32).

Mitozolomide did not therefore appear to generate an isocyanate in a biologically important manner. This conclusion was supported by an alkaline elution study on the type of DNA damage caused by mitozolomide and 2-chloroethyl isocyanate. The isocyanate was shown to produce many single-strand breaks in DNA, whereas mitozolomide did not (Gibson 1982). The lack of carbamoylating ability indicated that mitozolomide did not undergo a retro-cycloaddition reaction

in biological systems as suggested in A.5.

Reference has been made to cross-resistance studies performed with mitozolomide during its screening period (A.8). Resistance may be due to differential uptake of a drug as occurs in some cases of resistance to the mustards, whether this was also the cause of resistance to mitozolomide was examined. The uptake of radiolabelled mitozolomide into TLX5S and TLX5RT mouse ascities cells was measured. No significant difference in the equilibrium levels of [^{14}C]-imidazo mitozolomide were observed between sensitive and resistant cells (Fig 32). Further experiments using the imidazo labelled drug showed that the uptake was unaffected by metabolhc inhibitors (Table 7). The uptake kinetics were also non-saturable and the cell-medium ratio approximately 1 (Fig 31). From these results it was cnncluded that resistance was not due to differential uptake in the TLX5 system and that mitozolomide entered cells by simple passive diffusion. It may also be noted (Fig 32) that the drug uptake was very rapid, equilibrium at 37^{o}C being attained within lmin.

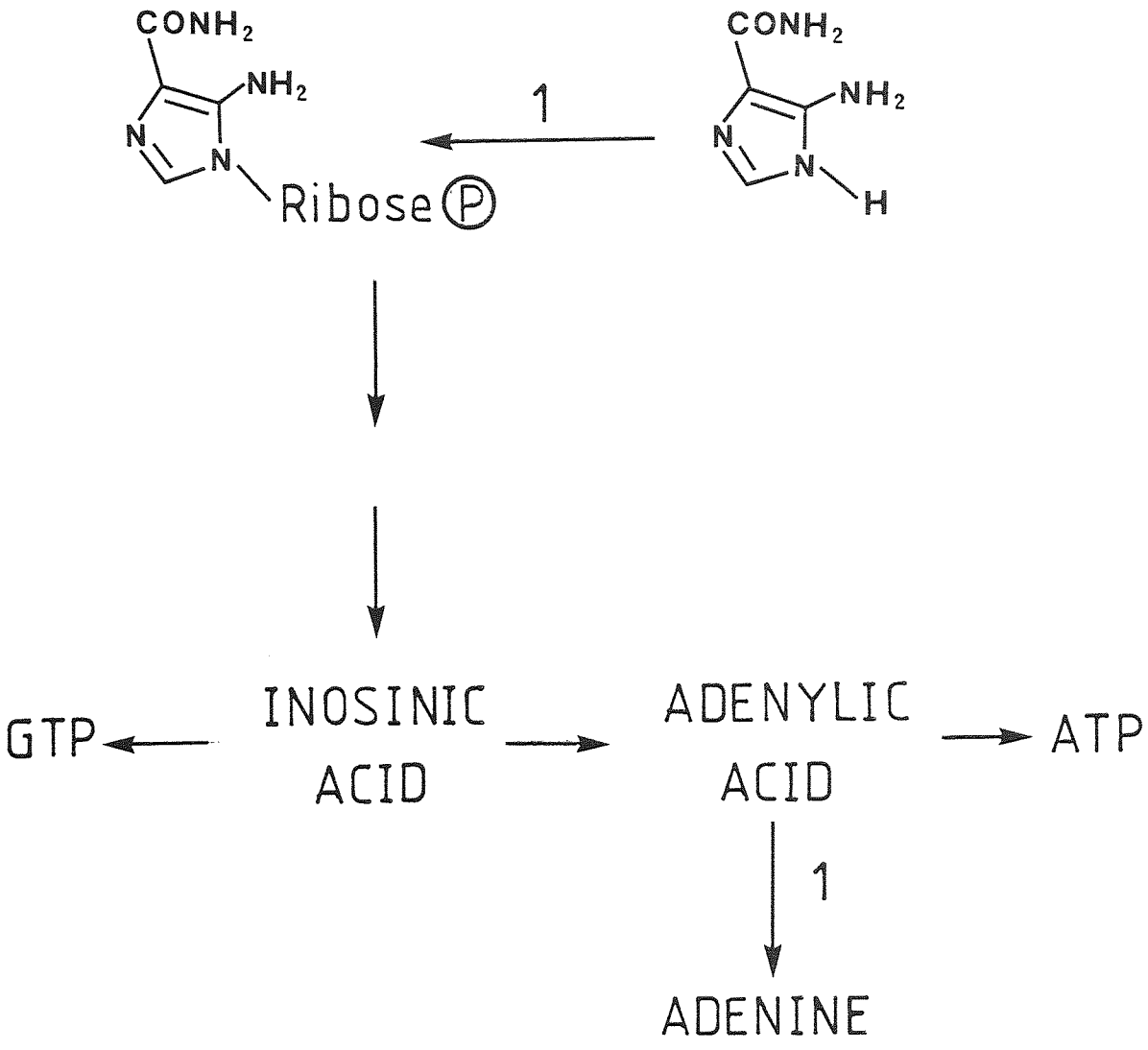
The fate of the imidazo label once inside the cell was also investigated. Acid precipitation of nucleic acids from [^{14}C]-imidazo mitozolomide treated TLX5 mouse ascites cells showed that radioactivity was associated with nucleic acids. Such labelling could be due to the 2-chloroethylation of DNA by mitozolomide without resulting decomposition. This hypothesis was considered highly unlikely, however, and it was believed that the labelling of nucleic acids was due to

the decomposition of mitozolomide to AIC via MCTIC. Salvage of AIC and its use in de novo purine biosynthesis would account for the subsequent labelling of nucleic acids (Fig. 48). Although this sequence of events would be non-toxic if proven to occur it would indicate that mitozolomide did qenerate MCTIC as previously considered (A.5.2.1), (Fig. 16).

On acid hydrolysis of labelled nucleic acids and subsequent separation using hplc, radioactivity wholly resides in the adenine and guanine bases of DNA and RNA (Fig 36). Exongenous AIC inhibited the incorporation of [^{14}C]-mitozolomide into nucleic acids (Fig 37) without interfering with the initial uptake of the drug (Fig 38). These results supported the suggestion that [^{14}C]-AIC was the vehicle by which the [^{14}C]-imidazo label of mitozolomide eventually reached DNA and RNA.

The incorporation of [^{14}C]-AIC per se has been previously demonstrated (Carlo and Mandel 1953, Conzelman et al. 1953) . The appearance of radioactivity in the RNA and DNA of cells on treatment with [^{14}C]-DTIC in the dark was rationalised by the suggestion of AIC generation from DTIC and its subsequent salvage for use in de novo purine synthesis (Gerulath and Loo 1972).

FIG. 48 Pathway of AIC salvage.



Enzyme 1 = Adenine phosphoribosyltransferase

Studies on the decomposition of mitozolomide using a hplc method developed at the University of Aston in Birmingham (Slack et al. 1983) proved that AIC was generated on mitozolomide decomposition. Examination of the decomposition of [^{14}C]- imidazo-mitozolomide confirmed the production of [^{14}C]-AIC (Fig 39). Generation of MCTIC by mitozolomide could be inferred from AIC generation but due to the instability of MCTIC, and since its absorption spectrum overlaps with that of mitozolomide , neither hplc or spectral methods could be used to follow the production of MCTIC in biological systems. However MCTIC has been trappped chemically on the decomposition of mitozolomide in aqueous Na_2CO_3. The decomposition of mitozolomide in aqueous conditions has also been shown to yield chloroethanol, as would be expected by the production of MCTIC and its subsequent reaction with water (Stevens et al 1984).

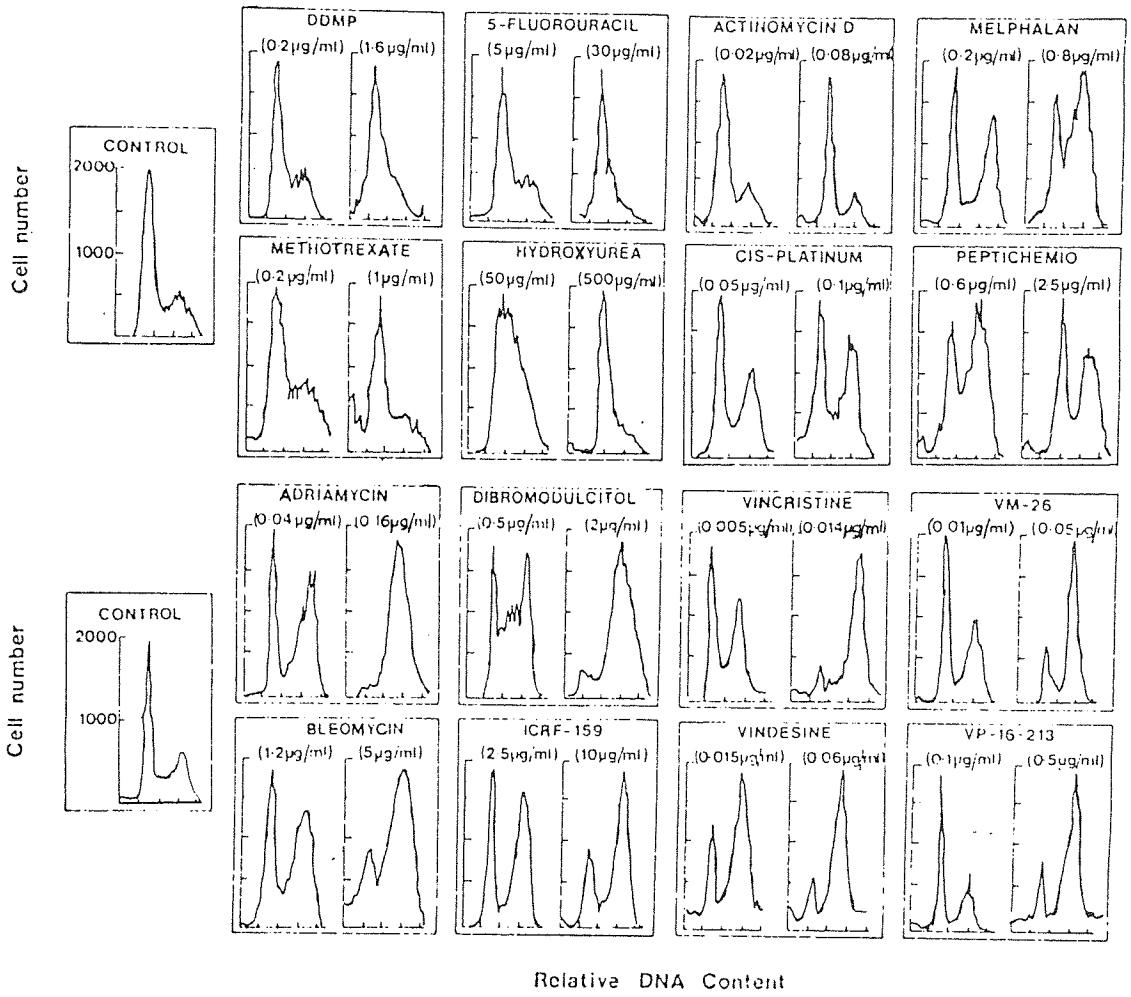
Precursor incorporation experiments had shown that mitozolomide affected particulary DNA synthesis, although to a lesser extent than BCNU. This result was suggestive of DNA alkylation as the basis of mitozolomide's mode of action. Experiments confirming the production of AIC implicated MCTIC as the likely alkylating agent. As explained monitoring of MCTIC production in biological systems is not possible at present, but the biological effects of alkylation may be investigated.

One common effect of alkylating agents, and especially agents whose cytotoxicity is due to the destruction of DNA integrity directly, is to block cycling

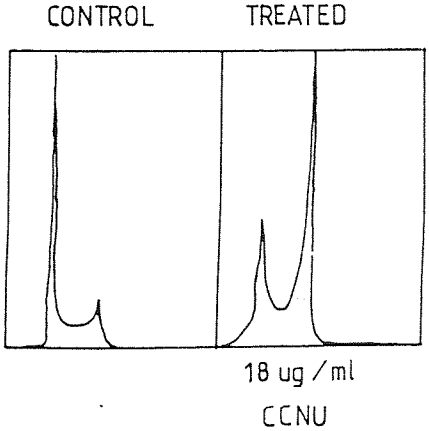
cells in the G_2/M phase of the cell cycle. Flow-cytometry provides a rapid means of investigating agents which produce such perturbations of the cell-cycle. The method produces DNA histograms which reflect the changes in the distribution of cells through the cell-cycle as previously explained (C.8).

A number of studies have attempted to relate the cell-cycle perturbative effects of cytotoxics to their mechanism of action (Tobey et al. 1979, Krishan et al. 1975). Generally only broad distinctions can be made using DNA histograms as indicators of mechanism of action; antimetabolites tend to cause accumulation at the G_1/S boundary with slippage into early S phase (Tobey and Crissman 1975), antimitotic agents produce a G_2 block (Krishan et al. 1975) and alkylating agents cause an accumulation of cells in late S and G_2/M, primarily as a result of a gross increase in the duration of these phases of the cell-cycle (Tobey and Crissman 1975, Hill et al. 1981), (Fig. 49).

FIG 49 Effect of various cytotoxic agents on the cell-cycling of dividing cells



Taken from Hill et al. (1981), murine neuroblastoma cells were exposed to low and high doses of the above drugs for 24hr. Low doses illustrate cell-cycle progression effects whilst high concentrations illustrate lethal effects.



Taken from Tobey and Crissman (1975) CHO cell suspensions were exposed to CCNU for 2hr and flow cytomatograms produced at 30 hr.

The excellent antitumour effect of mitozolomide is very clearly illustrated in the DNA histograms obtained when Lewis lung tumour was treated with mitozolomide in vivo (Fig 4Ø). In the final histogram (96hr), no tumour cells were detected. This series of histograms shows the disappearance of the G_1 cell population 24hr after treatment. There is an increase in the G_2/M population at 24hr but it is not immediately apparent. A few G_2/M cells were detected at 48hr. Presentation of the data as the number of cells in each stage of the cell-cycle as a percentage of the total shows clearly the increases in the G_2/M populations at both 24 and 48hr (Fig 41)

The antitumour activity of mitozolomide displayed in the above series of histograms is supported by the autopsy data obtained on day 21 (Table 8). All tumour parameters were significantely reduced in the treated animals compared to controls. The average tumour weight was 2.7g less in treated animals, only one animal in eight of the treated group had metatases and even then only one very small secondary tumour site was found (Ø.52g). Of the control animals 8/9 suffered multiple metastases of average weight 3.85 +/- 1.13g.

In an attempt to clearly define the cell-cycle perturbations produced by mitozolomide and to compare the effects with those of MCTIC a flow-cytometric examination of Lewis lung cells treated with mitozolomide and MCTIC <u>in vitro</u> was carried out. As the DNA histograms obtained using

drug treated cells are often dependant on the concentration of drug used (Hill et al. 1978) 40 μm, 4 μm and 0.4 μm drug concentrations were used. Cells were sampled by flow-cytometry after 8,12 and 24hr exposure to the drug containing medium and 24hr after drug removal by replacment of media (the 24hr recovery time-point). No effect was observed until the 24hr time-point at which time the G_1 peak deminished, and there was a clear enhanchment of the G_2/M peak in both mitozolomide and MCTIC treated cells. At 24hr recovery the effect was accentuated, both sets of drug treated cells showed a loss of the G_1 population, enhancement of the late S population and a peak of G_2/M cells.

The effects of mitozolomide and MCTIC as discovered by flow-cytometry were completely comparable with respect to type, extent and time-course of effect; they were also very similar to those of BCNU and other nitrosoureas (Fig 49). The production of the G_2/M block supported the hypothesis that both the drugs were acting directly on DNA, presumably by alkylation, and that mitozolomide could be exerting its effects via MCTIC. As previously pointed out such an effect could possibly be explained by some other cytotoxic action, so although more evidence "fitted" with the DNA alkylation hypothesis this was not yet proven.

There are a number of immediate implications consequent on acceptance of the hypothesis that mitozolomide alkylates DNA. Nitrosoureas alkylate DNA via the chloroethyldiazo species, in a directly analogous fashion to

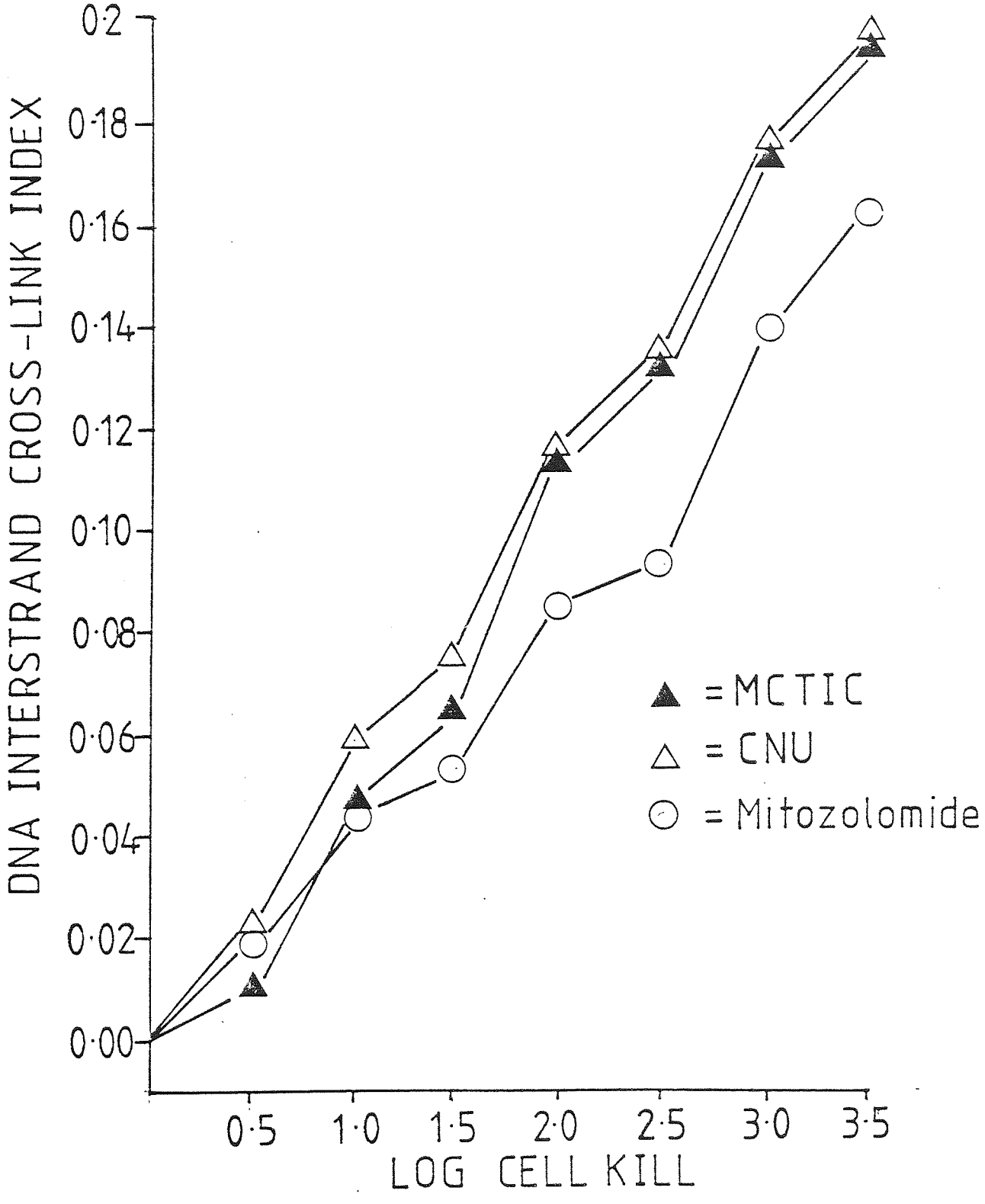
that postulated for mitozolomide. The mechanism by which this species alkylates DNA allows some reaction at the relatively inert base oxygen atoms, specifically O^6-guanine and O^4-thymidine. The relevance of the O^6-methyl transferase repair system to such adducts and to the cytotoxicity of the nitrosoureas has been discussed (A.7). It is a reasonable assumption that the resistance of mer+ cells to the nitrosoureas would also extend to mitozolomide if O^6-chloroethyl guanine production were equally important to its mode of action. Cell lines previously phenotyped mer+ and mer- at the Mill Hill laboratories, London, were obtained by courtesy of Dr. P.Karren and the growth inhibition of mitozolomide to these cell-lines tested (Fig 44). The mer+ cell-line (Raji) was approximately ten times more resistant to mitozolomide than the mer- cell-line (GMØ892A). This level of resistance falls within the range of that found by Erickson et al. (198Øb) for BCNU against various mer+ cell-lines.

Further evidence supporting the production of DNA cross-links, the importance of the alkylation at the O^6-guanine position and the envolvement of MCTIC in the cytotoxic reactions of mitozolomide was obtained by Gibson et al. (1984a and b). In a series of experiments using alkaline elution, mitozolomide, MCTIC and CNU were shown to produce similar levels of DNA interstrand cross-linking at equitoxic concentrations. At equimolar concentrations all the drugs possessed similar <u>in vitro</u> cytotoxicities as

measured by colony forming assays and cytotoxicity was found to correlate directly with the number of DNA cross-links produced (Fig 5Ø). Cross-links produced by CNU peaked at 6hr while both mitozolomide and MCTIC peaked at 9hr. this discrepancy between the time-course of effect of mitozolomide and MCTIC compared to that of the nitrosoureas was also observed in the precursor incorporation experiments mentioned earlier.

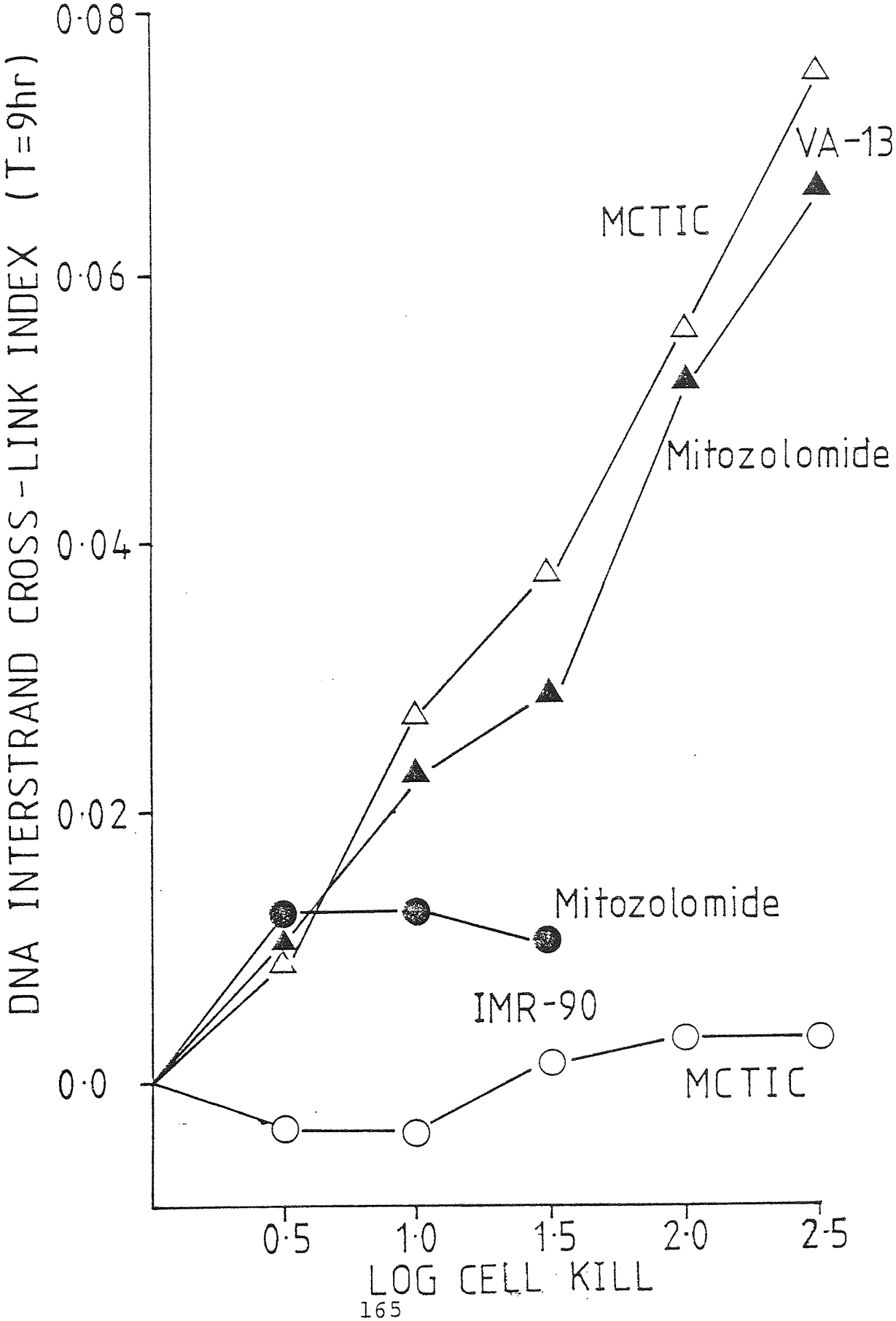
Following these experiments cross-linking and cytotoxicity were investigated by Gibson et al.(1984b) in mer+ and mer- cells, the IMR9Ø and VA19 cell-lines respectively. The results were directly comparable to those obtained earlier for the nitrosoureas; the mer+ cell-line being resistant to mitozolomide and MCTIC. Measurement of cross-links in the two cell-lines showed a direct correlation between the number of cross-links and cytotoxicity (Fig 51).

FIG. 50 Cytotoxicity versus the number of DNA cross-links produced by mitozolomide, MCTIC and CNU in L1210 cells.



DNA cross-links were measured at the time of maximal cross-linking for each drug i.e. CNU = 6hr, Mitozolomide and MCTIC = 9hr.

FIG. 51 Cytotoxicity and DNA cross-linking produced by mitozolomide and MCTIC in mer- (VA-13) and mer+ (IMR-90) cell-lines.



The O^6-methyl guanine transferase protein reacts stoichiometrically with DNA, exhibits suicide kinectics and is not inducible in mammalian cells (Foote and Mitra 1984, Demple and Karran 1983). Therefore in a mer+ cell there is a pool of the transferase protein which if exhausted must be resynthesised before the cell is again competent to detoxify lesions at the O^6-position of guanine. Recently it has been reported that the pre-treatment of mer+ cells (HT-29 human colon carcinoma cells and IMR-90 normal human fibroblasts) with the DNA methylating agent MNNG, saturates the monoadduct repair system (Zlotogorski and Erickson 1984). Similar results have also been reported for a human kidney carcinoma cell line by Day et al. (1981). If such MNNG treated cells are then exposed to a O^6-chloroethylating agent prior to the resynthesis of the mer+ protein pool then their resistance to these agents is removed (Zlotogorski and Erickson 1983). Similarly Gibson (personal communication) found that pretreatment of mer+ cells with MNNG prior to treatment with mitozolomide did increase their sensitivity to the drug.

The evidence therefore supports strongly the hypothesis of alkylation, particularly O^6-guanine alkylation via the production of MCTIC, as the mechanism by which mitozolomide exerts its cytotoxicity at the cellular level.

An examination of the differential growth inhibition of mitozolomide, MCTIC and a number of mitozolomide analogues, both inactive and active was also performed against mer+ and mer- cell-lines (Table 9).

Methazolastone the methyl analogue of mitozolomide showed the greatest difference between mer+ and mer- sensitivities. This is as expected as the mer repair system has a greater specificity for the methyl groups than the chloroethyl. Mitozolomide, MCTIC and BCNU produced very comparable results.

The resistance of mer+ cells to BCNU is slightly less than to mitozolomide and MCTIC. This could be due to a number of reasons. As mer+ cells can effectively repair the very lethal cross-links produced after alkylation at the O^6-guanine position their eventual demise is probably due to the production of lesions in other parts of the DNA genome. It is possible that BCNU produces a greater number of non-O^6 guanine alkylations than mitozolomide and MCTIC and hence is more cytotoxic to mer+ cells than these agents. It is also plausible that production of the 2-chloroethyl isocyanate is responsible for the increased sensitivity of mer+ cells. Erickson et al. (1980a), found that the differential cytotoxicity between mer+ and mer- cells was less in the case of carbamoylating congeners than non-carbamoylating congeners. Sariban et al. (1984) accounted for this phenonmenom by suggesting that the DNA lesions responsible for the death of mer+ cells, adducts other than O^6-chloroethyl guanine, were repaired by an excision mechanism. If isocyanates inhibited this mechanism, (as has been discussed previously) it could account for the increased sensitivity of the mer+ cell-lines to alkylating agents

which also possess carbamoylation ability.

The differential growth inhibition of chlorambucil, mustine, chloroethyl isocyanate and methotrexate to mer+ and mer- cell-lines was also investigated. These agents do not exert their cytotoxicity through alkylation at the O^6-guanine position and therefore any difference in toxicity is not directly due to the presence of the mer phenotype. The mer+ cell-line is infact consistently resistant to these agents, although the resistance is slight when compared to the known O^6-guanine alkylators, i.e. a 2-fold instead of a 10-fold resistance. It is possible that the mer+ phenotype is associated with other methods of protection against cytotoxics, such as co-induction of methyl-adenine glycosylase. An increase in the free thiol levels in mer+ cells could also be responsible for this general resistance although Brent (1984) reported that this was not the case in extracts taken from mer+ human leukemia lymphoblasts.

As the mer+ cell-line expressed 2-fold resistance to agents which are known not to produce alkylation at the O^6-guanine position of DNA, this level of resistance was taken as a "non-specific background" resistance. It was considered that if the mer+ repair capacity was envolved in resistance to an agent then resistance would be greater than 2-fold. Mitozolomide, MCTIC, BCNU, Methazolostone and the mitozolomide analogue no. 3, all fulfill the criteria of a greater than 2-fold resistance easily. From these results it would therefore appear that the production of O^6-chloroethyl guanine is an important facet of their mechanism of action.

The differential cytotoxicity of a number of mitozolomide analoques to the mer+ and mer- cell-lines were investigated. Namely the ester (analogue 3), sulphonamides (analoques 4,5,6,), and cyano (analoque 2) derivatives of mitozolomide (Table 9). All of these compounds possessed the 2-chloroethyl side-chain and initial chemical analysis had shown that all appeared to undergo the same ring-cleavage reaction as mitozolomide (Baig, personnel communication). Presumably all these compounds are therefore capable of producing a chloroethyltriazene moiety in an analogous fashion to the generation of MCTIC by mitozolomide. The only difference in the intermediates produced would reside in the imidazole ring. By comparision to the phenyltriazenes, in which the phenyl substituent is not critical to antitumour activity, the influence of different imidazole substituents to the antitumour activity of the imidazotetrazinones was expected to be minimal. Such an expectation proved to be unfounded.

The antitumour data for the analogues of mitozolomide did infact show that only the sulphonamides were active, both the ester and the cyanide being inactive (A.8). Inactivity <u>in vivo</u> may stem from many causes, particularly unfavourable pharmacokinectics, decomposition rates etc., and therefore may not be due to inherently different chemistry or mechanisms of cytotoxicity. When the analoques were tested against mer+ and mer- cell-lines, the results were unexpected. The sulphonamides which were active

in vivo showed no differential cytotoxicity to the two cell-lines whereas the mer+ cell-line was resistant to the inactive ester. The cyano analogue, inactive *in vivo* also showed no differential cytotoxicity.

Since the mer repair status of the rodent tumours used in the collection of screening data is unknown the lack of correlation between O^6-guanine chloroethylating agents with activity *in vivo* is potentially explicable. Similarly the *in vivo* activity of the apparently non-O^6-chloroethylating sulphonamides may depend on other DNA alkylation reactions. But it is difficult to accept that agents with such similar chemistry as the sulphonamides and mitozolomide would not have closely related mechanism of action. It is possible that the explaination once again lies with production of different spectrum of DNA alkylation products, although the lack of resistance of the sulphonamides might also be an artifact of the test system. Growth inhibition curves only allow estimation of cytotoxicity over a range of 1 log cell-kill. Cytotoxicity data for agents against mer+ and mer- cell-lines obtained using clonogenic assays, which normally cover 2-3 log cell kills, show that the greatest difference in the cytotoxicity to mer+ and mer- cell-lines often occurs once a 2 log cell kill has been acheived.

The half-lives of the analogues in phosphate buffers at different pH values were also determined (Table 1Ø). No correlation could be shown between half-lives and activity. This has also been the case for the nitrosourea

series (Weinkam and Lin 1982).

In conclusion the results presented in this thesis show that mitozolomide does not produce a chloroethyl isocyanate on decomposition; it does produce AIC which infers that decomposition occurs via MCTIC production. The similarity of the effects produced by MCTIC and mitozolomide in the parameters measured, precursor incorporation and cell-cycle perturbation support the hypothesis thay MCTIC is a metabonate of mitozolomide.

The importance of DNA alkylation at the O^6-guanine position to the mechanism of action of mitozolomide was illustrated by the resistance to the drug of cells capable of the repair of this lesion, i.e.mer+ cells. O^6-guanine alkylation has recently been suggested as of fundamental importance to the mode of action of the nitrosoureas. MCTIC, as a chlorodiazo species, should alkylate DNA in a manner directly analogous to the nitrosoureas. It is therefore proposed that the mode of action of mitozolomide at the cellular level is dependant on MCTIC generation and hence alkylation of DNA. This conclusion is supported by the cross-linking studies of Gibson et al (1984 a and b).

The mechanism of action as proposed for mitozolomide has implications for its success in the clinic. The absence of chloroethyl isocyanate production could be advantageous. The reactions of the isocyanate were outlined in section A.5.1.3, and the belief that despite much work, the designation of the precise role of the isocyanate as

either a non-selective toxin or an antitumour agent was still elusive. Recent work has concentrated on the ability of the isocyanate to interfere with nucleotide excision repair as mentioned previously. Sariban et al. (1984) expounded the argument that mer+ and mer- cells were more sensitive to carbamoylating than to non-carbamoylating nitrosoureas, possibly due to the inhibition of DNA excision repair. Other studies in which carbamoylation had not been shown to correlate with antitumour activity (Ahlgren et al. 1982) were carried out in rodents. As earlier work had shown that human cells employed nucleotide excision repair to a greater extent than rodent cells (Ahmed and Setlow 1977, Painter 1974), Sariban et al. (1984) concluded that investigations carried out in rodent systems might mask the importance of isocyanate production. It was suggested therefore that further non-carbamoylating nitrosoureas should be tested in the clinic. Mitozolomide would amply fulfill the criteria of a non-carbamoylating nitrosourea.

If the mode of action of mitozolomide does depend on O^6-chloroethylation of guanine then it is selectively toxic to mer- cells. Mitozolomide only becomes a selective antitumour agent if all tumour cells are mer- and all normal cells mer+, this is not the case. Many tumour cell-lines have been shown to be mer- (Yarosh et al. 1983, Sklar and Strauss 1983, Day et al. 1980 and Day and Ziolkowski 1979) but the opposite is also true.The Burkitt lymphoma derived cell-line, Raji, as used in this study, is mer+ (Harris et al. 1983) and Waldestein et al. (1982) have reported that

extracts of chronic lymphocytic leukemia have a high repair capacity for O^6-methylguanine lesions. Human lyphoblastoid lines, taken from the same individual, have also been shown to possess different abilities to repair O6-methylguanine (Sklar and Strauss 1983).

Variability of the mer phenotype is also found between normal tissues. Liver is generally very capable of removing O^6-guanine adducts whereas brain is not. The deficency of brain to repair such adducts has been shown to increase its susceptibility to the generation of brain tumours by ethylnitrosoureas (Muller and Rajewsky 1983). Heterogeneity also exists between the different cells that compose an organ, whereas hepatocytes are mer+, non-parenchymal cells have been shown to be mer- (Lewis and Swenberg 1980).

Obviously it will be difficult to utilise the new-found knowledge concerning the mer+ phenotype and cytotoxicity. As the primary toxicity of most alkylating agents expressed in the bone-marrow characterisation of the bone-marrow and tumour cells before treatment with cytotoxics seems the most obvious course to pursue. The best candidate for treatment with mitozolomide would therefore be a patient vith mer+ bone-marrow cells and mer- tumour cells. Unfortunately the problem of heterogeneity in both normal and tumour tissues, which has dogged the chemotherapeutist since the inception of cancer treatment may once again be the major obstacle to over-come.

F. REFERENCES

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