PHARMACEUTICAL STUDIES ON AROMATIC AZIDO COMPOUNDS

by

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A thesis submitted for the degree of

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SUMMARY

2,4-Diamino-5-(3-azido-4-chlorophenyl)-6-ethylpyrimidine [m-azidopyrimethamine, MZP] is a novel lipophilic antifolate and the first arylazide to be considered for clinical use. HPLC methods were developed for quantitative evaluation of MZP and its analogues. ^{13}C NMR spectroscopy studies revealed that, at physiological pH, protonation of the 2,4-diaminopyrimidines occurred predominantly on the heterocyclic N-1; in MZP, the pKa of this nitrogen was found to 7.19. The Log P of MZP was determined to be 2.81. MZP was found to have low aqueous solubility (22 $ugml^{-1}$) and hence the more water soluble MZP ethanesulphonate [MZPES] (17.6 $mgml^{-1}$) was chosen for formulation development.

Thermal degradation of 10 $mgml^{-1}$ aqueous MZPES solutions followed first order kinetics and the activation energy was calculated to be 139.3 $kJmol^{-1}$. Heating a 10 $mgml^{-1}$ aqueous MZPES solution at 145°C for 2 hours resulted in a 28% yield of m-aminopyrimethamine [MAP] as the major product. Aqueous solutions of MZPES were stable for at least 21 months when stored at -10, 4 or 20°C; but at temperatures >100°C degradation of the compound occurred rapidly.

Aqueous solutions of MZPES were shown to be photo-labile. The photolytic rate of MZPES, however, was slightly retarded in nitrogen-saturated solution. The nature and ratio of the photoproducts of MZPES formed in aqueous solution varied according to whether the solution was saturated with air, oxygen or nitrogen. Several of these degradation products have been identified and reaction schemes have been proposed to account for their formation. Structures and derivations of the unidentified products have also been proposed.

MZPES was formulated as a 10 $mgml^{-1}$ aqueous solution (pH 4.10) and recommended to be diluted in 500 mls 5% Dextrose infusion prior to administration. MZPES was shown to be stable in 5% Dextrose solution for at least 80 hours. MZPES injections were prepared under aseptic conditions and sterilized by filtration. The injections were packed in opaque containers and stored at 4°C to maximise stability.

The clinical pharmacokinetics of MZPES exhibited a biphasic profile. The distribution and elimination half-lives of the drug were determined to be 0.35 ± 0.22 and 37.42 ± 17.11 hours respectively. The average volume of distribution of MZPES was found to be 145.6 ± 34.8 L. No adverse haematological effects were observed to be associated with MZPES administration during the current study up to a dose of 250 mgm^{-2}. MZPES was shown to be less toxic than the prototype lipophilic antifolate metoprine.

Key words: m-Azidopyrimethamine, DHFR inhibitor, Thermolysis, Photolysis, Formulation

To My Parents

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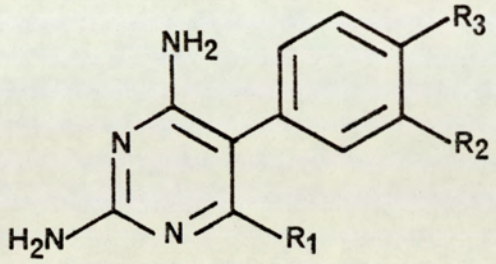


R_1	R_2	R_3	Compound
Et	N_3	Cl	m-Azidopyrimethamine [MZP]
Et	H	Cl	Pyrimethamine
Et	NH_2	Cl	m-Aminopyrimethamine [MAP]
Et	NO_2	Cl	m-Nitropyrimethamine [MNP]
Et	Cl	N_3	ISOMZP
Et	Cl	NH_2	ISOMAP
Et	Cl	NO_2	ISOMNP
Me	Cl	Cl	Metoprine
Et	Cl	Cl	Etoprine

ABBREVIATIONS

AUC	Area under the curve
AUC_t	Total area under the curve
BCNU	1,3-Bis(2-chloroethyl)-1-nitrosourea
CCNU	1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea
CSF	Cerebral spinal fluid
CV	Coefficient of variation
DHFR	Dihydrofolate reductase
DMA	Dimethylacetamide
DMSO	Dimethylsulphoxide
ESR	Electron spin resonance
HPLC	High performance liquid chromatography
IP	Intraperitoneal
IR	Infra-red
IV	Intravenous
K'	Capacity factor
K_i	Inhibition constant
LD_{10}	Lethal dose for 10% of test population
LD_{50}	Lethal dose for 50% of test population
Log P	Relative index of lipophilicity
MAPES	m-Aminopyrimethamine ethanesulphonate
MNPES	m-Nitropyrimethamine ethanesulphonate
MTD	Maximum tolerated dose
MTX	Methotrexate
MZPES	m-Azidopyrimethamine ethanesulphonate
MZPHCl	m-Azidopyrimethamine hydrochloride
MZPMS	m-Azidopyrimethamine methanesulphonate
NCI	The National Cancer Institute

NMR	Nuclear magnetic resonance
PACD	Photoinitiated autocatalytic chain decomposition
PFC	Perfluorocarbons
RES	Reticuloendothelial system
R_f	Relative index of movement from origin in TLC
TFA	Trifluoroacetic acid
TFSA	Trifluoromethanesulphonic acid (triflic acid)
THF	Tetrahydrofuran
TLC	Thin layer chromatography
Trap.AUC	Trapezoidal area under the curve
$T_{\frac{1}{2}\alpha}$	Distribution half-life
$T_{\frac{1}{2}\beta}$	Elimination half-life
V_B	Apparent volume of distribution
Vc	Apparent volume of central compartment
Vs	Versus
λmax	Wavelength of maximum UV absorbance

"What is impossible with man is possible with God"

Luke 18: 27

SECTION A

INTRODUCTION

1. AZIDES

All organic azides are synthetically derived, no naturally occurring compound has ever been discovered in this class. The synthesis of the first organic azide was described in 1864 by Griess (1) who reacted benzenediazonium tribromide with ammonia to obtain phenylazide [Eq. (1)].

$$\overset{+}{PhN_2}\overset{-}{Br_3} + 4NH_3 \longrightarrow PhN_3 + 3NH_4Br \qquad \text{Eq. (1)}$$

Initial research progress into this class of compounds had been slow. It was not until the 1950's that interest in the organic azides, or more particularly the reactive intermediates derived therefrom and the potential applications of these reactive species, suddenly burgeoned. This led to a rapid expansion on the literature of organic azides. The first comprehensive review on the chemistry and reactivity of arylazides was documented by Boyer and Canter (2) in 1954.

Various synthetic methods to arylazides were elucidated and these methods have been extensively reviewed (2,3,4). The most widely used method for the preparation of arylazides has been the diazotization of the corresponding arylamine, followed by addition of sodium azide [Eq. (2)].

$$ArNH_2 \xrightarrow{HNO_2} \overset{+}{ArN_2} \xrightarrow{NaN_3} ArN_3 + N_2 \qquad \text{Eq. (2)}$$

1.1. Decomposition of organic azides

Heat, mechanical shock, light or exposure to certain chemical reagents, e.g. concentrated sulphuric acid, can trigger off the decomposition of organic azides (5,6). This process is generally

characterised by the formation of molecular nitrogen and the release of large amounts of energy. During controlled decompositions in solution, the solvent acts as an energy sink and a heat transfer agent; thereby contributing to smooth reaction and safety. In the absence of a solvent an explosion may occur unless this energy is dissipated over the molecular fragment. The larger the fragment, the more effective the energy transfer becomes. Hence, arylazides are generally more stable to heat than alkyl or acyl-azides (6).

1.2. Arylnitrenes

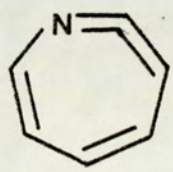
Decomposition of arylazides is believed to proceed generally via the formation of arylnitrenes, following the loss of molecular nitrogen (6,7,8). Arylnitrenes are uncharged, electron-deficient, reactive intermediates where the nitrogen atom possesses a sextet of electrons in its outer shell. These species may exist as an electrophilic singlet nitrene (I) or as a biradical triplet nitrene (II).

I II

Kinetic measurements of the thermolysis of arylazides that did not bear a reactive ortho-substituent in inert solvents showed a first order rate determining step for the disappearance of the azides or the the appearance of nitrogen (9,10,11) regardless of the ultimate products. This indicates a common initial step in the formation of an arylnitrene which subsequently undergoes a series of fast reactions such that a complex mixture of products results. It is very likely that the singlet arylnitrene is first formed because of

spin conservation. The temperature required to effect the thermolysis of arylazides lies in the region of 100 to 180°C.

Photolysis is usually the preferred method for studying these reactive intermediates because much milder conditions can be employed. The existence and the nature of the arylnitrenes have been extensively investigated by irradiating arylazides in solid matrices at low temperatures. Despite over 60 years of research, there are still many important details of these intermediates that are not completely understood. Triplet arylnitrenes have been observed by electron spin resonance (E.S.R.) at 4 - 77K and shown to be the ground state of arylnitrenes (12,13,14). The electronic absorption spectrum of a triplet nitrene has also been measured at low temperatures in frozen media and found to absorb broadly between 300 and 420 nm (15,16,17). In 1978, Chapman and LeRoux (18) obtained an infra-red spectrum of the intermediate formed from the photolysis of phenylazide in argon at 8K. They concluded that the major primary photoproduct is the didehydroazepine (III). Donnelly et al (19) also



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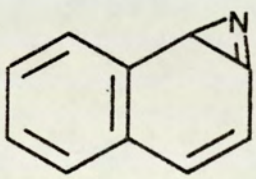

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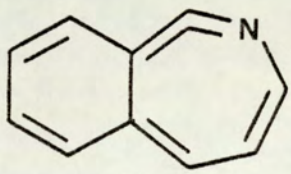
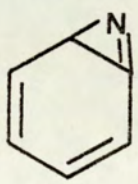


V



VI

reported the formation of didehydroazepines in the matrix photolysis of 3- and 4-substituted phenylazides at 12K. Substituents used in the study - fluoro, chloro, cyano, methyl and methoxy - had no influence on the formation of didehydroazepines. In 1980, Dunkin and Thomson (20) reported the photolysis of 1- and 2-azido-naphthalene in argon

or nitrogen matrices at 12K. Naphthazirines (IV) were detected first in the matrices and these were shown to undergo secondary photolysis to the corresponding didehydroazepines(V). Both the didehydroazepine (III) and benzazirine (VI) are the lower energy tautomeric forms of singlet nitrene which probably do not exist in high enough steady state concentrations to be detected.

Irradiation of phenylazide in inert and reactive solvents at room temperature has also been examined using low density continuous light sources or high power pulsed lasers (21). A remarkable decrease in the yield of triplet derived product, azobenzene, was observed when pulsed laser was used instead of continuous light source. The irradiating source, however, had no influence on the yield of azobenzene if a triplet sensitizer was included in the reaction. This suggests that triplet nitrene is not the first intermediate formed in the reaction. Irradiation with laser generates a much higher instantaneous concentration of transient products than does conventional photolysis. If the transient products from the photolysis of phenylazide were predominantly triplet nitrenes, then their rate of dimerization should increase under laser irradiation and give a concomitant increase in yield of azobenzene. The result, therefore, suggests that the triplet nitrenes must be formed only slowly from a singlet reservoir. As a result, one can postulate that photolysis of an arylazide leads to the formation of a singlet nitrene initially. This can intersystem cross-over to the triplet state or it can undergo rapid tautomerism to azirine or didehydroazepine, depending on the reaction conditions.

The nature of the substituents can also influence the formation of these reactive intermediates. In argon and nitrogen matrices, triplet nitrene formation appears to be a minor pathway, but in the

case of pentafluorophenylazide, nitrene rearrangement is suppressed, and the triplet nitrene is formed exclusively (22). It is likely that the electron withdrawing substituents prevent the singlet nitrenes from forming relatively long-lived azirines or didehydroazepines, and consequently, they intersystem cross rapidly and irreversibly to the triplet nitrenes.

In 1985, Leyva and Platz (23) examined the photolysis of phenylazide in a diethylamine matrix at low temperatures. They reported a significant change in product distribution with temperature. At 273 and 195K, the only significant product observed was a 3H-azepine [2-diethylamino-3H-azepine], a singlet derived product. At 173 and 153K considerably larger amounts of azobenzene, a triplet derived product, were formed than the 3H-azepine. Upon further cooling to 77K no significant yield of the 3H-azepine was observed. Instead, a very high yield of triplet derived products was obtained. It is difficult to rationalise this observation with those discussed above. It is possible that singlet nitrenes are formed at 77K in the diethylamine matrix but cannot react with the nucleophiles under these conditions; hence on standing they gradually revert to the ground state triplet. Alternatively, the diethylamine matrix may hinder the rearrangement of singlet nitrene.

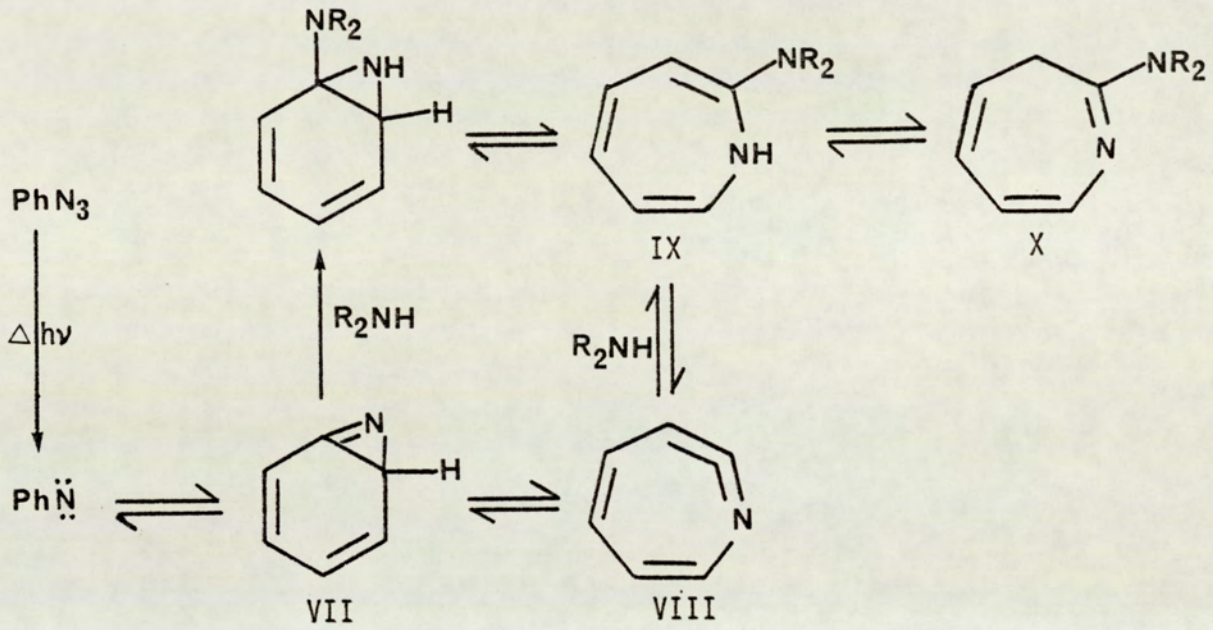
It can, therefore, be concluded that arylnitrenes are formed in the thermolysis or photolysis of arylazides. The nature of the intermediates is still the subject of much debate. Singlet transient products, didehydroazepines and benzazirines, and triplet nitrenes have been identified and their relative formation is greatly influenced by the nature of the matrix or solvent, temperature, substituents, external nucleophiles and the use of sensitizers.

1.21. Reactions of arylnitrenes

The chemistry of arylnitrenes is a vast and highly complex subject. Only the more common reactions of the simple azides will be briefly described. A more detailed survey on the subject has been compiled by Abramovitch and Kyba (6), Smith (7) and Scriven (8).

1.21A. Ring expansion to azepines

Arylazides undergo ring expansion to azepines on decomposition in nucleophilic solvents, such as amines (24,25) and alcohols (26,27), or in neutral solvents where nucleophiles are present (28,29). The pathway by which azepines are derived is still a subject of controversy. There are two contenders for the intermediates in the reaction. Substituted azepines may be formed *via* the addition of nucleophiles to either an intermediate benzazirine (VII) (24,25) or didehydroazepine (VIII) (18,30). Both these species have been detected in low temperature matrix photolysis (20) and they are probably in equilibrium with each other (19). The reaction is summarised in Scheme 1. Sundberg et al (31,32) showed that the reaction of singlet state intermediate did not give the 3H azepine (X) directly but proceeded via the 1H azepine (IX).



Scheme 1. Azepine formation

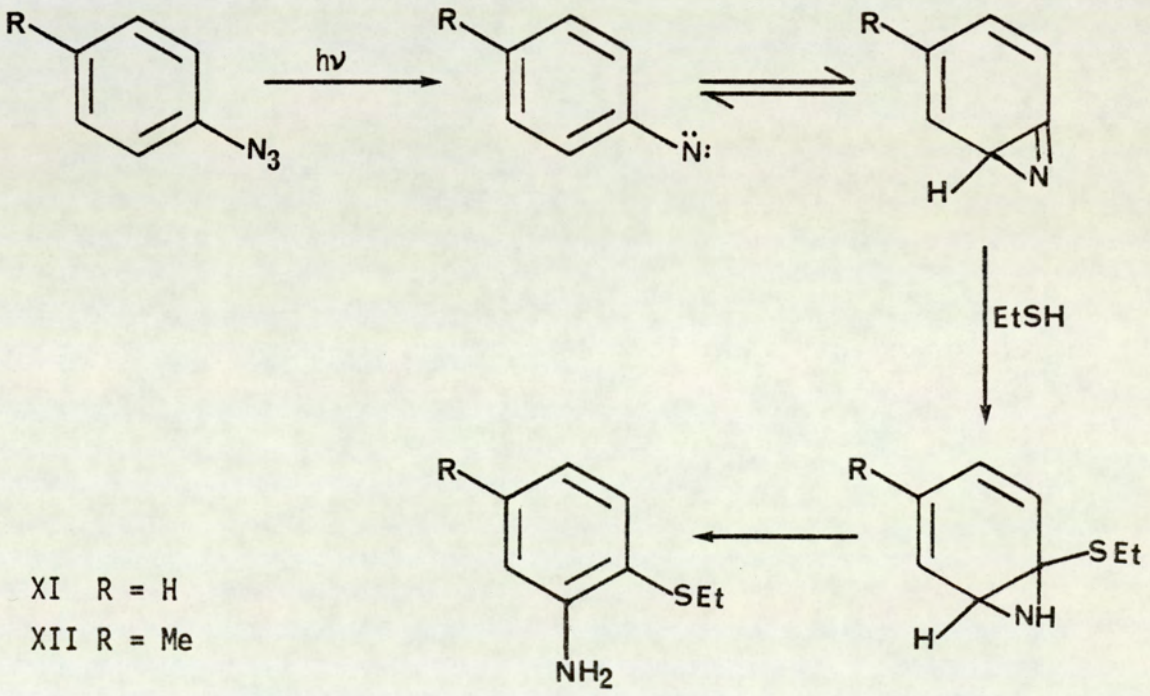
The yield of azepines may be increased by stabilising the singlet nitrenes and/or suppressing the intersystem crossing to triplet nitrenes. This has been achieved by carrying out the reactions in cosolvents with lone pairs e.g. tetramethylethylenediamine (35), dioxane (28,29) and tetrahydrofuran (36). Ortho substituents such as carbonyl, cyano or trifluoromethyl also promote azepine formation by increasing the electrophilicity of the singlet nitrene (37). Para- and meta-substitution can also result in azepine formation, although much lower yield is obtained and longer photolysis time is needed.

Some nitrenes, such as p-nitrophenyl, o- and p-cyano, and o-chloro phenylnitrenes, give no azepines under conditions where

others do (33). This may be attributable to the acceleration of intersystem crossing to the triplet state caused by these substituents (34).

The use of oxygen and sulphur nucleophiles has not proven as rewarding as that of amines in promoting ring expansion to azepines. Photolysis of phenylazide in methanol (26) or hydrogen sulphide/ diethyl ether (24) resulted in only a very modest yield of the substituted azepines.

Photolysis of phenyl and p-tolyl azide in ethanethiol does not give rise to azepines; instead o-ethylthioanilines XI and XII are the major products (38, Scheme 2). The 1,2 nitrogen walk observed in the photoproduct of p-tolyl azide indicates that the reaction does not occur via the radical attack by ethanethio (Ets•) radicals. The reaction probably proceeds via nucleophilic addition of ethanethiol to benzazirine.



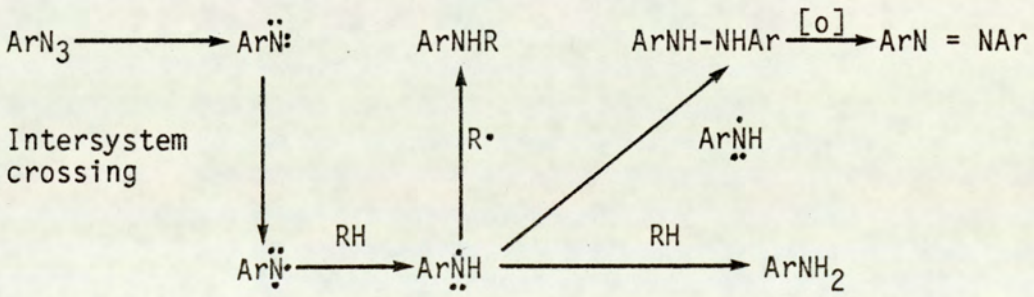
XI R = H
XII R = Me

Scheme 2. Reaction of an arylazide with ethanethiol

1.21B. Hydrogen abstraction

When arylazides that do not bear a reactive ortho substituents are decomposed in inert solvents, e.g. benzene or acetonitrile, a mixture of arylamines, azo componds and amorphous polymeric materials is produced (9,39). The relative amount of these products varies widely depending on the nature of the substituents, solvents, irradiation wavelengths and perhaps temperature. The yield of arylamines is increased in triplet-sensitized decompositions of arylazides (21,40), indicating that these products are derived from the reactions of triplet nitrenes.

Arylamines are formed as a result of hydrogen abstraction by triplet nitrenes from the solvent or the nitrene precursor (9,41,42). Hydrazo compounds may also be produced in the process by dimerization. Photolysis of arylazides in hydrocarbons also results in the formation of N-substituted arylamines in addition to arylamines (41). Formation of N-substituted arylamines has been shown by Hall et al (31) to arise from the reaction of anilino radicals and not from the direct insertion of singlet nitrene into C-H bonds (Scheme 3). There is no evidence in the literature to suggest that a nitrene will abstract molecular hydrogen.



Scheme 3. Hydrogen abstraction process

Smith and Hall (10) studied the effect of substituents on the yield of arylamines by decomposing a series of *para*- and *meta*-substituted phenylazides in decalin solution. A chloro substitution was found to have very little effect on the yield of arylamines. Methoxy and bromo-substituents increase the yield dramatically, whereas the yield drops sharply with nitro substituted phenylazides.

The effect of solvent on the yield of arylamines can be correlated qualitatively with the hydrogen donating quality of the solvent. Thermolysis of phenylazide in benzene or p-xylene (43) gave rise to 20-25% of aniline. In decalin (9,10) and thiophenol (44) yields of 44-79% and 52% were reported respectively. Reiser and Leyshon (39) studied the photolysis of phenylazide in deoxygenated solvents and reported the relative product formation after 2% conversion of the azide. The yield of aniline varied very slightly between benzene (5%), cyclohexane (4%), methanol (7%), ter-butyl alcohol (5%) and 2-propanol (12%). In this instance, the change of solvent had more effect on the formation of azobenzene. The yield of azobenzene was 50% in benzene, 51% in cyclohexane, 55% in ter-butyl alcohol and only 3-5% in 2-propanol and methanol. A short irradiation time was reported to favour azo formation (46).

1.21C. Azo compound formation

Azobenzene is formed by decomposition of arylazides in inert or deoxygenated solvents (9,21) *via* the reaction of triplet nitrenes (21,39). In de-aerated solvents, the azobenzene formed is exclusively the *trans*-isomer (46).

Unsymmetrical azobenzenes are obtained when two different aryl-nitrenes are generated simultaneously (45). This indicates that one of the mechanisms for the formation of azo compounds is *via* the

direct dimerization of two arylnitrenes. It has also been suggested that azo compounds may arise from the attack of nitrene on the azide (42) but no concrete evidence exists to support this mechanism. In fact phenylazide was recovered almost quantitatively from the triethylphosphite deoxygeneration of nitrosobenzene in the presence of this azide (47). Another mechanism proposed for the formation of azo compounds is via the hydrogen abstraction process, scheme 3 (9). This involves an initial abstraction of one hydrogen by a triplet nitrene to form an anilino radicals which subsequently dimerises into a hydrazo compound. Oxidation of the hydrazo compound affords the azo compound (48).

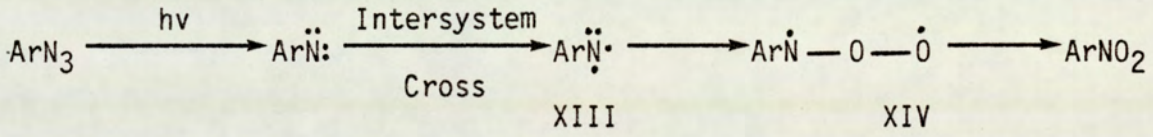
p-Methoxyphenylazide had been used as the substrate in a study of the effect of solvent on azobenzene formation (45). In benzene, the yield of dimethoxyazobenzene by photolysis was only 18%, but in solvents having O,S or N atoms, such as tetrahydrofuran or acetonitrile, it increased to 82-91%. By employing short irradiation times and phenylazide as substrate, Reiser and Leyshon (39) reported a much higher yield of azobenzene in benzene (50%) than in methanol (5%). Substituents, irradiation wavelength and time of exposure can all influence product formation. The report on the effect of substituents on azo formation is one of great variation; a systematic approach to the subject is lacking. Generally, those arylazides that give low yields of arylamines result in high yields of azo compounds (39). The yield of the polymeric materials also varies substantially.

1.21D. Reaction with molecular oxygen

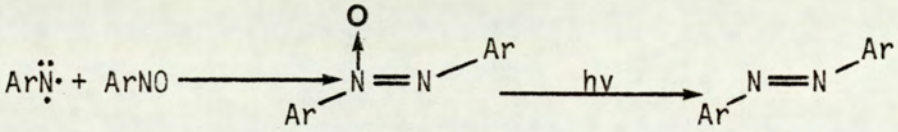
Azides are normally inert to air, and thermolysis or photolysis of arylazides does not generally involve atmospheric oxygen in the reaction. Deliberate introduction of molecular oxygen during photo-

lysis of phenylazides resulted in the formation of nitrobenzene via nitrosobenzene (46,49). With para-substituted arylazides, azoxy compounds were also isolated (49). Traces of arylamines and azo compounds were also formed. Hitherto, there is no report of nitrobenzene formation following thermolysis of arylazides.

The reaction of phenylnitrene with oxygen was evidently of the triplet nitrene (49); triplet sensitizer and quencher greatly influenced the formation of nitrobenzene. Inclusion of singlet oxygen sensitizer or quencher, however, had no effect on the yield of the nitro compound. Support for the trapping of triplet nitrene by triplet oxygen came from E.S.R. observation of the biradicals XIII and XIV during photolysis of p-diazidobenzene and oxygen in a frozen glass matrix at 77K (50).



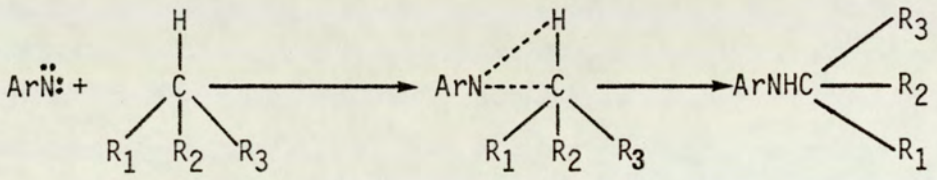
Formation of azoxy compound was ascribed to the reaction of triplet nitrene with nitroso compound (46,49). Irradiation of azoxybenzene gave rise to azobenzene.



1.21E. Insertion into C-H bonds

Intramolecular insertion of arylnitrenes is generally a poorly competitive process. However, it was observed that decomposition of arylazides in hydrocarbon solvents such as cyclohexane, pentane and 2-phenylbutane gave rise to N-substituted arylamines in addition to primary arylamines (41,48,51,52).

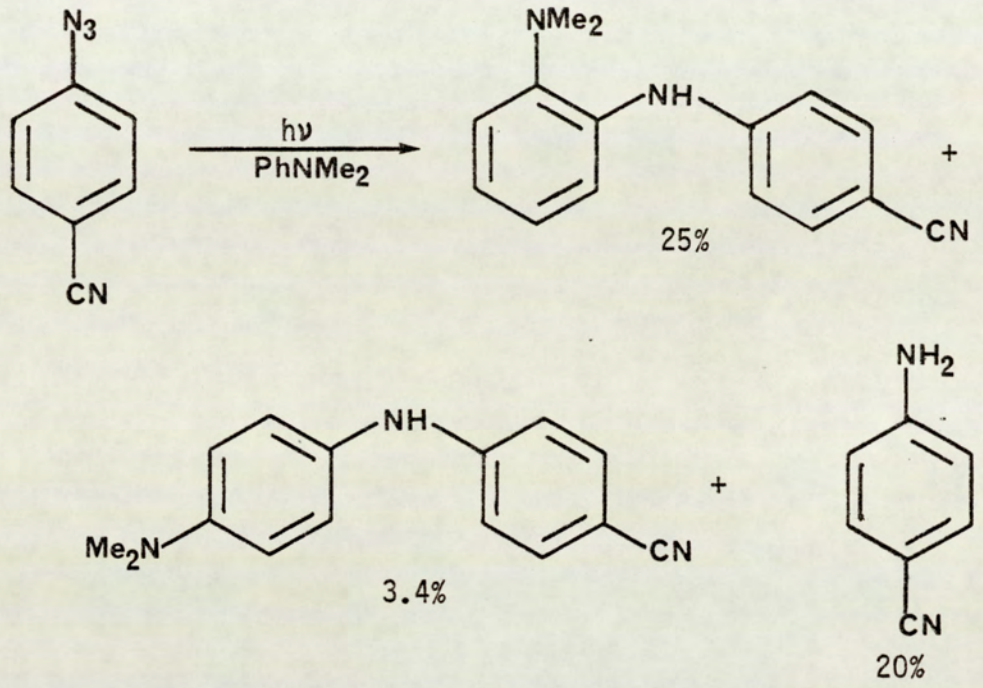
The formation of N-substituted arylamines can occur *via* the direct insertion of a single nitrene into the C-H bond (Scheme 4) or a two steps reaction of triplet nitrene by hydrogen abstraction followed by radical coupling (Scheme 3).



Scheme 4. Insertion of singlet nitrene into C-H bond

Evidence provided by Hall *et al* (41) suggests that the above reaction proceeds *via* the triplet pathway rather than the singlet pathway. Thermolysis of phenylazide in cyclohexane with varying quantities of neopentane as an inert diluent had no effect on the product ratio of aniline to N-cyclohexylaniline, indicating that the two products arose from the same intermediate. Evidence also came from thermolysis of the azide in 2-methyl-2-deuteriopropane and 2-methylpropane at 160°C. An isotope effect of K_H/K_D 4.1 was observed, suggesting that the C-H bond was broken before N-C bond was formed. However, this does not rule out the possibility that insertion of singlets into C-H bond can occur under other reaction conditions.

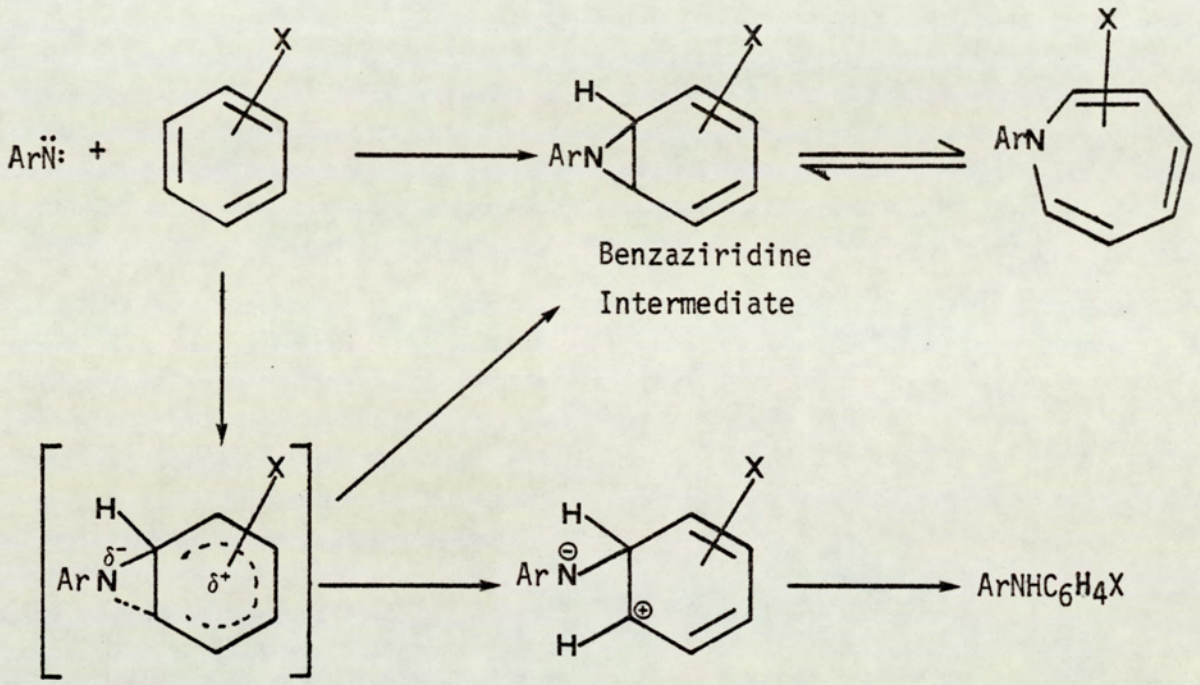
Insertion of arylnitrenes into aryl C-H bonds had also been observed when arylazides with strongly electron withdrawing *para*-substituents (e.g. nitro, cyano or trifluoromethyl) were thermolysed in the presence of arenes that were highly activated towards electrophilic substitution (53). The yield of diphenylamine was about 13-38% [Eq. (3)]. This reaction was not observed with phenylnitrene which was probably not sufficiently electrophilic to attack the aromatic substrate.



Eq. (3)

Phenylnitrene (54) and pentafluorophenylnitrene (55), generated from the deoxygenation of the precursor nitroso compounds using triethylphosphite, were observed to be more electrophilic than the reactive species generated by thermolysis or photolysis. They attacked even benzene and gave rise to substituted 1H-azepines in addition to the N-substituted compounds.

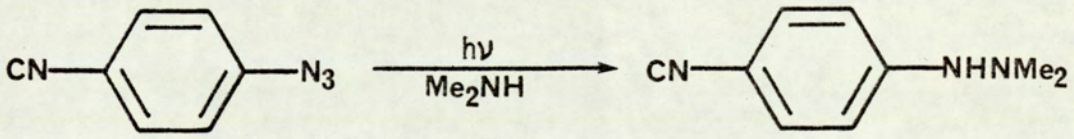
The attack on the aromatic substrate is probably carried out by a singlet nitrene. Abramovitch et al (47) proposed two mechanistic pathways through which insertion of a nitrene into an aryl substrate may occur (Scheme 5). The extent to which each of these will be followed depends on the nature of the aromatic substrate, the substituents and the reaction conditions.



Scheme 5. Insertion of nitrene into aryl C-H bond.

1.21F. Insertion into N-H bonds

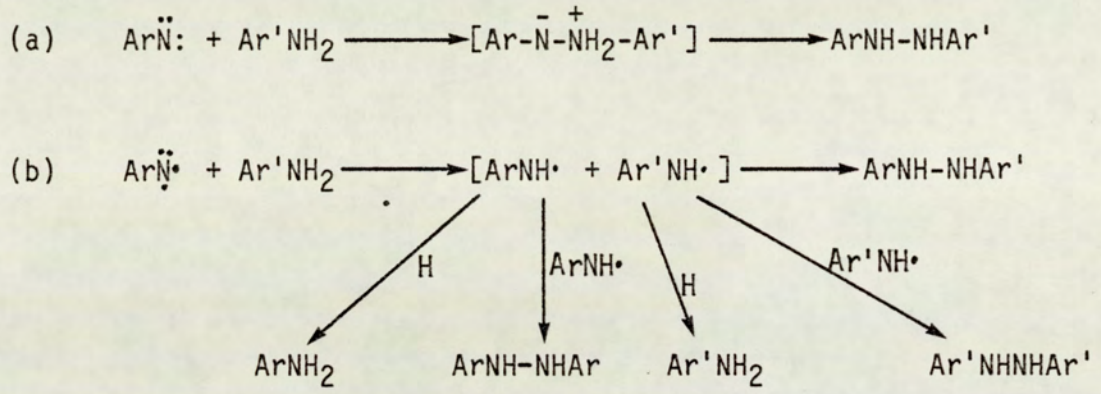
Insertion of arylnitrenes into N-H bonds to give hydrazines [Eq. (4)] has been reported by Odum and co-workers (34,56). The two reports gave conflicting evidence as to whether a singlet or triplet



Eq. (4)

nitrene was involved in hydrazine formation. The relative yield of hydrazine was found to be dependent on the irradiation wavelength (56).

One can postulate that the formation of hydrazine may involve either a direct insertion of singlet nitrene into an N-H bond or a two steps free radical reaction of the triplet nitrene (Scheme 6), similar to C-H insertion.



(a) Singlet pathway

(b) Triplet pathway

Scheme 6. Hydrazine formation

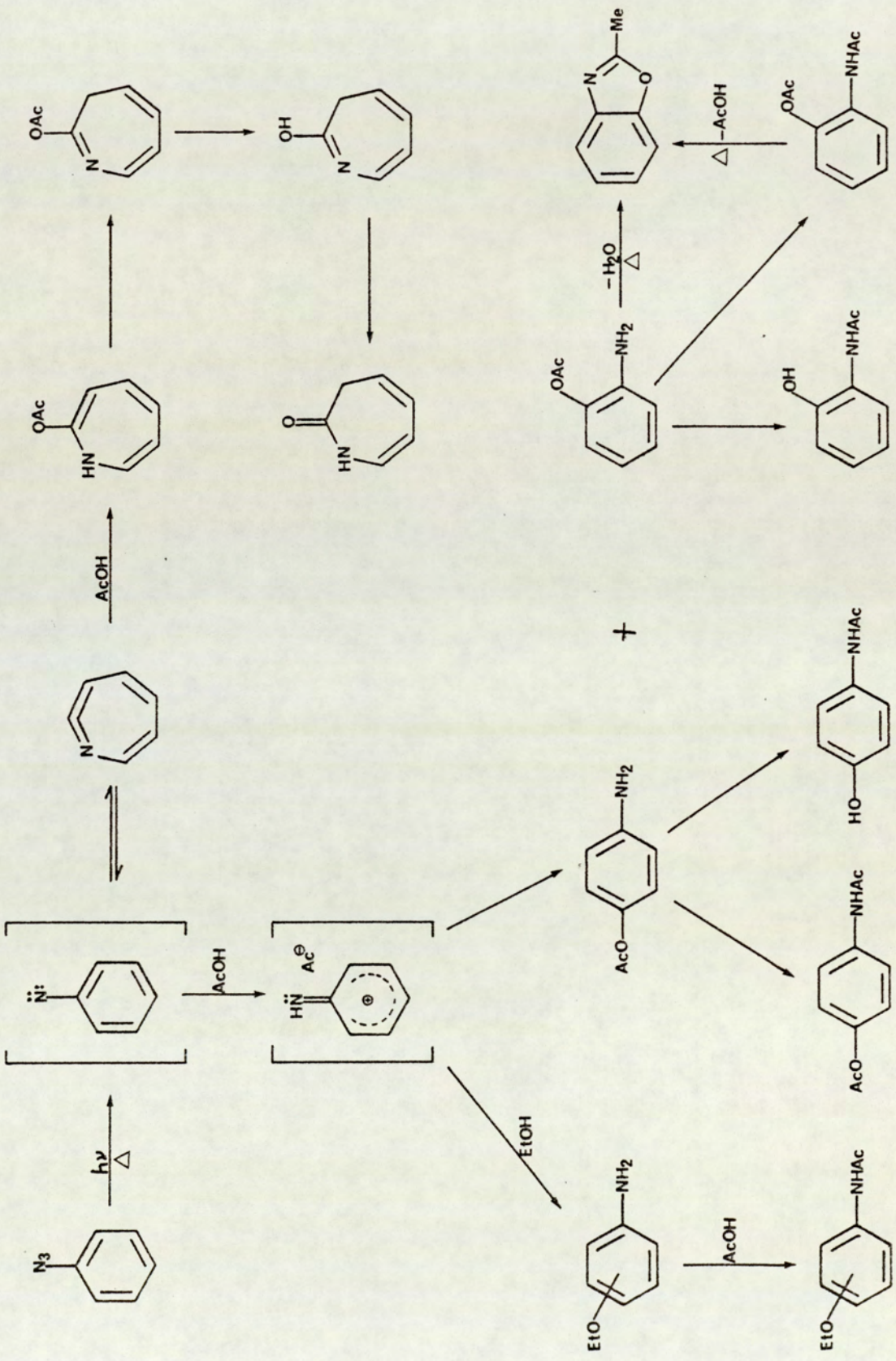
When certain arylazides were thermolysed in the presence of arylamines, small amounts of unsymmetrical azo compounds (~5%) were formed in addition to the usual arylamine, symmetrical azo compounds and tars (57,58). Therefore, thermolysis of arylazides in the presence of arylamines may result in azo compunds formed from insertion of the arylnitrene into N-H bonds in addition to the azo compounds formed by the usual dimerization of nitrenes.

1.21G. Decomposition of arylazides in acids

Acid-catalyzed decomposition of arylazides gives rise to ortho- and para-substituted arylamines (59). Takeuchi and Koyama (60,61) studied the photolysis and thermolysis of phenylazide in acetic acid

and reported the formation of azepinones together with a wide range of aminophenols. Upon irradiation of phenylazide in phenol (61), only aniline and 2-phenoxy-3H-azepine were obtained. The absence of the ring disubstituted products suggests that these must be formed via a different pathway to azepine in the presence of acid. The yield of these preoducts was not influenced by the presence of a triplet sensitizer, indicating that the reaction proceeds via a singlet pathway. Takeuchi and Koyama (60,61) concluded that the singlet arylnitrene formed could either ring expand or was protonated by acetic acid to give a phenylnitrenium-acetate ion pair (Scheme 7). The latter reacted with a variety of nucleophiles, e.g. water, ethanol or acetate, in the reaction media giving rise to the substituted anilines. Decomposition of an azide in both acetic acid and dioxane proceeded with similar rate and activation parameters, suggesting that nitrene formation must be the rate-determining step and that protonation occurred on the nitrene and not the azide.

Scheme 7. Decomposition of phenylazide in acetic acid

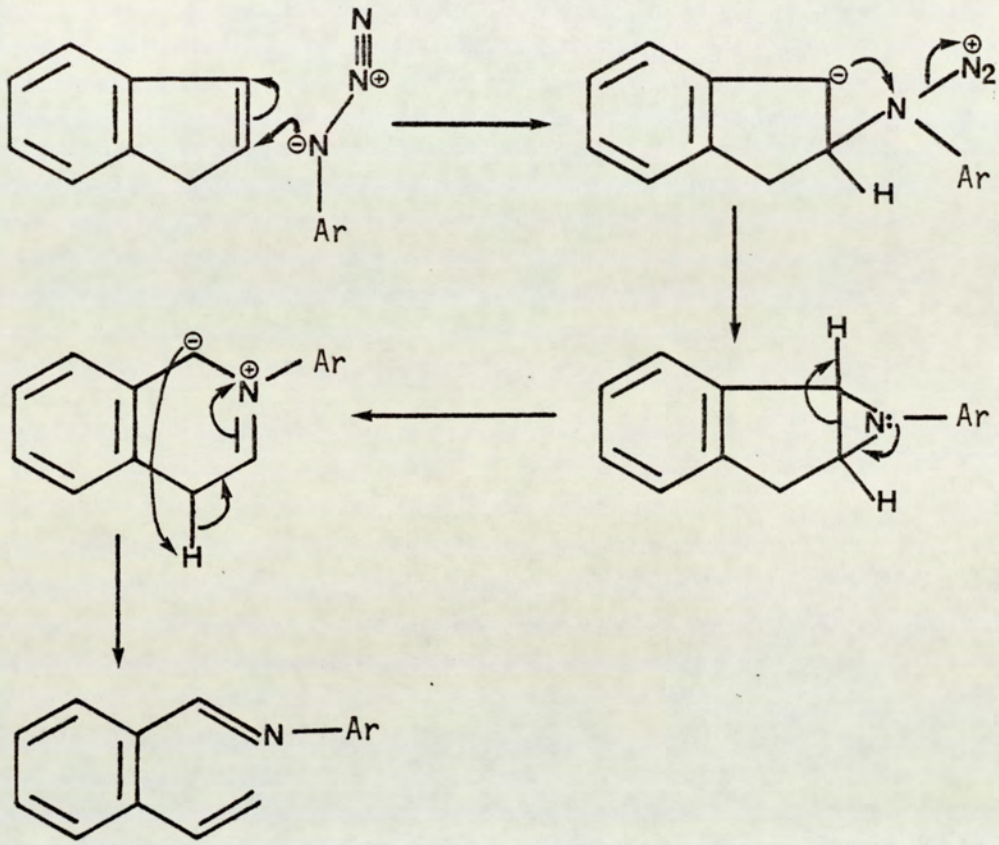


1.3. Thermolysis

Thermolytic decomposition of arylazides generally follows first order kinetics (9,10,11,62). Deviation from this first order reaction has been observed and can be attributed to (a) induced decomposition of azide at high concentration by nitrene or free radical species (11); (b) contaminants, e.g. oxidising agents, in the reacting solvents (63); and (c) interaction between azide and unsaturation in the solvent generated by hydrogen abstraction (10). At concentrations where induced decomposition of azides was absent, the rate of reaction was independent of concentration (9,64). The rate of thermolysis of azides is dependent on both the activation energy and the entropy change (64).

1.31. Effect of solvents

Walker and Waters (9) studied the decomposition of phenylazide at 132°C in a range of solvents. With the exception of indene, there was very little variation between the rate of reaction in the various different solvents. The solvents used included tetrachloroethylene, tetralin, paraffin, cumene, decalin, ethylbenzoate, benzene, cyclohexane and aniline. The first order rate constant for decomposition of azide in these solvents ranged from 0.54 to 1.41 x 10^5 sec^{-1}. In indene, however, the rate of reaction increased dramatically, with a rate constant of 150 x 10^5 sec^{-1}. Similar results were obtained from decomposition of p-methoxyphenylazide except that another group of solvents providing an intermediate rate of decomposition of the azide, with rate constant between 20 and 45 x 10^5 sec^{-1}, were identified. These solvents were benzylalcohol, diphenylmethanol and trans-stilbene. The fast reaction in indene was thought to arise from interaction between the azide and the solvent leading to a concerted loss of nitrogen and the formation of an aziridine (Scheme 8).



Scheme 8. Reaction of an arylazide with indene.

The absence of nitrene involvement in indene was supported by the fact that only one product was obtained and no azo compound was isolated.

Patai and Gotshal (65) reported the thermal decomposition of p-substituted-2-nitrophenylazide in solvents of varying polarity. No significant difference in the rate of pyrolysis was observed between the non-polar solvents, e.g. heptane, benzene, xylene, and the very polar solvents, e.g. dimethylformamide and formamide. Solvents of intermediate polarity, e.g. cyclohexanol, 95% ethanol, also gave similar results.

There is no simple interpretation of solvent effect on azide decomposition. In general, solvents seems to have only a slight influence on reaction rate but they may affect the ratio of product

formation. The rate of reaction is only accelerated markedly if the azide reacts concertedly with the solvent.

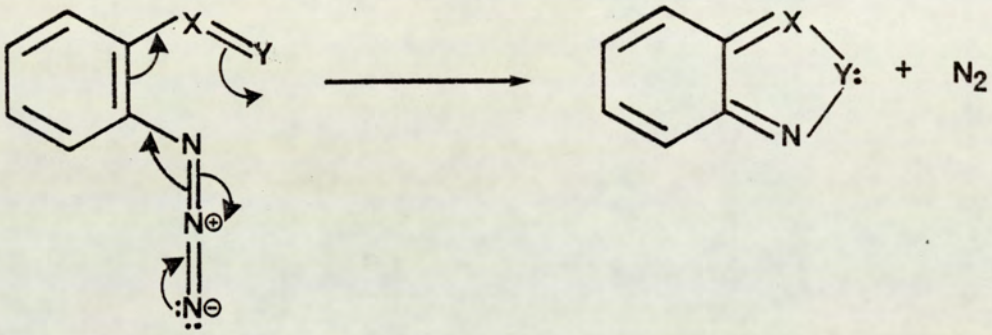
1.32. Substituent effect

The rate of pyrolysis of phenylazide was increased by all substituents in the phenyl residue (10,11). Ortho-substitution produced rate enhancement from 1.27 fold for methyl to five fold for chloro, cyano and methoxycarbonyl (11). Only slight increase in rate of less than two fold was observed for all meta-substituents (10). With para-substituents the rate increase was in the order of 1.55, 1.84, 2.28 and 4.70 fold for chloro, nitro, cyano and methoxy respectively (10,11). The rate enhancement was attributable to the inductive and mesomeric effect of the substituents. However, there was no simple correlation between the rates of reaction and the Hammett's substituent constants (10).

Certain ortho-substituents with α,β-unsaturation, e.g. phenylazo, nitro, acetyl and benzoyl, led to an extremely large rate increase in the thermolysis of phenylazide (11,63). These reactions were generally characterised by the formation of cyclized products. Rate enhancement of 21180, 738, 287 and 70 fold had been reported for ortho substituents of phenylazo, nitro, acetyl and benzoyl respectively. These results cannot be explained by simple inductive, mesomeric or steric effects. Therefore, these substituents must have participated in a specific role in the transition state to assist pyrolysis (11,63). Dyall and Kemp (63) concluded that the driving force for the reaction must be provided by concerted π bond reorganisation leading to the new heterocyclic ring. The activation energies of azides where pyrolysis was assisted by ortho-substituents ranged from 95 to 113 KJ mol^{-1}, whereas the activated energies for

the pyrolysis of o-substituted phenylazides which proceeded without anchimeric assistance ranged from 116 to 129 KJ mol^{-1}.

Dyall et al (11,63,66) proposed an electrocyclic mechanism to account for the concerted process in which the neighbouring group assisted in the fragmentation of the azido group (scheme 9).



Scheme 9. Decomposition of an arylazide assisted by participating ortho substituents

This mechanism requires that both the azido group and the participating neighbouring group are in the plane of the aromatic ring to facilitate bond re-organisation. Introduction of another substituent ortho to either of the groups would lead to steric hindrance and the twisting of one of the groups out of the ring plane. Consequently, a decrease in thermolytic rate was observed for 3- and 6-methyl-2-nitrophenylazide, compared to 2-nitro-phenylazide (63). A para-substituent with respect to the azido group will also influence the rate of assisted pyrolysis via a mesomeric effect (66,67). An electron withdrawing group at this position increases the

rate while an electron releasing substituent decreases it.

Alternative mechanisms have also been proposed to account for the vast differentials observed between the various participating ortho groups (68,69). The validity of any of these mechanisms still remains to be elucidated. Spagnolo et al (70) studied the decomposition of 2-azido-2'-arylbiphenyls and reported products derived from both the nitrene intermediate and a concerted cyclization mechanism.

1.4. Photolysis

The results of a photochemical reaction are frequently expressed in terms of quantum efficiency or quantum yield (Ø). This is defined as the number of moles of light absorbing substance that react for each einstein of the absorbed radiation. By the law of photochemical equivalence, one mole of the substance should be involved in the reaction as a direct result of the absorption of one einstein of radiation. Therefore, if the only reaction which occurs in a given process is the one associated with the light absorption, the quantum yield should be unity. Deviation from Einstein's law of photochemical equivalence can occur if the products formed in the primary process are involved in a secondary reaction with the substrate (71,117).

1.41. Photoinitiated autocatalytic chain decomposition (PACD)

The quantum yield of disappearance of phenylazide $\emptyset(-PhN_3)$ was highly dependent upon concentration (71,72). A $\emptyset(-PhN_3)$ value of 0.5 was obtained upon irradiation of 2×10^{-5}M phenylazide in deoxygenated acetonitrile. However, at concentrations $>3\times10^{-3}$M, the measured $\emptyset(-PhN_3)$ values greatly exceeded unit efficiency (71). The observed increase in $\emptyset(-PhN_3)$ with increasing phenylazide concentration

followed an exponential function (72). Trans-azobenzene was the only photoproduct (71,72). An autocatalytic branching chain mechanism was proposed to account for this behaviour (71,72). This mechanism involved the reaction of phenylnitrene with phenylazide to afford two phenylnitrenes, with or without the involvement of an intermediate [X] [Eq. (6)]. The chain termination steps consisted of the reaction of phenylnitrene and phenylazide and/or dimerization of two phenylnitrenes to afford trans azobenzene [Eq. (7) and (8)].

$$PhN_3 \xrightarrow{h\nu} PhN + N_2 \qquad \text{Eq. (5)}$$

$$PhN + PhN_3 \longrightarrow [X] \longrightarrow 2PhN + N_2 \qquad \text{Eq. (6)}$$

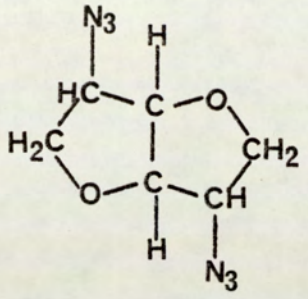
$$PhN + PhN_3 \longrightarrow Ph\text{-}N{=}N\text{-}Ph + N_2 \qquad \text{Eq. (7)}$$

$$2PhN \longrightarrow Ph\text{-}N{=}N\text{-}Ph \qquad \text{Eq. (8)}$$

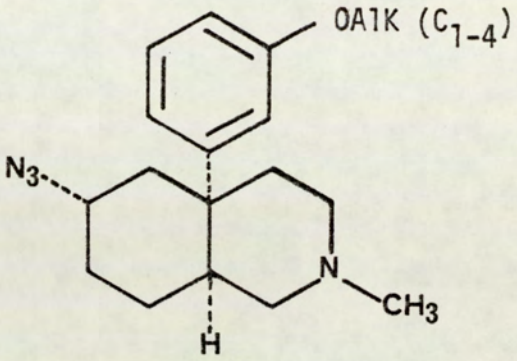
This chain reaction is quenched effectively by dissolved oxygen in solution (73).

1.5. Pharmaceutical applications of azides

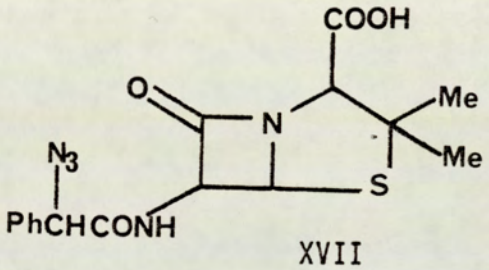
Several azides have been patented as potential pharmaceutical agents. Sulphamoyl azides of formula $R_1R_2NSO_2N_3$ were claimed to have hypotensive activity (74). β-styrene sulphamoyl azide (75) and 2-azidoacetophenone oxime (76) have also been implicated as antihypertensive agents with low toxicity. Claim of sedative activity has been made for certain azides, e.g. 1,4:3,6-dianhydro-2,5-diazido-2,5-dideoxyhexitols (XV) (77). According to Sandoz (78), substituted isoquinolines of the formula (XVI) possess both sedative and analgesic activity.



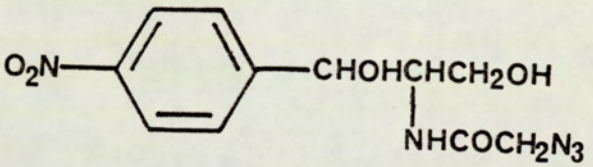
XV



XVI

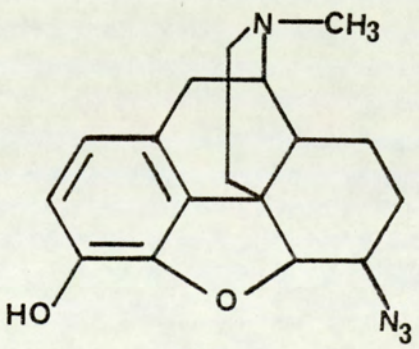


XVII



XVIII

Three azido compounds were documented in the 28th edition of the Martindale Pharmcopeia (79). These compounds which have found clinical applications are α-azidobenzylpenicillin (XVII), azidoamphenicol (XVIII) and azidomorphine (XIX). α-Azidobenzylpenicillin has the same chemotherapeutic and antibacterial activity as the parent compound but it is more stable to acid hydrolysis (80), thereby introducing the possibility of oral administration. Introduction of the azido group into morphine renders it more active than the parent compound (81), probably due to more effective blood-brain barrier penetration as a result of the lipophilic azido group.



XIX

2. PRODUCT DEVELOPMENT

Once the therapeutic efficacy of a compound is established in *in vitro* and *in vivo* laboratory studies and demonstrates potential clinical application, the candidate drug will proceed through a drug development programme. This involves preformulation, formulation and manufacture of the required dosage form(s) for clinical consumption. Ideally, acute and chronic animal toxicology studies should be carried out on the formulated product. This is, however, not always possible because of the time scale involved.

2.1. Preformulation

Preformulation is the process of establishing the optimal quality of a drug through the determination and/or definition of those physical and chemical properties considered important in the formulation of a stable, effective and safe dosage form. A preformulation study, however, is not a mere mechanical routine of performing a list of tests and gathering of data but requires a flexible approach and a critical evaluation of the study programme. The major direction of the investigation is determined by the type of compound under study and the intended route of administration. Different dosage forms require different approaches to the study programme, resulting in different emphasis being made on the in-depth study of particular physicochemical properties. For example, particle size analysis and the flow properties of powdered drug are crucial factors to be considered when formulating tablets or capsules but they are not as relevant when injections or oral liquids are the intended dosage forms.

The physicochemical properties of a compound that are commonly considered in preformulation studies include solubility, ionisation

constant, partition coefficient, decomposition profile, stability, thermal and photo effects and the kinetic studies of the degradation process. These properties would provide a better understanding of the new compound and the inter-relationship between molecular structure and drug action. These data would also provide information regarding handling precautions and optimal storage conditions for the compound.

One of the first requirement in any preformulation programme is to characterise the new drug substance by physical means such as infra-red, ultra-violet-visible or mass spectroscopy, melting point etc. Reference standards for the new compound can then be established from these specifications. Limits for purity and drug content must also be set.

Development of analytical profiles and analytical methods is of primary importance before any studies can be carried out on the new compound. The analytical methodology must be purity and stability indicating, hence it must be specific for the compound under investigation. It must also be sensitive enough to detect the presence of small amount of impurities in the parent compound. Impurities may be derived from the synthetic route or decomposition. The two major sources of impurities derived from the synthetic route are (a) the reagents and (b) the intermediates. The first category rarely poses any major problem and they are usually readily identifiable. Even if they cannot be completely removed toxicology data on these reagents are usually available to enable limits of their presence to be established. However, there are exceptions. The presence of trace amounts of certain solvents used in the preparative process is unacceptable in pharmaceuticals, especially for intravenous dosage forms. Consequently, they must be removed entirely before the raw material can be used. These unacceptable solvents

include methanol, chloroform, methylene chloride and denatured alcohol. The use of reagents of poor analytical grade can lead to impurities in the new drug substance which is very difficult to predict and hence should be avoided. Impurities due to intermediates in synthesis are more troublesome. Unless adequate separation techniques are available, small amounts of the intermediates will always be present. Limits for the presence of these compounds can only be set on a rational basis if toxicological data on them are available. The third category of impurities consists of degradation products. Attempts to isolate and identify these products are usually carried out during degradation studies under accelerated conditions.

2.2. Formulation

Formulation is the process of converting a medicament or chemical compound into a pharmaceutically acceptable dosage form, based on the information accumulated during the preformulation programme so that maximum effectiveness of the compound can be elicited. The aim of formulation is to design a preparation which is stable (>2 years shelf-life), does not alter the efficacy of the active ingredient and has a minimum amount of adverse effects. Formulation of drug dosage forms requires that attributes of drug identity, strength, dose uniformity, stability and bioavailability be taken into account, together with those properties that permit their manufacture on high speed machinery, shipment in commercial channels and storage. At the simplest, the dosage form may be a solution of the drug in water. On the other hand, it could be a complex drug delivery system. The complexity is usually not intentional but is a result of the composition that is required to achieve the objectives of obtaining maximum efficacy from the product.

For the phase I clinical trial of an antitumour agent, intravenous administration appears to be the ideal route of choice. This route would remove any variation due to inter-individual bioavailability. Therefore, any therapeutic or toxicological responses can be directly related to the injected dose. It is desirable that the formulation of clinical trial product for antitumour agents be kept as simple as possible to exclude any possible influence exerted by additives or excipients, e.g. solvents such as DMSO and DMA have been shown to have possible differentiating activity against some tumours (163). Addition of another compound may affect the efficacy or toxicity of the drug and it may also complicate the degradation process (117). Should it be necessary to include another compound such as preservative, excipients etc. in the formulation, its interaction with the drug must be evaluated and its influence on the stability of the parent compound studied.

Intravenous dosage form can be either a solution, a freeze-dried powder to be reconstituted just prior to administration or more rarely an emulsion where the particle size of the dispersed phase is carefully controlled to prevent embolism. It is required that these products must be sterile, isotonic, or nearly isotonic, if a large volume is to be infused, and free from pyrogens if the injection volume is greater than 15 ml (82). The pH of the injections must also be monitored with due care. The normal acceptable pH range for non-buffered solution is about 3.5 to 9, and for buffered solution it is about 5.5 to 8. Tonicity is not always a crucial factor to be considered, especially if the volume of injection is very small or in the case where the injection is to be added to an infusion fluid that the volume of injection added is relatively small compared with the volume of the infusion fluid. The solvent systems suitable for intra-

venous products are limited to those that produce little or no tissue irritation. By far the most frequently employed vehicle for sterile intravenous products is water. If the compound under investigation is unstable in solution a sterile freeze dried powder can be used as an alternative dosage form to overcome the problem. However, the production of a sterile freeze dried powder is more complicated and requires the use of special facilities to ensure that the lyophilized powder in the final container is sterile with the acceptable level of moisture content.

2.3. Solubility

The solubility of solids in liquids is a very important aspect in the formulation of many types of dosage forms, particularly those intended for intravenous administration. A parenteral solution avoids the disadvantages inherent in suspensions such as non-uniform dose, caking and possible slow release of the medicament when it is not required. All drugs, whatever route of administration, must possess some limited intrinsic aqueous solubility to be effective in the physiological media. The final solubility of a drug in water is governed by the sum total of the possible forms of water-water (WW), drug-drug (DD) and drug-water (DW) interactions involved in the transferral of the solute particle from the solid phase to the solution phase. The relationship between these interactions and the activity coefficient of the drug in water, γ_w, was described by Yalkowsky (83) [Equation 9].

$RT \ln \gamma_W = DD + WW - 2DW$ Equation 9

R = Gas constant T = Temperature

If DD + WW - 2DW >0, e.g. for electrolytes in water, there will be less than complete mixing and the drug will have a finite aqueous solubility; the greater the difference between the adhesive and cohesive interactions, the lower the solubility. Manipulation of any of these interactive forces can be exploited to bring about solubilization (87).

The melting point of a solid provides a good indication of the extent that drug-drug interaction is contributing to low aqueous solubility. For solids with high melting point, e.g. >200°C, drug-drug interaction is probably a significant factor in reducing solubility. However, if a drug melts below 100°C it is not likely that crystal interactions have any significant effect on solubility. The most effective way of increasing the aqueous solubility of a low melting drug is to alter the solvent characteristics in such a way as to decrease solvent-solvent and increase solvent-drug interactions by using agents such as surfactants, cosolvents, soluble complexing agents etc. Highly crystalline solutes, however, requires an additional mechanism to enhance solubility (87).

The solubility of basic or acidic organic compounds in water can often be increased by formation of more soluble salts e.g. atropine sulphate is about 2600 times more soluble than atropine base in water (84). This is probably due to more effective interaction between the charged form of the drug with water molecules. Manipulation of the pH of the vehicle can also result in enhanced solubility of an ionisable compound.

Organic solvents, e.g. dimethylformamide, dimethylacetamide or

dimethylsulphoxide, have also been employed to increase the solubility of drugs but the use of these solvents is limited by their physiological toxicities (85).

2.31. Cosolvents

Cosolvents have found important applications in parenteral dosage forms as a means of solubilising drugs for both intravenous and intramuscular administration. Parentral products on the market that are formulated in cosolvents include Valium and Dilantin, in which the drug is solubilized in 10% ethanol and 40% propylene glycol (86,87). The most frequently used cosolvents are propylene glycol, ethanol, glycerine and polyethylene glycol. The degree to which the solubility of a drug can be increased for a particular cosolvent is dependent upon the non-polarity of the drug and the cosolvent.

The use of cosolvents, however, can lead to problems of toxicity, reduced blood compatibility and injection difficulty because of increased vehicle viscosity. Therefore, intravenous formulation should contain a minimum amount and low concentration of the cosolvents to reduce these effects, and in particular, to prevent haemolysis <10% ethanol (88), <32% propylene glycol (89), <40% polyethylene glycol (90) in large volume infusion fluids are required. Precipitation of drugs solubilized in cosolvents is a potential problem when the formulation is diluted into infusion fluid or in the blood stream (87). Precipitation at the injection site can cause local irritation or embolism and it also reduces the amount of drug delivered to the required site. To overcome this problem, a solubility curve can be constructed to predict the behaviour of the drug and cosolvents on dilution (87,91).

2.32. Surfactants

Poorly water soluble drugs can often be solubilized in surfactant solution above the critical micelle concentration (92,93); the concentration at which the individual surface active agents form molecular aggregates or micelles, with the hydrophobic groups of the individual molecules orientated towards the centre of the micelle. Solubilization is achieved by adsorption or incorporation of the drug molecules onto or into the molecular aggregates. Although surfactants are included in a wide range of pharmaceuticals as minor adjuvants, very few products actually utilise micellar solubilization in their formulation.

Micelles are not static entities and their sizes, and consequently their solubilization capacity, change with changes in the physicochemical environment. Factors such as temperature (94,95); pH (86,97); presence of other substances, e.g. polymers (98,99), alcohol, electrolytes (93) etc., can markedly affect the solubilizing efficiency of a surfactant. Dilution of a micellar solution to below the critical micelle concentration results in deaggregation of the micelles and release of the solubilized drug. If the drug is in excess of its aqueous solubility, precipitation ensues.

One of the limiting factors for the use of micellar solubilization in formulation is that relatively large concentrations of surface active agents are required to solubilize small amount of drug: Althesin, an intravenous anaesthetic, contains 20% w/v of a non-ionic surfactant cremophor EL (79). Therefore, surfactants can only be effective for solubilizing high potency drug. In addition, the possible short or long term adverse toxicological effects of the surfactants on the body must be taken into account when considering micellar solubilization in the formulation of new compound.

Surfactants are more toxic when given intravenously than orally. Anionic and cationic agents are strongly haemolytic, which precludes their use in solubilized parenteral preparations (92,93). Another disadvantage of using surfactants is the possible concomitant solubilization of other ingredients, such as preservatives, thus reducing their efficiency.

Soluble surface active polymers have also been used as solubilising agents (93,100). However, their mechanisms of solubilization is still ambiguous (93). It is thought that these hydrophilic polymers form monomolecular micelles at low concentrations but at high concentrations they form aggregates of varying size which have the ability to solubilize drugs.

2.33. Complexation

Reversible complexation between drug and ligand has been employed to overcome pharmaceutical solubility problems. The total solubility of a drug through complexation depends on (a) the inherent solubility of the substrate, (b) the stability of the complex, and (c) the concentration of the ligand. When the concentration of free ligand in solution equals its inherent solubility in that medium, further addition of ligand has no effect on the total solubility (101). The application of complexation to the solubilization of drug is generally limited, since complexation between organic molecules usually results in only a small increase in apparent solubility.

The apparent solubility of an antitumour agent, hexamethylmelamine, in aqueous solution was increased by complexation with gentisic acid (102). The solubility enhancement ranged from 5 to 90 fold, depending on the pH of the medium and total gentisate ion concentration. 3-Methylgentisic acid was used to enhance the

solubility of another cytotoxic agent, acronine, by >400 fold (103). Short term toxicity of gentisate ion injected intravenously into rabbits was studied and found to cause mild vaso-irritation (103).

Cyclodextrins have also been investigated as solubilizing agents. They are oligosaccharides that are capable of forming inclusion complexes by taking up various drug molecules into their central cavity. These complexes are generally stable in aqueous solution. The use of β-cyclodextrins to solubilize drugs is, however, frequently limited by their own low aqueous solubility. A marked improvement in solubilization efficiency was reported when the more water soluble alkyl-derivatives of β-cyclodextrins were used (104). Solubilization of diazepam, digitoxin, hydrocortisone and indomethacin was achieved using these derivatives and a linear increase in the solubility of these compounds with concentration of the cyclodextrin derivatives was observed (104).

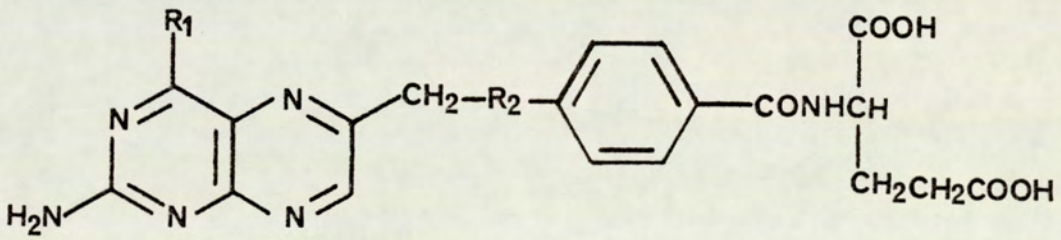
2.34. Oil in water emulsions

Parenteral oil in water emulsions have been used to overcome the solubility and stability problems of selected drug substances for which intravenous formulations are desired but difficult to develop. Methyl CCNU (105) and an investigational drug NSC No. 278214 (106) are both antineoplastic agents of low aqueous solubility and the latter compound is also liable to hydrolysis. Intravenous administration of these drugs was made possible by employing Intralipid fat emulsion as a vehicle. The drug was first dissolved in absolute alcohol (105) or dimethylacetamide and/or cremophor (106) and then incorporated slowly into the emulsion. The disadvantage of such preparations is that they are unstable and consequently have to be reconstituted just prior to administration. Alternatively, oil in

water emulsions can be prepared by dissolving the drug in a fixed oil, followed by emulsifying the oil in water (107). This preparation, however, is limited by the number of drug substances that will dissolve in the oil and the toxicity of the oil when given intravenously (85). For intravenous administration, it is crucial that the oil droplets in the emulsion are sufficiently small to prevent embolism; size of the droplets must be monitored carefully. Preparation, properties and quality control of sterile intravenous oil in water emulsions have been well described by Hansrani et el (108).

3. ANTIFOLATES

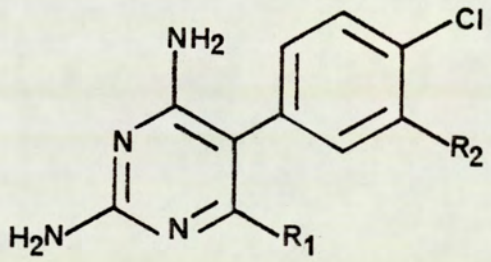
The use of antifolates in cancer chemotherapy was pioneered in 1948 by Farber and coworkers (111) who introduced aminopterin (XX) into the clinic and obtained temporary remissions in acute childhood leukaemia. Further research led to the discovery of the structurally similar but less toxic methotrexate (MTX, XXI) (112). Both these compounds exert their antitumour activity *via* the inhibition of dihydrofolate reductase (DHFR), a key enzyme involved in the transfer of one carbon units required for the *de novo* biosynthesis of nucleic acids and several essential amino acids (113). Even after ~40 years in the clinic, MTX remains one of the most important agents in cancer treatment. It is currently used in the treatment of a variety of tumours, e.g. acute leukaemia, small cell lung carcinoma, breast cancer, lymphoma, osteosarcoma etc. (114). Both aminopterin and MTX are termed "classical" antifolates because they have structures that closely resemble the substrate, folic acid (XXII).



		R_1	R_2
XX	Aminopterin	NH_2	NH
XXI	MTX	NH_2	NMe
XXII	Folic acid	OH	NH

Compounds that do not bear a structural resemblance to folic acid were also shown to be capable of inhibiting DHFR. The diamino-pyrimidines were the first examples of "non-classical" antifolates to

be synthesized (115). Amongst the compounds discovered in this class were pyrimethamine (XXIII), metoprine (XXIV) and etoprine (XXV) (116). Subsequent structure activity studies revealed that a lipophilic substituent at position 5 of the 2,4-diaminopyrimidine moiety greatly enhanced the affinity of the compounds to DHFR (117,118). Protonation at the N-1 position is essential for tight binding between the drug and enzyme (116). Metoprine and etoprine underwent clinical trials in the 1950s, but had to be withdrawn because of unacceptable toxicities despite significant antileukaemic effect obtained (119). Other "non-classical" antifolates synthesized include triazines (120), 2,4-diaminoquinazolines (121), adamantylpyrimidines (122) and pyridopyrimidines (123).



		R_1	R_2
XXIII	Pyrimethamine	Et	H
XXIV	Metoprine	Me	Cl
XXV	Etoprine	Et	Cl

Despite its extensive applications, the effectiveness of MTX is limited by (a) lack of substantial activity against solid tumours, (b) minimal penetration of blood brain barrier or cerebral spinal fluid (CSF), (c) broad spectrum of toxicities, and (d) development of drug resistance (114,116). Since MTX is a polar and non-lipid soluble molecule, it cannot penetrate cells adequately by simple passive diffusion but has to rely on the folate carrier active transport system (114). Impairment of this transport system is, therefore, one of the mechanisms by which the cells can acquire resistance to MTX

(124,125). Acquired resistance can also be mediated through elevated levels of DHFR (126) and altered affinity between the drug and enzyme (127). In vitro studies of MTX resistance revealed that the initial step was often the impairment of transport mechanism, followed by changes in DHFR resulting either in an increase in activity or an altered affinity, or both (128).

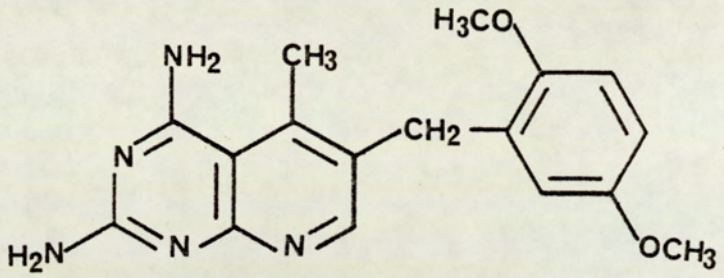
Many analogues of MTX were synthesized in an attempt to improve the therapeutic index of the drug. Nearly all bind as well to DHFR, some even better, but as anticancer drugs, they offer little or no improvement (116). Attention was then focussed on the "non-classical" liphophilic antifolates as potential candidates to overcome some of the limitations encountered by MTX. The rapid entry of these agents into cells, without dependence on carrier mediated transport, may allow drug delivery to cells or body compartments where MTX enters very slowly or is excluded, e.g. in transport mediated resistant cells, CSF, brain and other lipid rich tissues etc. (129). These compounds are, therefore, potentially useful in treating brain or solid tumours. The higher levels of these drugs accumulated intracellularly offers further potential usage in treating resistant cells with elevated DHFR level. Moreover, some "non-classical" antifolates were found to inhibit a different site, e.g. thymidine synthetase, in the folic acid metabolism (130,131,132). Certain diaminopyrimidines were also reported to have a second folate independent site of inhibitory action (122). A different site of inhibitory action is potentially useful in overcoming drug resistance mediated through the altered affinity between drug and DHFR.

Interest in the "non-classical" antifolates, particularly the diaminopyrimidines, was rekindled in the 1970's because of their aforementioned potential applications. Attempts were made to exploit

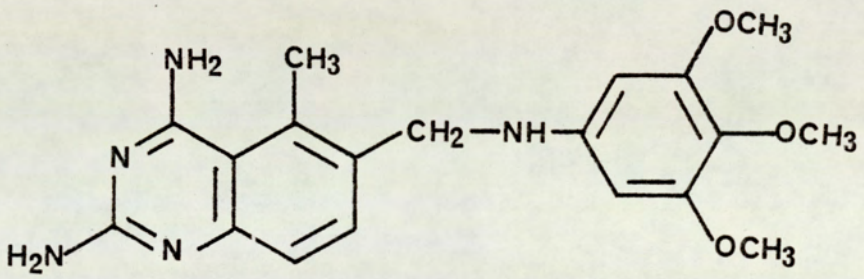
the liphophilic nature of these compounds in cancer treatment. Pyrimethamine was used successfully to induce remission of >6 months in meningeal leukaemia (133). Metoprine was re-evaluated as an antitumour agent (134). New understanding of its pharmacokinetics (129,135) suggested that the toxicities of metoprine associated with earlier trials could be a result of unsatisfactory dosing schedule. Therefore, the effectiveness of metoprine as an anti-neoplastic agent was again tested in the clinics (136,137,138). However, the efficacy of the drug at tolerated dose was again found to be limited. The untoward effects of metoprine at limiting doses included severe headache, skin rash, gastrointestinal discomfort, nausea, leucopenia and thrombocytopenia. Part of the observed toxicities is believed to be mediated through the inhibition of the enzyme histamine-N-methyl-transferase (139). Other diaminopyrimidines were also reported to interfere with histamine metabolism (139,140). The additional disadvantage of metoprine is its long and protracted plasma half-life, $\sim$ 216 hours, (129) which not only prevents satisfactory achievement of optimum schedule of drug administration but also contributes to the observed toxicities.

Therefore the search is on to discover a "non-classical" anti-folate which would overcome the limitations encountered by MTX, but devoid of the toxicities observed in metoprine. Amongst the novel liphophilic antifolates currently under investigation are BW301U (XXVI) (132), trimetrexate (XXVII) (141,142) and DAMP (XXVIII) (143). BW301U is a potent inhibitor of DHFR and under experimental conditions the toxicities associated with metoprine administration were not observed (132). Both trimetrexate and DAMP, however, have the disadvantage that they interfere with histamine metabolism (140). Moreover, trimetrexate does not penetrate brain tissues or CSF to any

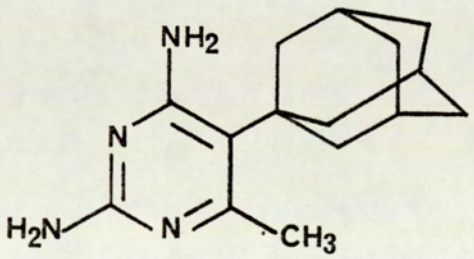
greater extent than MTX despite its improved lipophilicity (141,142). There are, therefore, avenues open for developing novel lipophilic antifolates.



XXVI BW 301U



XXVII Trimetrexate

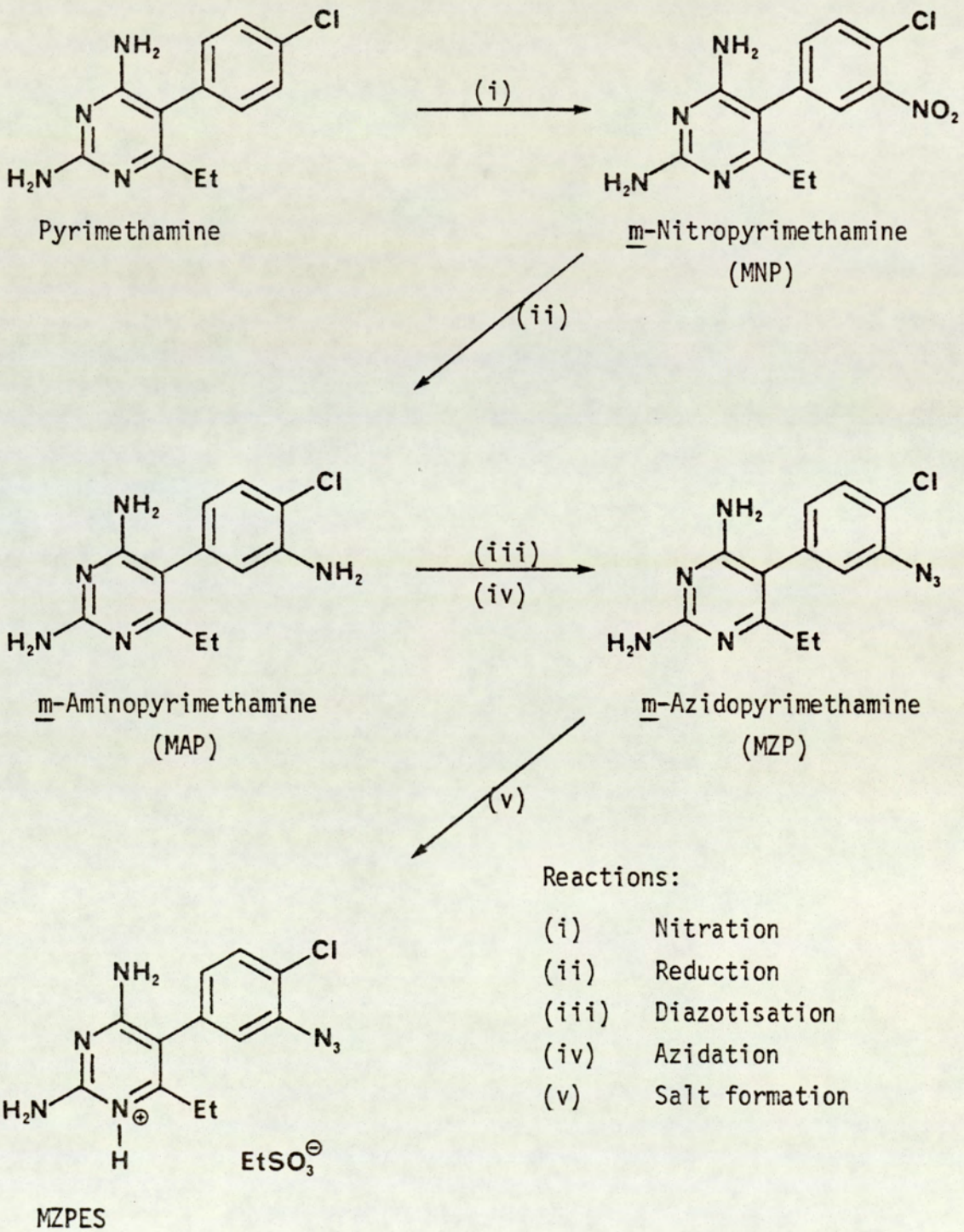


XXVIII DAMP

3.1. m-Azidopyrimethamine (MZP)

m-Azidopyrimethamine [2,4-diamino-5-(3-azido-4-chlorophenyl)-6-ethylpyrimidine] is a novel "non-classical" lipophilic antifolate synthesized by Stevens and coworkers (144) at Aston University in 1978. The azido group is introduced into the antimalarial drug, pyrimethatmine, via a simple three steps reaction (scheme 10). The ethanesulphonic acid salt of the compound was synthesized to overcome anticipated pharmaceutical problems of the low aqueous solubility of the free base. The electronic effect of the azido substituent resembles that of halogens (7) and therefore MZP is expected to have similar lipophilic characteristics of metoprine and etoprine. The plasma half-life of MZP, however, would hopefully be short since the azido group is very susceptible to degradation and biotransformation (6,7). The potential of the azido group to be transformed to reactive nitrene species (6,7), themselves capable of versatile interactions with biological substrates, makes this agent an intriguing prospect for study.

Scheme 10. Synthesis of MZPES



Pyrimethamine

m-Nitropyrimethamine (MNP)

m-Aminopyrimethamine (MAP)

m-Azidopyrimethamine (MZP)

MZPES

Reactions:

(i) Nitration
(ii) Reduction
(iii) Diazotisation
(iv) Azidation
(v) Salt formation

3.11. Antitumour activity

The antifolate activity of MZP was evaluated using L1210 leukemia DHFR and an inhibition constant of 2.4 ± 0.16 nM was found between inhibitor and enzyme (144). This compares well with the activity of etoprine which has a ki of 0.6 ± 0.06 nM (144). *In vitro* studies showed that MZP is a hundred times less cytotoxic than MTX against the L5178 cells but ~twenty times more effective at killing MTX-resistant L5178 cells (145). This is, therefore, one avenue of activity that can be exploited to justify the introduction of MZP into the clinic.

MZP was tested against a panel of experimental murine tumours at the National Cancer Institute (NCI) (146) and the results are summarised in Table 1 (147). Test results for metoprine (147) and MTX (148,149) are also included for comparison. MZP appears to be a more superior antitumour agent than metoprine, against the panel of tumours tested. Although B16 melanoma and M5076 reticulum cell sarcoma were resistant to MTX, they were most responsive towards MZP. Both MZP and MTX were active against the leukemia cell lines; MZP, however, shows very little activity against Lewis lung carcinoma, $CD8F_1$ mammary and colon 38 tumours.

Table 1. Experimental murine antitumour activity of MZP

Tumour	Optimum T/C[a](%) MZP	Assessment [b] MZP	Metoprine	MTX
P388 leukaemia	151	+	+	++
L1210 leukaemia	158	++	+	++
B16 melanoma	157	++	-	-
TLX5 lymphoma	135	+	-	NT
M5076 reticulum cell sarcoma	174	++	NT	-
Lewis lung carcinoma	<140	-	-	+
$CD8F_1$ mammary	24	-	-	+
Colon 38	>42	-	-	-

a The ratio of the median survival time (days) for treated (T) and control (C) animals expressed as a percentage value.

b NCI activity criteria in "Chemical Structures of interest to the division of cancer treatment" vol III, 1983.

NT Not tested.

3.12. Toxicology

The toxicology of m-azidopyrimethamine ethanesulphonate (MZPES) was evaluated at Elm Farm Laboratories of Life Science Research (150). The acute intravenous (IV) and intraperitoneal (IP) toxicity of MZPES was investigated in a group of male mice of the Charles River CD-1 strain at dosages of 15,22,33,42 and 50 $mgkg^{-1}$ or 19,33,57,80,100 and 173 $mgkg^{-1}$ respectively. The acute median lethal

dose (LD_{50}) and 10% lethal dosage (LD_{10}) together with their 95% confidence limits were determined (Table 2). From the LD_{10} value in mice, the starting dose in humans for Phase I clinical trials was calculated and found to be 5.4 mgm^{-2}. This represents 1/10 of the LD_{10} dose in mice.

Table 2. Lethal doses of MZPES in mice

Route		Dose ($mgkg^{-1}$)	95% Confidence interval
Intravenous	LD_{50}	44	29-59
	LD_{10}	18	9-27
Intraperitoneal	LD_{50}	68	60-76
	LD_{10}	53	43-64

The majority of deaths by both routes of administration occurred within 5 minutes and 0.25 hour of dosing, respectively, without any observed *ante mortem* signs. The clinical signs amongst mice surviving treatment with MZPES included decreased motor activity and/or muscle tremor following IV or IP administration of all test dosages, and clonic convulsions at the highest of the IV dosage levels. No indication of delayed toxicity associated with either route of administration was observed. There was no evidence of obvious toxicity in the blood or bone marrow observed, following a 28 days' study. Macropathological examination of the principle organs and tissues of mice treated with MZPES at an IP dosage of 55 $mgkg^{-1}$ revealed no significant lesions other than a very minor change of the semiferous tubules of a single animal.

The effect of MZPES on the inhibition of histamine-N-methyltransferase was also investigated. It was found to be a hundred times less active than metoprine in inhibiting the enzyme (151) (Table 3). The activity of MZPES resembles that of pyrimethamine which produces no clinical problems via the interference of histamine metabolism. Therefore, certain toxicities associated with metoprine administration should be absent in MZPES.

The antitumour activity of MZP and its minimal activity against histamine-N-methyltransferase render it a very promising compound which warrants investigation at the clinical trial level.

Table 3. Activity of MZPES and analogues against histamine-N-methyltransferase

Drug	Concentration	% Inhibition
MZPES	1×10^{-4}M	71
Pyrimethamine	1×10^{-4}M	69
Metoprine	1×10^{-6}M	81

3.2. Aims and scope of current study

The current study focussed on one particular arylazide, MZPES. This is the first aryl azide to undergo clinical trial for any indication. Hence, it is imperative to fully understand the chemistry and the chemical and physical stability of this class of compound. The main aim of the project was to develop a safe, effective and stable parenteral dosage form of MZP to be utilized in the Phase I clinical trials. This involved an in-depth study of the physical and

chemical characteristics of the compound. To facilitate the evaluation of these properties, an analytical methodology was developed for quantitative and qualitative studies.

For reasons mentioned earlier, the physicochemical properties of a drug that are considered important in the formulation of parenteral dosage forms include solubility, ionisation constant and partition coefficient (Section A, 2). Various methods of solubilising MZP and/or its salts were investigated to provide a general guideline on the solubilization of lipid soluble drugs. The effects of substituents on ionization and partition coefficient were evaluated by studying compounds related to MZP.

It was also the aim of the present study to investigate the stability of MZPES, the salt of MZP chosen for formulation development, in a pharmaceutical system and to identify those factors which might affect this stability. Studies with other arylazides revealed that this class of compounds was particularly sensitive to thermolysis and photolysis. Therefore, the effect of heat and radiation on MZPES was investigated extensively.

Part of the present project also followed the progress of MZPES in Phase I clinical trials. The untoward effects associated with MZPES administration were identified. The pharmacokinetics of the compound was compared with that of other lipophilic antifolates.

SECTION B

MATERIALS AND METHODS

1. CHEMICALS AND SOLVENTS

1.1. 2,4-Diamino-5-(3-azido-4-chlorophenyl)-6-ethylpyrimidinium ethanesulphonate [m-azidopyrimethamine ethanesulphonate, MZPES; Ref: 26W83WA] and 2,4-diamino-5-(4-chlorophenyl)-6-ethylpyrimidine [Pyrimethamine] were kindly supplied by the Wellcome Foundation Ltd., U.K.

1.2 2,4-Diamino-5-(3-amino-4-chlorophenyl)-6-ethylpyrimidine [m-aminopyrimethamine, MAP], 2,4-diamino-5-(3-nitro-4-chlorophenyl)-6-ethylpyrimidine [m-nitropyrimethamine, MNP], 2,4-diamino-5-(3-amino-4-chlorophenyl)-6-ethylpyrimidinium ethanesulphonate [MAPES], 2,4-diamino-5-(3-nitro-4-chlorophenyl)-6-ethylpyrimindinium ethanesulphonate [MNPES], 2,4-diamino-5-(4-chlorophenyl)-6-ethylpyrimidinium ethanesulphonate [pyrimethamine ethanesulphonate], 2,4-diamino-5-(3-chloro-4-aminophenyl)-6-ethylpyrimidine [ISOMAP], 2,4-diamino-5-(3-chloro-4-nitrophenyl)-6-ethylpyrimidine [ISOMNP] and 2,4-diamino-5-(3-chloro-4-azidophenyl)-6-ethylpyrimidinium ethanesulphonate [ISOMZPES] were generously supplied by Dr R J Griffin of Aston University.

1.3. Dipotassium hydrogen orthophosphate, BDH Analar grade, and orthophosphoric acid 88% SG 1.75, Fisons SLR grade, were used in the preparation of phosphate buffers.

1.4. Citric acid, tri-sodium citrate, L(+)lactic acid and sodium lactate, used in the preparation of citrate and lactate buffers, were supplied by Sigma Chemical Company Ltd.

1.5. The perfluorocarbons used in the present project were kindly supplied by Dr P Coe of Birmingham University.

1.6. Methanol used in high performance liquid chromatography (HPLC) analysis was either supplied by Fisons or May and Baker. HPLC grade of the solvent was employed in all the analyses carried out in this project

1.7. All other chemicals used in this work were obtained commercially as Analar grade and used as supplied.

2. INSTRUMENTS

2.1. HPLC Analysis

Several HPLC systems were employed in the analysis of MZPES and its degradation products. Routine quantitative HPLC assay of the compound was accomplished using a Waters Associates trimodular system consisting of a M720 system controller, M710B WISP with an automated injector and a carrier system accommodating 40 sample stations, and an M730 data module. Delivery of mobile phase was achieved using two of the model 6000A or 510 Waters solvent delivery systems. A Lambda-max 480 variable wavelength spectrophotometer was used as a detector. Throughout the entire study, Waters C_{18} reversed phase radial compression columns were used with C_{18} guard pak inserts. RCM-100 radial compression modules were used to hold the columns. Towards the end of the present project, this system was improved by introducing a Waters 840 data and chromatography control system with a Waters interface module instead of M720 system controller and M730 data module. A Waters 490 programmable multiwavelength 4 channels UV detector was also introduced. This new system enabled both simultaneous monitoring at several wavelengths and data generated to

be stored on disc so that the integration parameters for each sample could be optimized.

Part of the development of analytical methodology was carried out on a composite system consisting of two Altex 100A pumps, an Altex system programmer and a rheodyne loop injector with a sample loop size of 20 ul, together with a Lambda-max 480 variable wavelength detector, RCM-100 radial compression module and a LKB 2210 1-channel recorder.

The majority of the analyses of degradation products and method development were performed using a system comprising an Altex 100A pump, a rheodyne loop injector (20 ul), a RCM-100 radial compression module, a Pye Unicam LC-UV detector and a LKB 2210 1-channel recorder.

2.2. Electronic absorption

The UV absorbance measurements in the solubility experiments were achieved using a Beckman DU7 spectrophotometer. In the determination of ionization constants, the UV absorbance was monitored using a Unicam SP800 UV spectrophotometer.

2.3. Weighings and volume measurements

Accurate weighing of compounds was accomplished using a Sartorius 1207 MP2 four decimals electronic balance. Weighings >500 mg, however, were performed on a Sartorius 1219 MP balance. Volume of solutions was measured using Eppendorf fixed volume pipettes of 10,20,50,100,200,500 and 1000 ul. Volumes of 1 to 5 mls were pipetted using the Gilson pipetteman pipette of variable volume.

2.4. pH measurements

All the pH measurements in this project were accomplished using a Philips PW9410 digital pH meter. BDH buffer tablets of pH 4.0 and 7.0 were used to produce solutions for the calibration of the pH meter.

2.5. Centrifuge

Where centrifugation was required as a separation technique, the Heraeus Labofuge 6000 centrifuge was employed.

3. DEVELOPMENT OF ANALYTICAL METHODOLOGY

Attempts were made to develop analytical methods that would separate MZPES from the related compounds such as pyrimethamine, MAP and MNP.

3.1. Thin layer chromatography (TLC)

Solutions of MZPES and its analogues were prepared in absolute ethanol. A solution consisting of a mixture of these compounds was also prepared. The solutions were spotted onto TLC plates with the aid of micropipettes (~50 ul). The TLC plates were chosen to include a variety of adsorbents. Amongst the TLC plates used in the study were:-

(a) Silica gel plates - Plastikfolien Kieselgel $60F_{254}$

(b) Freshly prepared 0.25 silica gel plates (30g Kieselgel $60F_{254}$ in 60 ml water)

(c) Freshly prepared alumina plates (30g aluminium oxide in 60 ml water)

(d) Cellulose plates

The solvent systems were chosen to include as wide a variety of solvents and polarities as possible. A base, e.g. pyridine, triethylamine or ammonia, was added to improve the development of the compounds on the chromatograms.

3.2. Electronic absorption spectra

UV-visible spectra of MZPES and its analogues were determined in the range 200-700 nm. The absorption maxima for each compound were recorded.

The use of UV absorbance as a method of quantifying drug content of MZPES in solution was evaluated. Solutions of MZPES at different concentrations were prepared. The concentration range used was between 0.01 and 0.08 $mgml^{-1}$. UV absorbance of the solutions at 280 nm was measured. The absorbance of the solutions was then plotted against their concentrations, and the relationship between the 2 parameters determined. Several samples at each concentration were prepared to determine the accuracy of the method.

3.3. High performance liquid chromatography (HPLC)

An HPLC method was developed to separate MZPES and some of its analogues which were potential impurities in the sample. This method was based on that described by Levin and coworkers (152). The conditions chosen were such that optimum separation between peaks and a relatively short analysis time were achieved (see Results Section).

This method was also evaluated for quantifying drug content in solutions. Solutions of MZPES at various concentrations, 0.1 to 0.8 $mgml^{-1}$, were prepared. To each ml of MZPES solution, a known volume of pyrimethamine solution was added as an internal standard. Several samples were prepared at each concentration so that error incurred

during dilutions or instrumental error could be evaluated. The peak height or peak area ratios of MZPES to pyrimethamine were calculated. These values were then plotted against the concentration of MZPES in the samples. The linearity of the relationship between the two parameters was evaluated.

An HPLC method was also developed to study the decomposition products of MZPES. A photo-degraded solution of MZPES was used to evaluate the efficiency of this method. Attempts were made to separate the different peaks present as decomposition products. Gradient elution of various compositions was tried. Different isocratic mobile phases were also employed to try to optimise peak separation. Eventually, an isocratic system derived from a change in the composition of the mobile phase in the above quantitative method was employed. All mobile phases used in HPLC analysis were passed through a scinter glass filter size 4 for degassing and removal of any particulate matters.

4. SOLUBILITY

The solubility of MZPES and related compounds in water and/or other solvents at room temperature was determined by UV absorbance measurements. Calibration curves of absorbance vs concentration were constructed for each compound. Two main methods were employed to achieve a saturated solution. The first method involved shaking excess solid in solution on a Fisons Whirlimixer at near maximum speed for 5 to 10 minutes. The violent vibration employed was similar to ultra-sonificatin and hence should aid dissolution. However, it was later found that for drugs with very slow dissolution rates, this method of agitation was not sufficient to produce a saturated solution within the time employed; another method was, therefore,

used. This involved heating the solid/drug in solution at 80-100°C for 5 to 10 minutes with constant stirring. Solid compound was added until excess was present. The solution was cooled to room temperature to allow precipitation of excess drug in solution as a result of elevated temperature. The solution was then centrifuged at 3000 r.p.m. for 10 minutes. UV absorbance of the supernatant or a dilution of the supernatant was measured. The solubility of the compound was then determined from the calibration curve.

5. IONISATION CONSTANT

The ionisation constants of MZP and its analogues in aqueous solution were measured using a spectrophotometric method (153). This method exploits the differences in the UV absorption spectra between the neutral molecules and the ionized species. Phosphate buffers of 0.02 or 0.05M with pH ranging from 2.8 to 9.14 were prepared. The compound under study was dissolved in distilled water at a concentration such that, when diluted 1 in 2, would give an absorbance value near to 1.0 at the absorption maximum with the highest extinction coefficient. This was not always achieved because of the limitations imposed by the low aqueous solubility of certain compounds. The aqueous drug solutions were then mixed with equal volume of each of the buffer solutions. The pHs of the resulting solutions were measured. The UV absorbance of the solutions was scanned between 450-200 nm. From the absorption spectra, the pKa of the compound was calculated.

6. PARTITION COEFFICIENT

The lipophilicity of MZP and related compounds was evaluated by studying their partition characteristics between octanol and an

aqueous phase. Initially, distilled water was used as the aqueous phase. This was, however, found to be unsatisfactory because the pH of the distilled water obtained in our laboratory ranged from 5 to 7. Consequently, variation in log P of the same compound was observed from day to day. Therefore, phosphate buffer 5 mM at pH 7.4 was chosen as the aqueous phase. The compound under study was dissolved in the aqueous phase. An aliquot of this solution was analyzed by HPLC. 250 mls of the remaining solution was then shaken with 10 or 20 mls of octanol in a 500 mls separation flask. The immiscible phases were brought into close contact by constant inversion of the flask for 20-30 minutes. Prolonged agitation was not necessary as Leo et al (109) reported that equilibrium was achieved for many substances with a mere 50 simple repeated inversion. The two phases were allowed to stand for a further hour before the aqueous phase was collected. An aliquot of this phase was centrifuged and the clear solution analyzed by HPLC. The knowledge of the amount of solute present in the aqueous phase before and after shaking with octanol enabled the log P of the compound to be calculated using the formula:-

$$\text{Log P} = \text{Log} \frac{\text{concentration of solute in octanol}}{\text{concentration of solute in phosphate buffer}} \times \frac{\text{volume of buffer}}{\text{volume of octanol}}$$

7. THERMOLYSIS

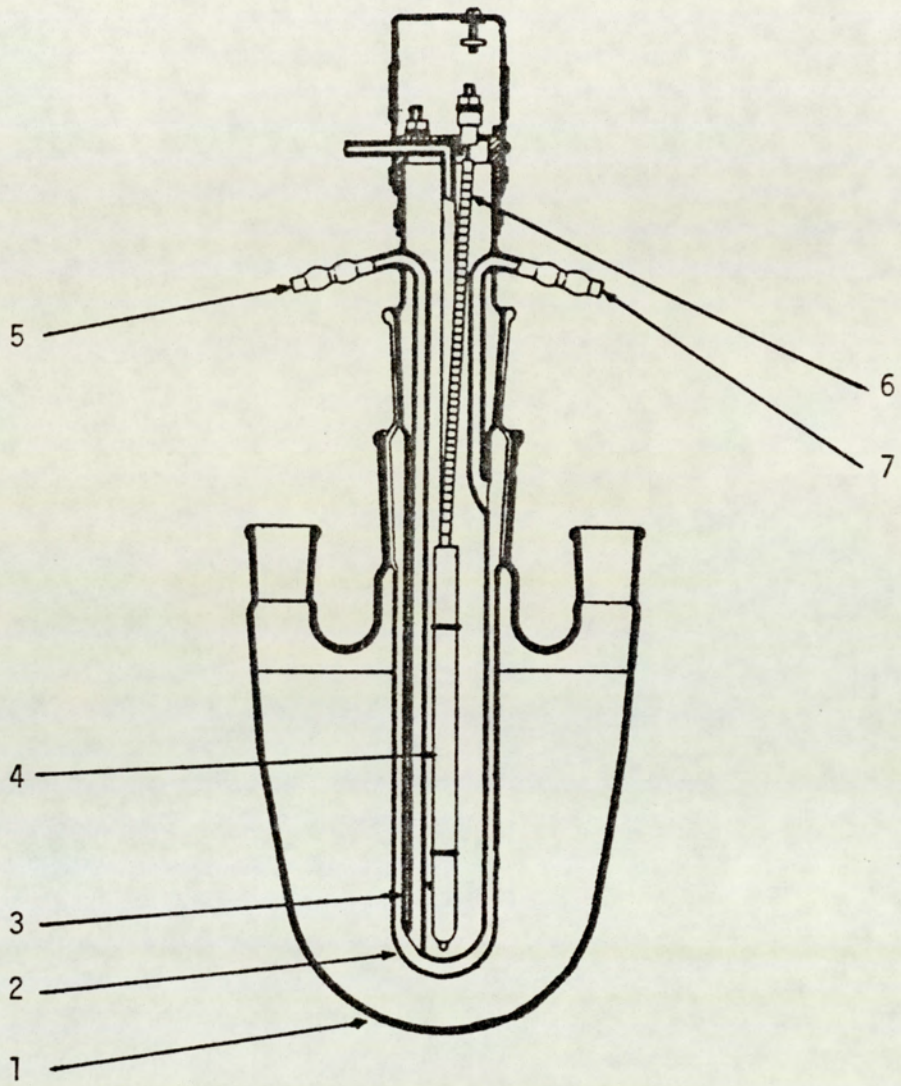
Solutions of MZPES in distilled water at concentrations 1,5 or 10 $mgml^{-1}$ were prepared. The solutions were then packed into 1 ml amber glass ampoules and sealed. Initial studies investigated the effect of autoclaving MZPES solutions at 115 or 121^{o}C. The effect of heating MZPES in buffer solutions of different pHs was also investigated.

In accelerated stability studies, ampoules of aqueous MZPES solutions were heated in a thermostated oil bath at elevated temperatures, 120-160^{o}C. Uniform temperature was ensured by means of a constant speed mechanical stirrer. Once the required temperature was attained, the ampoules were lowered into the bath in a wire basket. The temperature of the bath was monitored constantly with a thermometer. The ampoules were sampled in pairs at 5 minutes' interval initially. The sampling interval, however, was increased as heating time progressed. The samples were analyzed for MZPES content in solution by HPLC.

8. PHOTOLYSIS

Photolysis of MZPES or related compounds was carried out in a Hanovia photochemical reactor (Fig. 1). A medium pressure arc tube was used. This emitted radiation predominantly at 254, 265, 297, 313 and 366 nm. Cooling water was continuously passed through the space between the inner and outer jacket to maintain the temperature of the reagents as low as possible, 15-20^{o}C, in order to avoid any complications due to thermal reactions.

The compound under study was dissolved in water to produce a litre solution. The concentration of the solution varied between 0.1 and 1.0 $mgml^{-1}$ as required. The solution was then placed in the reaction vessel and constant agitation was achieved by means of a magnetic stirrer. The solution was saturated with either oxygen, nitrogen or compressed air by bubbling the gas through the solution for 20-30 minutes prior to irradiation. The passage of gas through the solution was continued throughout the course of irradiation. Samples of the solution were taken from the reaction vessel at



1. Pyrex flask 1L
2. Outer quartz jacket
3. Inner quartz jacket
4. Mercury vapour arc tube
5. Cooling water inlet
6. Ceramic insulated leads from terminals to arc tube
7. Cooling water outlet

Fig. 1. Hanovia Photochemical Reactor

appropriate time intervals. Initially, samples were taken at 1 or 2 minutes' interval but as photolysis progressed the sampling interval was increased to 15-30 minutes. The samples were analyzed for MZPES content by HPLC.

9. SYNTHESIS OF SALTS OF MZP

MZP phosphate was synthesized by heating equimolar quantities of MZP and orthophosphoric acid in distilled water. The amount of water added was such that it was just sufficient to bring MZP into solution. MZP Phosphate was recrystallized from water and characterized by IR (Fig. 2) and elemental analysis [Found: C,36.7%; H,3.91%; N,24.41%. $C_{12}H_{14}N_7ClO_4P(H_2O)_{\frac{1}{2}}$ requires C,36.33%; H,4.06%; N,24.71%]. The infra-red spectra of MZP (Fig. 3) and MZPES (Fig. 4) are included for comparison.

MZP citrate was prepared by the same method using citric acid instead of phosphoric acid. MZP citrate was characterized by IR (Fig. 5) and elemental analysis [Found: C,45.14%; H,4.29%; N,20.61%. $C_{18}H_{19}N_7ClO_6(H_2O)_{\frac{1}{2}}$ requires C,45.53%; H,4.46%; N,20.65%].

MZP lactate was prepared by heating equimolar quantities of MZP and L(+) lactic acid in absolute ethanol. Sufficient ethanol was added so that the compounds just dissolved on heating. MZP Lactate was recrystallized from ethanol and characterized by IR (Fig. 6) and elemental analysis [Found: C,47.18%; H,4.94%; N,25.57%. $C_{15}H_{18}N_7ClO_3$ required C,47.44%, H,4.78%; N,25.82%].

10. SYNTHESIS OF MNPES

m-Nitropyrimethamine ethanesulphonate (MNPES) was synthesized by heating equimolar quantities of MNP and ethanesulphonic acid in distilled water. The amount of water added was such that it was just

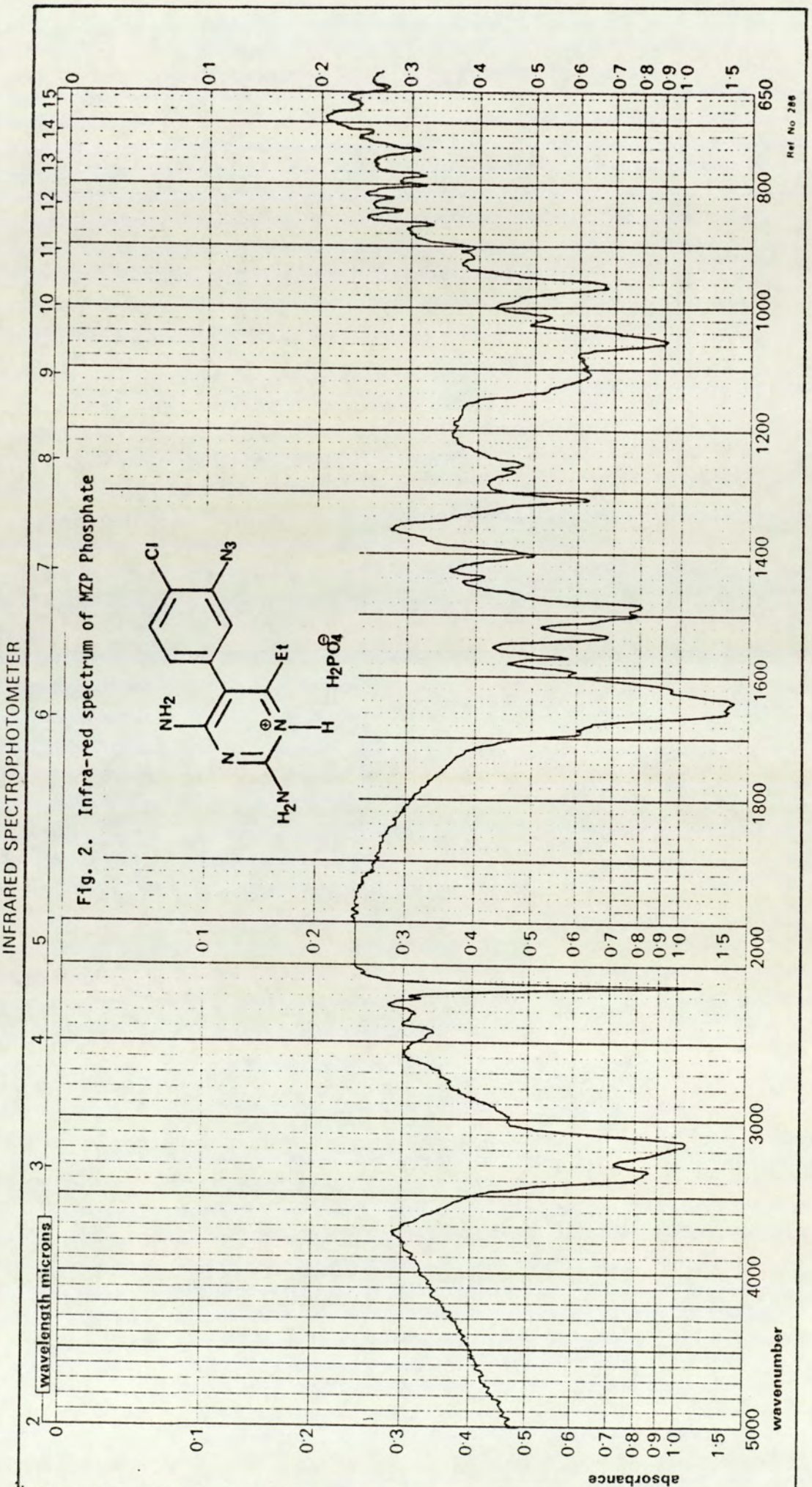


Fig. 2. Infra-red spectrum of MZP Phosphate

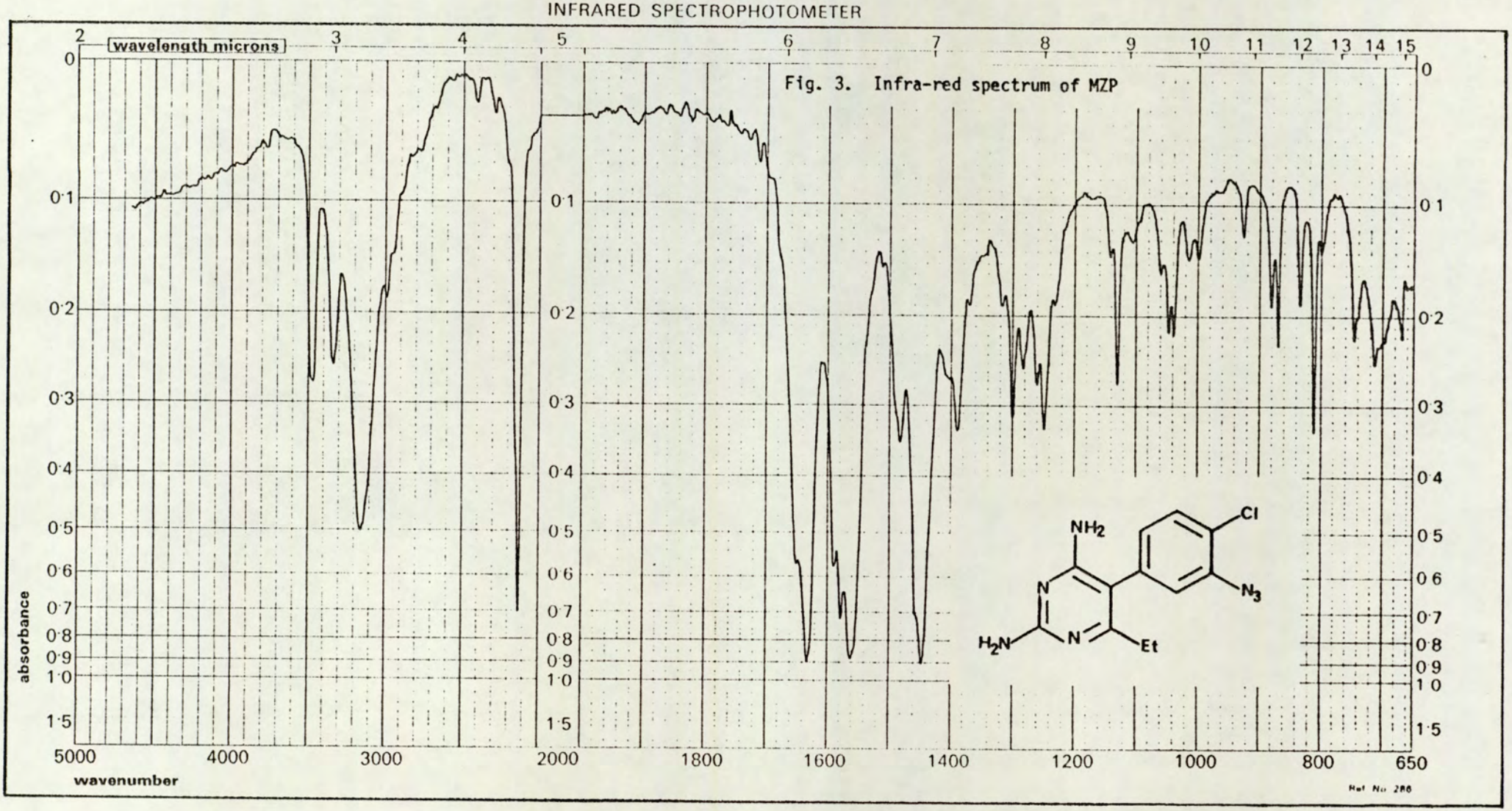


Fig. 3. Infra-red spectrum of MZP

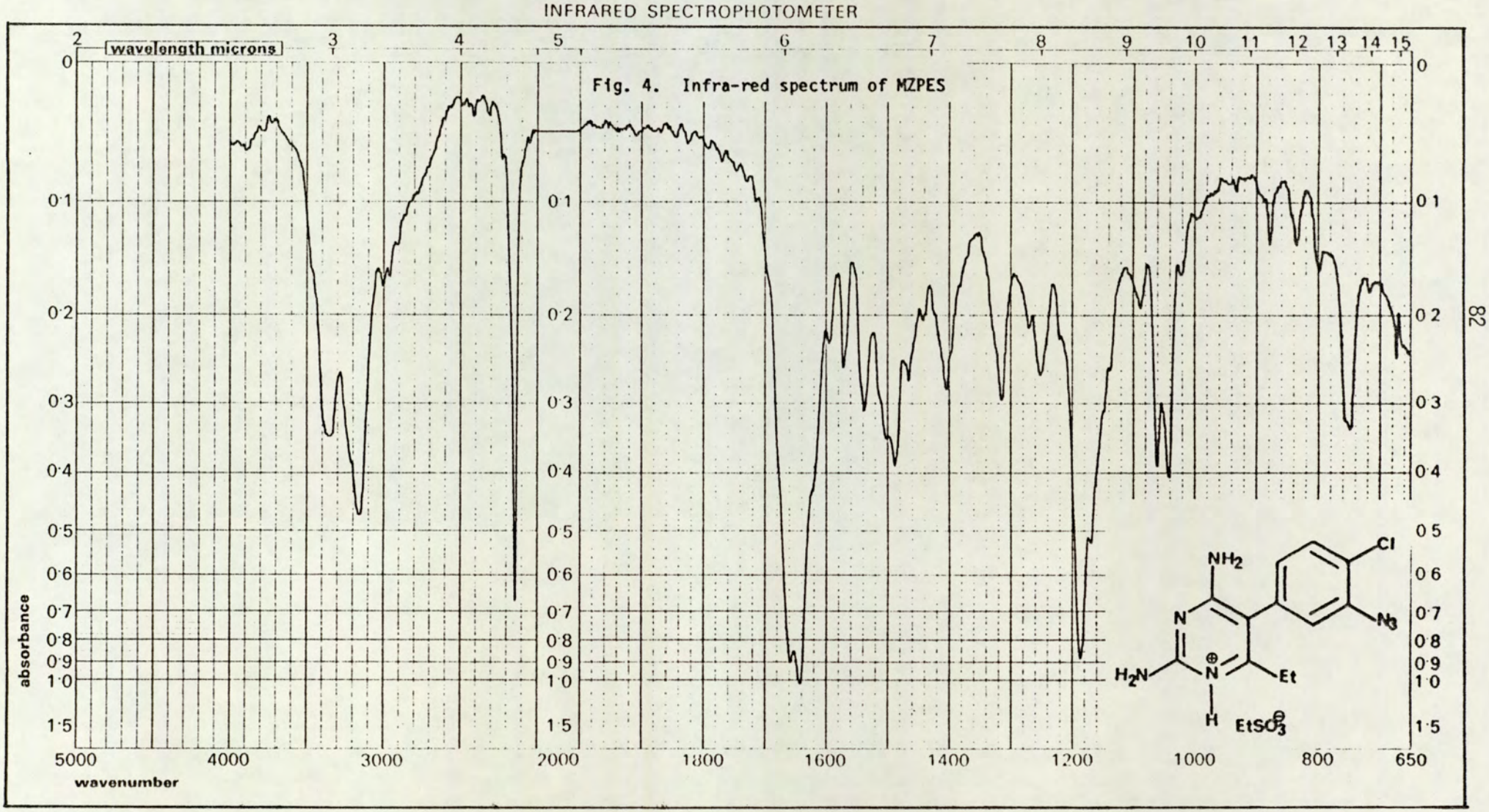


Fig. 4. Infra-red spectrum of MZPES

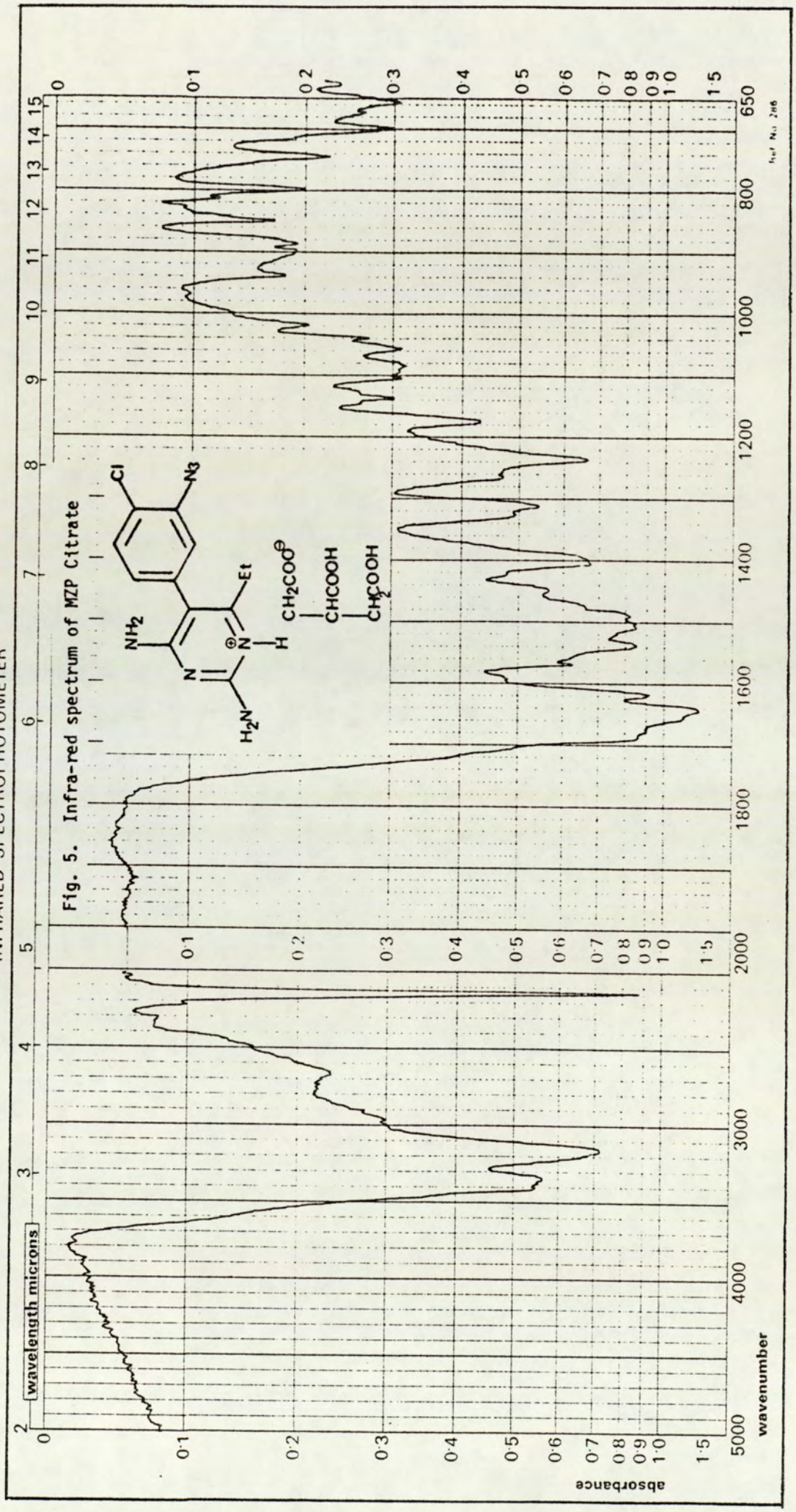


Fig. 5. Infra-red spectrum of MZP Citrate

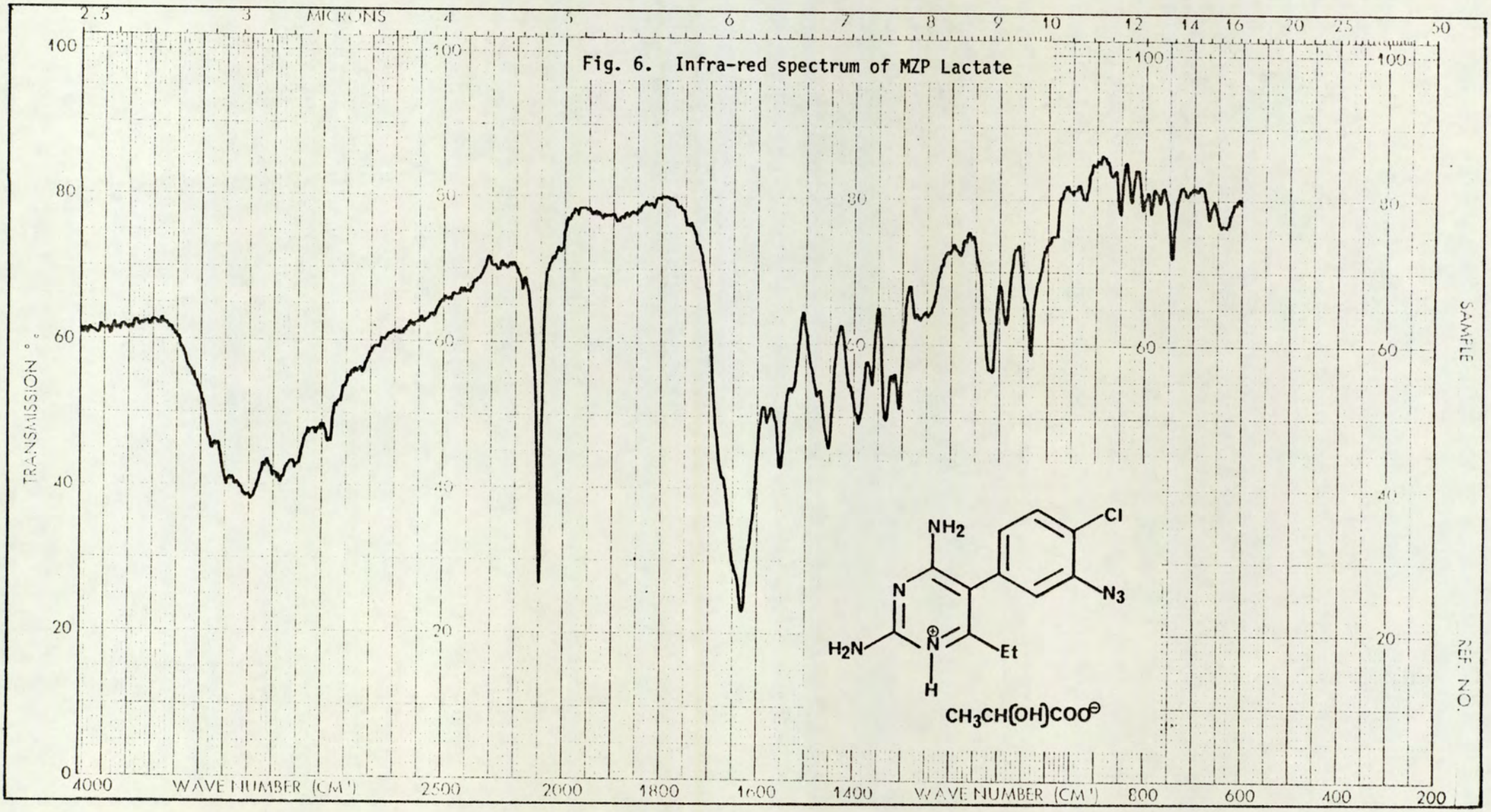


Fig. 6. Infra-red spectrum of MZP Lactate
2.5
3
MICRONS
4
5
6
7
8
9
10
12
14
16
20
25
50
100
80
60
40
20
0
TRANSMISSION %
4000
WAVE NUMBER (CM')
2500
2000
1800
1600
1400
WAVE NUMBER (CM')
800
600
400
200
SAMPLE
REF. NO.
NH2
Cl
N
N3
H2N
N
Et
H
CH3CH(OH)COO⊖

sufficient to dissolve MNP in heated aqueous ethanesulphonic acid solution. MNPES was recrystallized from water. MNPES was characterized by elemental analysis [Found: C,41.19%; H,4.51%; N,17.01%. $C_{14}H_{18}N_5ClSO_5(H_2O)_{1/4}$ requires C,41.18%; H,4.57%; N,17.15%].

11. SYNTHESIS OF MAP DIETHANESULPHONATE

$MAP(ES)_2$ was synthesized by Professor M F G Stevens. One mole of MAP was allowed to react with two moles of ethanesulphonic acid in ethanol, with gentle heating. The amount of ethanol added was such that it was just sufficient to produce dissolution. $MAP(ES)_2$ was characterized by elemental analysis [Found: C,38.84; H,5.41%; N, 14.18%. $C_{16}H_{26}N_5ClS_2O_6(H_2O)_{1/2}$ requires C,38.98%; H,5.52%; N,14.21%].

12. SYNTHESIS OF AZO COMPOUNDS

Attempts were made by Dr M D Threadgill to synthesize 5,5'-bis-(2,4-diamino-6-ethylpyrimidin-5-yl)-2,2'-dichloroazobenzene. Several methods (154-155) were tried and found to be unsatisfactory. In each case, a mixture of products was obtained, with the azo compound as one of the minor products (156).

13. PHARMACOKINETIC CALCULATIONS

The clinical pharmacokinetic parameters of MZPES were calculated from a ln concentration vs time plot (Fig. 36). The calculations were based on a two compartment open model.

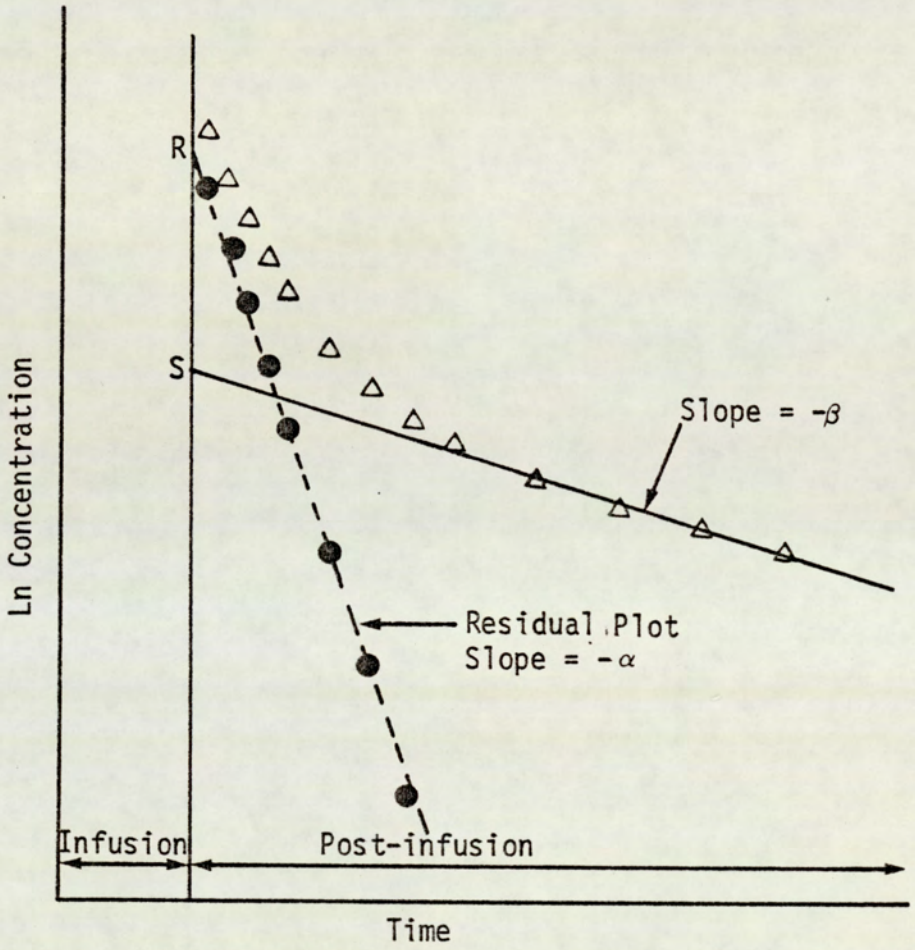


Fig. 36 Treatment of a ln concentration vs time plot to calculate the pharmacokinetic parameters

From the slope of the elimination phase, the value of the constant β is obtained. The elimination phase is extrapolated to the end of the infusion time and the intercept gives the value of the constant S (Fig. 36). A residual line is plotted between the distribution phase and the extrapolated elimination phase. From the slope of this line the value of the constant α is obtained. The

intercept produced by this line at the end of the infusion time gives the value of the constant R. (Fig. 36). The coefficients R and S can then be related to A and B, the zero time intercepts following intravenous injection, by the following expression:-

$$A = \frac{R\, X_0\, \alpha}{K_0(1 - e^{-\alpha T})}$$

$$B = \frac{S\, X_0\, \beta}{K_0\,(1 - e^{-\beta T})}$$

where Xo = administered dose and equals the product of the infusion rate (Ko) and the infusion time (T) i.e. KoT.

These constants can then be used in the calculations of the distribution constants, K_{12} and K_{21}, and the elimination constant, K_{10}, using the following equations:-

$$K_{21} = \frac{A\beta - B\alpha}{A + B}$$

$$K_{10} = \frac{\alpha\beta}{K_{21}}$$

$$K_{12} = \alpha + \beta - K_{21} - K_{10}$$

where K_{12} is the distribution constant for the distribution of drug from the central compartment to the peripheral compartment and K_{21} is the distribution constant for the distribution of drug from the peripheral compartment back to the central compartment.

The apparent volume of the central compartment (Vc) and the

apparent volume of distribution (V_B) of the drug in the body is determined by:-

$$V_c = \frac{Xo}{A + B}$$

$$V_B = \frac{V_c K_{10}}{\beta}$$

The total area under the curve (AUC_t) is calculated using the following equation:-

$$AUC_t = \frac{A}{\alpha} + \frac{B}{\beta}$$

The distribution and elimination half-lives of the drug are calculated from the following expressions:-

$$t_{\frac{1}{2}\alpha} = \frac{0.693}{\alpha}$$

$$t_{\frac{1}{2}\beta} = \frac{0.693}{\beta}$$

SECTION C

RESULTS AND DISCUSSION

1. DEVELOPMENT OF ANALYTICAL METHODOLOGY

1.1. Thin layer chromatography (TLC)

Thin layer chromatography was evaluated as a method of separating MAP, MNP, pyrimethamine and MZP. Each compound, including the ethanesulphonic acid salt of MZP, was run separately. A mixture of all the compounds was also run simultaneously in order to evaluate the effect of inter-compound associations and interactions on separation (188). Fifty solvent systems were evaluated as suitable mobile phases. The mobile phases tested were obtained by varying the solvent composition within the following systems:-

(a) Toluene, acetone and ethanol

(b) Toluene, acetone, ethanol and ether

(c) Acetone, ethanol and water

(d) Ethylacetate and ethanol

(e) Ethylacetate, ethanol and ether

(f) Ethylacetate, ethanol and water

(g) Toluene, methanol and acetone

(h) Toluene, ethanediol and acetone

All the systems investigated produced very poor separation of the compounds. None of the systems produced a difference of > 0.2 between the R_f values of the least and the most polar compound under investigation. When a mixture of compounds was run, only 3 spots were distinctly identified under ultra-violet light. This suggested that two of the compounds were inseparable in all the systems tested. Certain solvent systems failed to produce any marked separation between the compounds. These systems included (a) toluene and acetone, (b) acetone and ethanol, (c) toluene and ethylacetate, and (d) toluene, ethanediol and acetone.

Of the TLC plates used, Silica adsorbent proved to be superior

to alumina or cellulose adsorbent in separating this series of compounds. Cellulose plates in general produced extremely poor separation. The inclusion of a base in the solvent systems gave rise to better separation suggesting that perhaps the compounds were better separated under alkaline conditions where the neutral molecules predominated.

Hubbell and coworkers (157) reported the use of TLC methods to separate pyrimethamine and its metabolites, and, metoprine and its metabolites. They employed silica plates and the following solvent systems:- (a) n-butanol [60%], water [10%], acetic acid [10%] and pyridine [10%], and (b) chloroform [25], isopropanol [20] and ammonium hydroxide [1]. Although these compounds were structurally very similar to the compounds in the present study, the solvent systems did not produce sufficient separation between MAP, MNP, pyrimethamine and MZP to be of use as an analytical method.

The systems evaluated in the present study did not produce sufficient separation between the compounds to enable the use of TLC as an analytical or stability indicating method for this series of compounds. A section of the results of the present study is included in appendix I.

1.2. Electronic absorption spectra

The UV-visible spectra of MAP, MNP, pyrimethamine and MZPES in water revealed that all these compounds absorbed over the same wavelength range of 200-350 nm. The absorption maxima of these compounds were also very similar. Therefore, UV absorption would not be a very selective method for analysing MZPES because it would fail to identify the presence of impurities of structurally similar compounds.

The UV absorbance of MZPES solution and its concentration followed a linear Beer-Lambert relationship (Fig. 7). Therefore, UV absorption would be a very useful method for rapid assaying of the content of MZPES in solution, provided the purity of the compound is established.

1.3. HPLC

HPLC had been the main chromatographic method used throughout the course of this study. The method used for routine analysis of MZPES is depicted in Fig 9. This method is specific for MZPES and the presence of any impurities can be readily identified. Pyrimethamine was employed as an internal standard. The ratio of peak height or peak area of MZPES/pyrimethamine was found to vary in a linear relationship with the concentration of MZPES in solution (Fig 8). Therefore, HPLC has been used to quantify the amount of MZPES in solution as well as being employed as a purity and stability indicating assay. Alkaline pH > 7.0 was found to be necessary for satisfactory resolution between the compounds. This method is also highly sensitive and the presence of MAP and MNP in MZPES solution can be detected at limits of 0.1 and 0.2% w/w respectively.

A HPLC method was also developed for the study and isolation of decomposition products. Various gradient elution or isocratic systems of different solvent compositions were evaluated but were not found to produce any improvement over the method described in Fig 10.

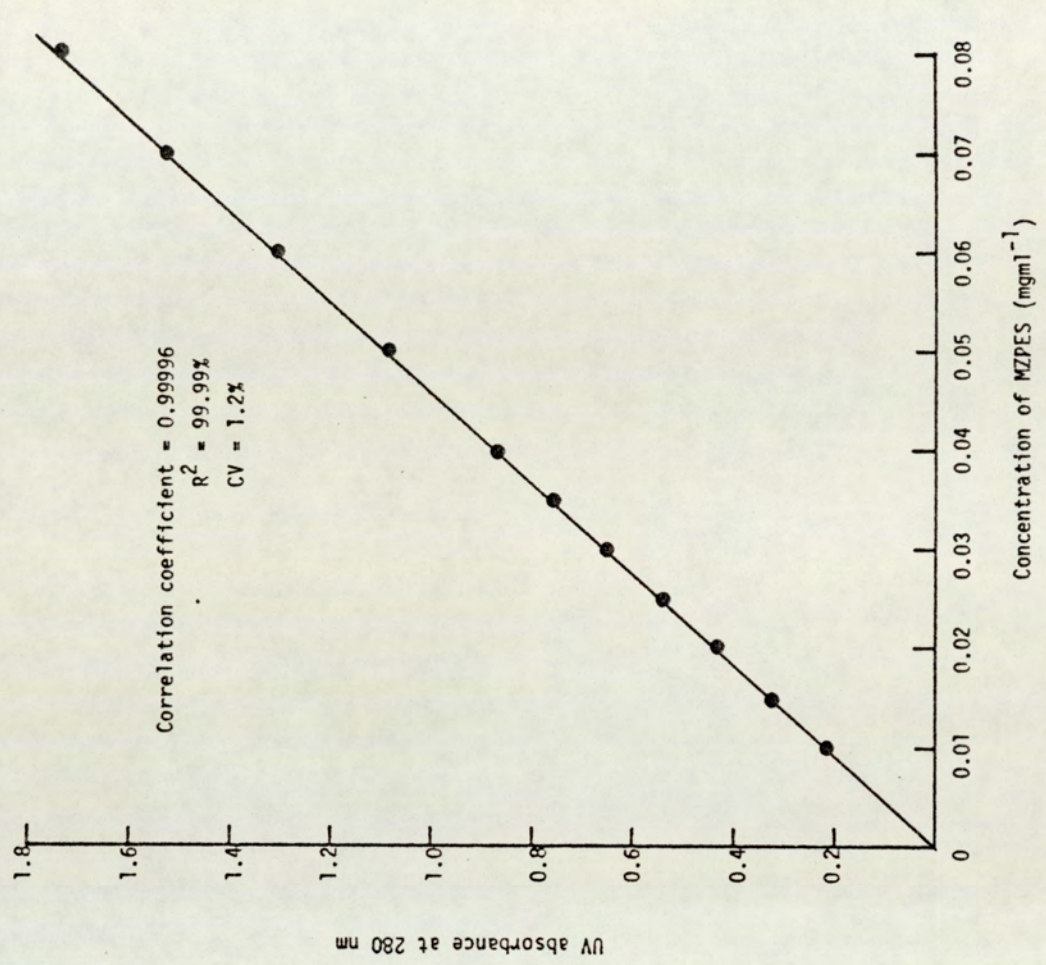


Fig. 7. UV absorption of MZPES Vs Concentration

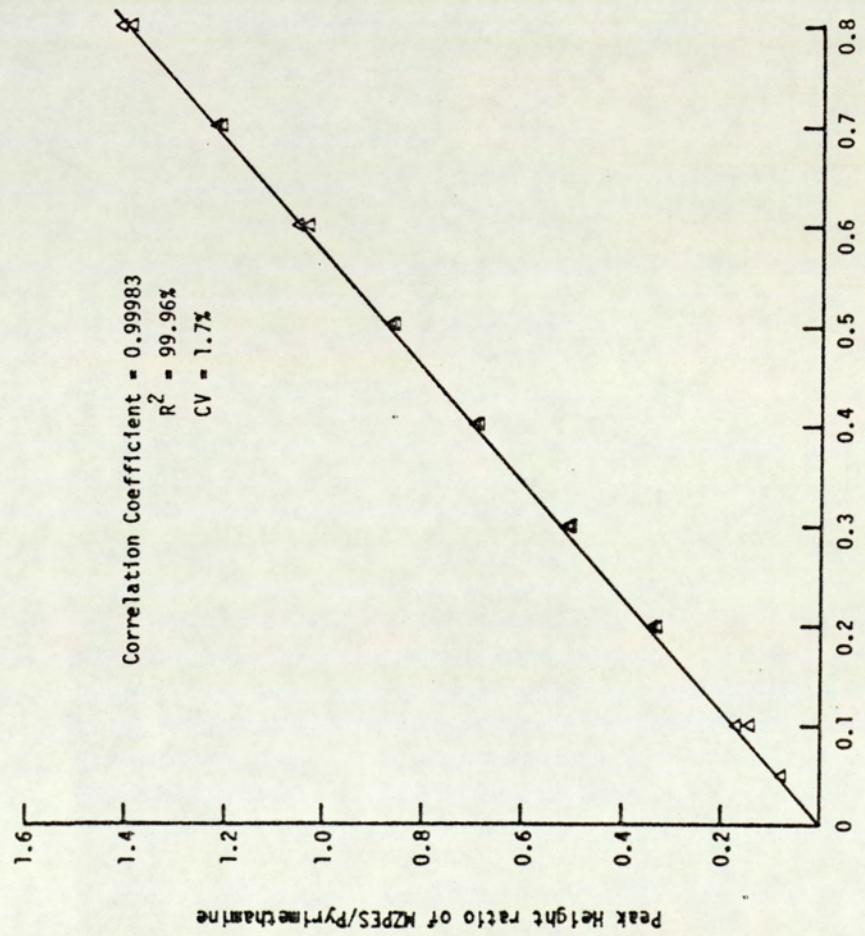
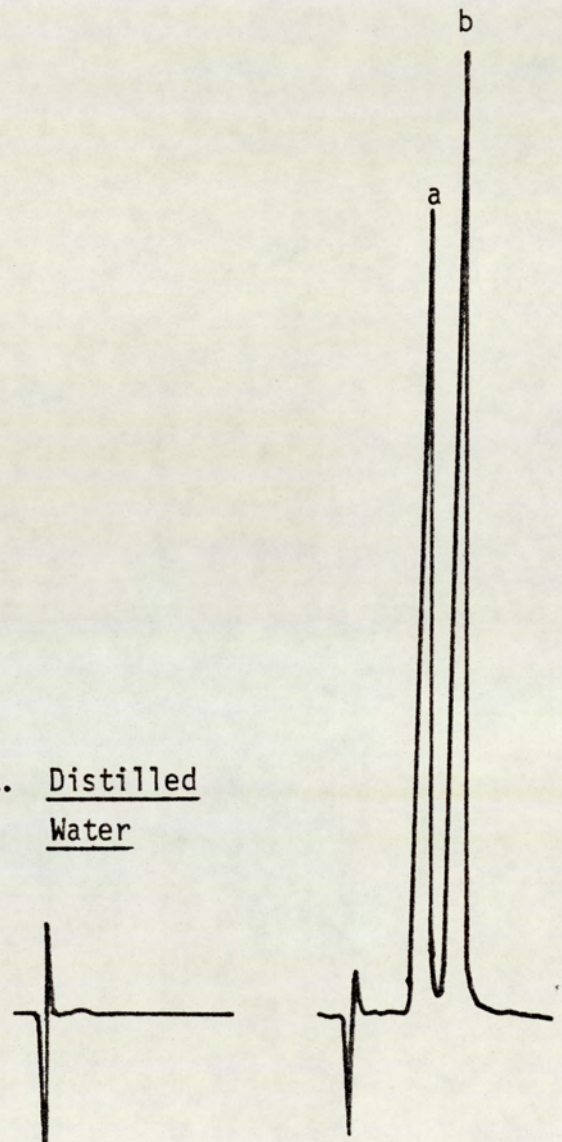


Fig. 8. HPLC Peak Height ratio MZPES/Pyrimethamine (internal standard) Vs Concentration of MZPES

Fig. 9. HPLC chromatograms of MZP and Pyrimethamine

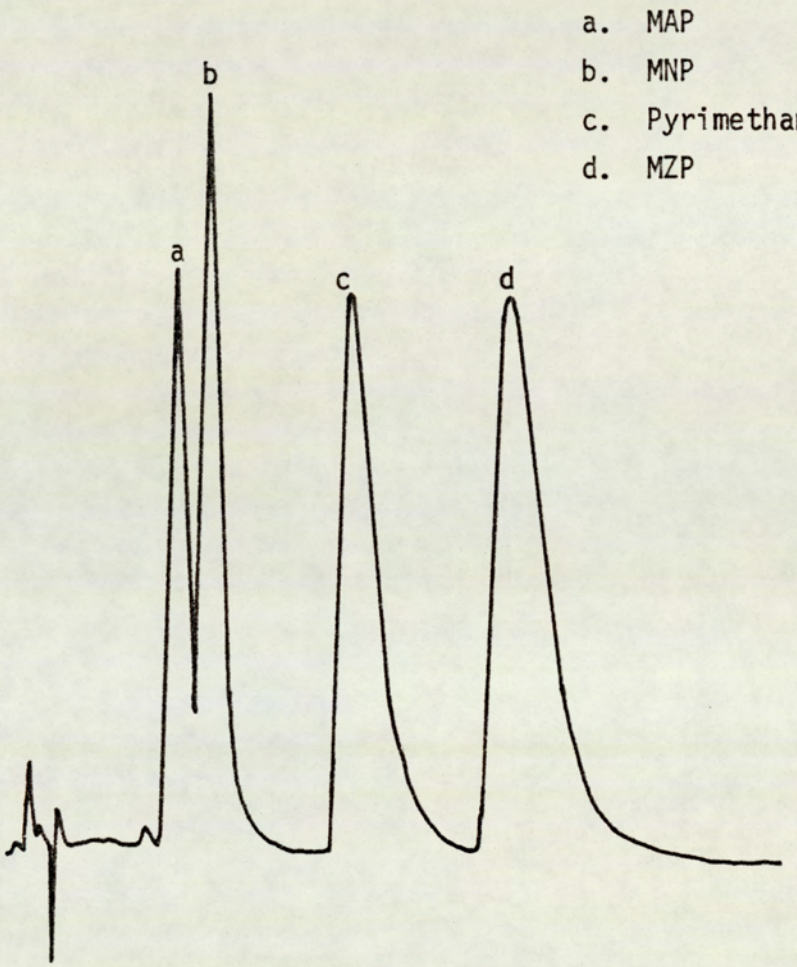
B. Sample



HPLC Conditions:

1. Mobile Phase: Methanol 75%, Phosphate buffer 0.005M(pH 7.5) 25%
2. Flow Rate: 1.5 $mlmin^{-1}$
3. Detection: UV, 250 nm
4. Chart Speed: 5 mm min^{-1}
5. C_{18} column, 0.5 x 10 cm

Fig. 10. HPLC chromatograms of MAP,MNP, Pyrimethamine and MZP



HPLC Conditions:

1. Mobile Phase: Methanol 65%, Phosphate buffer 0.005M (pH 7.5) 35%
2. Flow Rate: 1.0 $mlmin^{-1}$
3. Detection: UV 250 nm
4. Chart Speed: 5 $mm\ min^{-1}$
5. C_{18} column, 0.5 x 10 cm

2. PREFORMULATION EVALUATION

2.1. Physical Characteristics

The physical properties of MZP and MZPES were characterized. The data generated (Table 4) serves as a useful reference for the identification of the compounds. Together with the HPLC assay of drug content and purity, these data can be used as criteria for the quality control of new batches of MZP or MZPES.

Table 4. Physical Characteristics of MZP and MZPES

Property	MZP	MZPES
Appearance	Microprisms	Off white crystals
Crystallisation solvent	Ethanol	Water
Melting point	185-188°C	190-191°C (decomposed)
UV spectrum (λ_{max},H_2O)		215,252,287 nm
IR spectrum (KBr, ν_{max}) (Figs. 3 and 4)	3470,3320,3150,2120, 1630,1560,1450	3350,3160,2140, 1640 cm^{-1}
^{1}HNMR spectrum (in DMSO-d_6) (δ)	1.0 (3H,t,CH_3)	1.09 (3H,t,CH_3), 1.15 (3H,t,CH_3)
	2.18 (2H,q,CH_2)	2.29 (2H,q,CH_2) 2.59 (2H,q,CH_2)
	5.80 (2H,S,NH_2)	7.04 (1H,S,NH)
		7.17 (1H,dd,H-6')
	5.96 (2H,S,NH_2)	7.45 (1H,d,H-2')
	7.02 (1H,q,H-6)	7.68 (1H,d,H-5')
	7.19 (1H,d,H-2)	7.84 (2H,S,NH_2)
	7.59 (1H,d,H-5)	8.21 (1H,S,NH)
Mass spectrum	m/z 291,289,263,262,226,65	
X-ray crystallography	-	Structure confirmed[a]

[a] Please see reference 161

2.2. Solubility

Three methods were employed to study the aqueous solubility of MZPES and related compounds at room temperature. The first method involved shaking an excess of the compound in water in a centrifuge tube with the aid of a Fisons Whirlimixer for 5-10 minutes. This was not a satisfactory method of producing saturated solutions. The results (Table 5) revealed that for most compounds, especially those with a slow rate of dissolution, e.g. MZP Phosphate, MZP citrate etc., saturation was not attained within the time of agitation employed. The variation in rate of solution is probably a result of differences in particle size, crystal form, solvation energy etc. between the different compounds. The second method of achieving saturated solutions involved heating excess of the solid in boiling water and then cooling the solution to room temperature before analysis. This method, however, has the potential risk of producing super-saturated solutions. To avoid this, the solutions were allowed to stand at room temperature for an hour to permit precipitation and establishment of equilibrium between solute and solution. The third method was used to confirm that the level of supersaturation occurring in the second method was not significant: this method involved shaking an excess of the compound in water with a mechanical shaker over a prolonged period of time, e.g. 72 hours, until equilibrium was established. This was a more accurate method of determining solubility but was very time consuming. The solubility of MZP determined by both method 2 and 3 was not significantly different (Table 5). The first method, however, gave a much lower solubility. Therefore, the second method was chosen for the determination of solubility of the compounds in the present study.

The solubility of MZP free base was found to be extremely low,

~ 20 ug ml^{-1} (Table 5). This would not be sufficient to produce a satisfactory solution formulation for intravenous administration. The toxicological data on the ethanesulphonic acid salt of the compound revealed that the starting dose in human trials would be 5.4 mgm^{-2}. During dose escalation this could reach ~300 mgm^{-2} at the maximum tolerated dose. Therefore, the final formulation must be able to deliver between 500 and 600 mg of the drug at each administration. A solubility of about 10 $mgml^{-1}$ would, thus, be required.

There are various options by which the solubility of a drug can be enhanced (83-108). The use of cosolvents and surfactants was not considered appropriate for the solubilization of MZP because of the inherent toxicities of these agents (88,89,90,92,93) in intravenous administration and the potential risk of drug precipitation when these formulations were diluted (87). These effects need to be considered carefully especially when large amounts of surfactants or cosolvents would be needed to achieve a 500 fold increase in solubility. Complexation would not be a suitable method for solubilizing MZP either because this method normally only produced a mdoerate enhancement of solubility (102,103). Therefore, the options left for solubilization of MZP included salt formation, oil in water emulsion or the use of organic solvents. The production of a stable sterile oil-in-water emulsion of MZP would be a complicated process requiring specialised equipment and it was decided that this avenue of solubilization would only be resorted to if all else failed! The use of organic solvents in solubilization of MZP was a viable option but again, as in the case of surfactants, the use of these agents is limited by their toxicities (85). Salt formation was favoured as a solubilizing method bcause it is simple and it removes the potential

Table 5. continued ....

MZPES	Lactate buffer		
	0.01M	14.447 ± 0.767	20.600 ± 0.093
	0.025M	13.819 ± 1.578	
	0.05M	13.331 ± 0.397	7.253 ± 0.014
	0.1M		4.081 ± 0.006
	0.2M		4.790 ± 0.002
MZPES	DMSO	380.95 ± 0.35	
MZPES	DMA	194.68 ± 0.28	
MZPES	Ethanol	8.49 ± 0.01	

---

a MZP HCl = m-azidopyrimethamine hydrochloride salt

b MZPMS = m-azidopyrimethamine methanesulphonic acid salt.

c All buffer solutions were at pH 4.0

Table 5. continued...

Method 1. Saturated solution produced by shaking an excess of compound in water in a centrifuge tube with the aid of Fisons Whirlimixer for 5-10 minutes at 20°C.

Method 2. Saturated solution produced by heating an excess of compound in water until boiling and then cooling to 20°C.

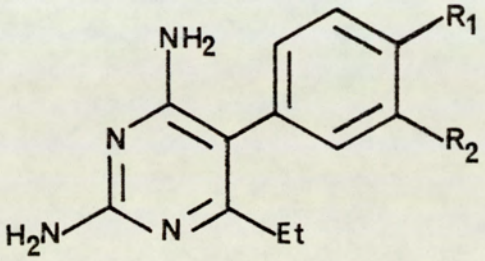
Method 3. Saturated solution produced by prolonged shaking of compound in water with a mechanical shaker for 72 hours at 20°C.

problems, such as altered efficacy of the drug, altered stability of the preparation etc, due to the presence of an additional compound.

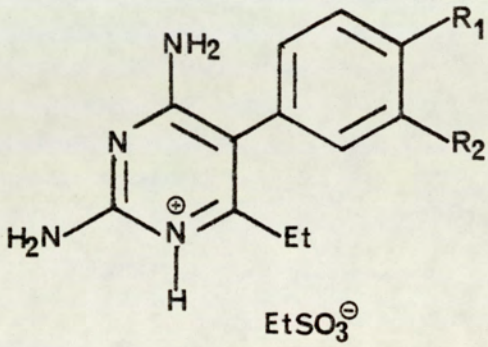
All the salts of MZP produced an enhancement of solubility. The hydrochloric, methanesulphonic and lactic acid salts only produced moderate increase in the solubility of MZP. More marked enhancement of solubility was observed with salt formation between MZP and phosphoric, citric or ethanesulphonic acids. The aqueous solubilities of MZP phosphate, citrate and ethanesulphonate, in terms of molar dissolution, were 41.7, 35.3 and 43.8 x 10^{-3} M respectively. MZPES was, therefore, the most water soluble salt formed and it was chosen for further development.

Attempts were made to study the effect of changing solvent systems on the solubility of MZPES. The use of citrate or lactate buffers was not found to produce any improvement (Table 5). In fact, as the concentration of buffer solutions was increased, the solubility of MZPES decreased. The decrease in solubility was more marked at lower buffer concentrations, whereas at concentrations of 0.1M and 0.2M the solubility of MZPES was almost identical; suggesting the attainment of a plateau phase in the relationship between decrease in MZPES solubility and the concentration of buffer solutions. No satisfactory explanation can be offered to account for this rather peculiar behaviour. The use of organic solvents greatly increased the solubility of MZPES. A 22 and 11 fold increase in solubility of MZPES were obtained using dimethylsuphoxide and dimethylacetamide respectively, when compared to the aqueous solubility of MZPES. The solubility of MZPES in water was considered sufficient to produce a 10 $mgml^{-1}$ solution formulation for Phase I clinical trials. The simple formulation has the advantage that any pharmacological effects or toxicities observed can be directly

Table 6. Aqueous solubility of 2,4-diaminopyrimidine analogues at room temperature



XXIX



XXX

Compound	R_1	R_2	Solubility (mg/ml^{-1}) Method 1[a]	Method 2
XXIX				
MZP	Cl	N_3	0.0037 ± 0.0002	0.0224 ± 0.004
MAP	Cl	NH_2	0.118 ± 0.019	0.784 ± 0.005
ISOMAP	NH_2	Cl	0.208 ± 0.03	0.870 ± 0.002
MNP	Cl	NO_2	0.0098 ± 0.002	0.0310 ± 0.008
Pyrimethamine	Cl	H		0.0288 ± 0.003
XXX				
MZPES	Cl	N_3	13.999 ± 1.287	17.596 ± 0.447
ISOMZPES	N_3	Cl	3.908 ± 0.127	22.758 ± 0.106

[a] For the conditions used in the methods see Table 5.

attributed to the drug.

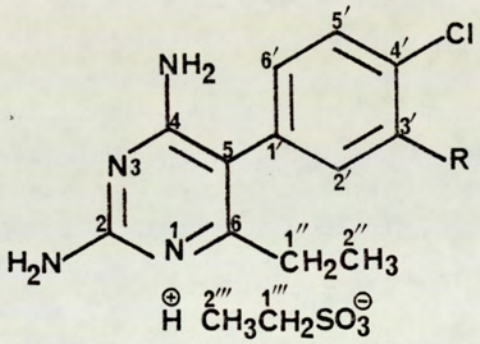
The introduction of an amino group instead of azido group into the 3-phenyl moiety greatly increased the aqueous solubility of the compound. Presumably the amino group has the potential of forming hydrogen bonding with water and thereby increases the solute-solvent interaction. The presence of a nitro group did not result in significant alteration in the solubility of the compound. The aqueous solubility of MNP was only 1.4 times that of MZP (Table 6). The aqueous solubility of pyrimethamine was even closer to that of MZP. The interchange of substituents of R_1 and R_2 in XXIX and XXX to produce compounds of the iso-series did not result in any significant alteration in the solubility of the compounds (Table 6).

2.3. Ionisation Constants

There are several acidic and basic centres in a complex molecule such as MZP. The amino groups in the pyrimidine ring have the potential of losing a proton but this has been reported to occur only in non-physiological conditions (158,159). The pKa of the amino-substituent in pyrimidines and other nitrogen heterocycles was found to vary between 13 and 24 (158,159). Therefore, ionisation of the amino substituents was not likely to occur under the experimental conditions employed in the present study. On the other hand, there are several centres in the MZP molecule that are likely to be protonated under suitable conditions. These centres include the two heterocyclic nitrogen, the amino substituents and conceivably the aromatic azido group. It was therefore important to determine the site and sequence of protonation in the molecule so that the pKa determined can be associated to a particular centre.

In order to study the nature of protonation in this series of

compounds, proton-coupled ^{13}C NMR spectra of MZP, MAP, Pyrimethamine and their salts in deuterated dimethylsuphoxide were obtained. It was not possible to perform these studies in deuterated water because of the low aqueous solubility of the free bases. It is believed that the nature of protonation of these compounds in both dimethylsulphoxide and water would be similar. The results obtained are tabulated in Tables 7-11. The chemical shifts were assigned by Dr. M.D. Threadgill. The numbering of the atoms is as in Fig 11.



R	Compound
H	Pyrimethamine
NH_2	MAP
N_3	MZP

Fig. 11. Pyrimidine Analogues

When pyrimethamine was protonated with one equivalent of ethanesulphonic acid, an upfield shift of 7, 12 and 4 units occurred in the chemical shifts of carbon 2, 6 and 1" respectively, compared to that of unprotonated pyrimethamine (Table 7). No significant change occurred in the chemical shifts of the aromatic carbons. This indicated that protonation in solution occurred predominantly on N-1 nitrogen when pyrimethamine was monoprotonated.

Similar chemical shift patterns were observed when MAP was protonated with one equivalent of ethanesulphonic acid (Table 8),

[a] s singlet, d doublet, t triplet, q quartet, m multiplet, dd doublet of doublets, tq triplet of quartets, qt quartet of triplets.

[b] Coupling constants shown to the nearest integer were estimated graphically, others were taken from the numerical listing generated by the spectrometer.

[c] $\Delta\delta_{1-0}$, δ(1 x $EtSO_3H$)-δ(free base).

[d] Assignment uncertain between the resonances indicated.

[e] $\underline{J}$ < 10 Hz but poorly resolved.

Table 8 continued ....

2''	13.53	qt	127.1, 5.0	12.88	qt	128.7, 4	- 0.65
1'''				45.35	tq	132.4, 4.6	
2'''				9.72	qt	128.3, 4	

---

[a] s singlet, d doublet, q quartet, m multiplet, dd doublet of doublets, tq triplet of quartets, qt quartet of triplets.

[b] Coupling constants shown to the nearest integer were estimated graphically, others were taken from the numerical listing generated by the spectrometer.

[c] $\Delta\delta_{1-0}$: δ(1 x $EtSO_3H$)-δ(free base).

[d] Assignment uncertain between the resonances indicated.

[e] J < 10 Hz but poorly resolved.

[f] Decoupled by irradiation of proton resonance centred at 7.273 (5'-H).

[g] J reduced by ca. 10 Hz by irradiation as in (f).

indicating that protonation occurred on N-1 in solution. When MAP was treated with two equivalents of ethanesulphonic acid, very little change occurred in the chemical shifts of the carbons in the pyrimidine ring when compared to the mono-protonated species (Table 9). This indicated that in the pyrimidine ring protonation only occurred on N-1 under the condtions employed. Protonation, however, also occurred in the aromatic ring as indicated by the slight changes in the chemical shifts of the aromatic carbons (Table 9). One equivalent of ethanesulphonic acid caused an upfield shift of 0.31 units on C-2' whereas two equivalents of the acid resulted in a downfield shift of 1.35 units. The chemical shift of C-3' was also affected, an upfield shift of 1.98 units was observed when two equivalents of acid was used. A downfield shift of 2.70 and 1.53 units was also observed for C-4' and 6', whereas only a slight change was detected in the chemical shift of C-5'. Faure et al (160) studied the change in chemical shifts of ^{13}C NMR spectra of aromatic amines and reported that protonation of the amino substituent resulted in a large upfield shift of 12 units on the ipso carbon, a moderate downfield shift on the ortho carbons, a large downfield shift of ~12 units on the para carbon and an insignificant change in chemical shift of the meta carbons. The results above, therefore, showed that there was only partial protonation of the aromatic amino substituent in solution despite it being fully protonated when the compound was in the solid state (Section B, 11).

Protonation of MAP was also studied in a strong acid, deuterated trifluoroacetic acid (Table 10). More pronounced chemical shift changes were observed in the aromatic ring. An upfield shift of 17 units occurred on C-3' and a downfield shift of 10.7, 15.2, 4.15 and 15.1 was observed for C-2', 4', 5' and 6' respectively. This

Table 9 continued ....

2''	13.53	12.79	qt	127.0, 5.0	- 0.74	- 0.09
1'''		45.38	tq	128.8, 4.6		+ 0.03
2'''		9.54	qt	128.2, 4.2		- 0.18

---

[a] s singlet, d doublet, m multiplet, dd doublet of doublets, tq triplet of quartets, qt quartet of triplets, tm triplet of multiplets.

[b] Coupling constants shown to the nearest integer were estimated graphically, others were taken from the numerical listing generated by the spectrometer.

[c] $\Delta\delta_{2-0}$: δ(2 x $EtSO_3H$ salt)-δ(free base), $\Delta\delta_{2-1}$: δ(2 x $EtSO_3H$ salt)-δ(1 x $EtSO_3H$ salt).

[d] Assignment uncertain between the resonances indicated.

[e] $\underline{J}$ <10 Hz but poorly resolved.

Table 10 continued ....

1''	27.60	24.78	tq	132.5, 5	- 2.82	+ 1.09
2''	13.53	10.55	qt	130.6, 5.1	- 2.98	- 2.33

---

a s singlet, d doublet, m multiplet, dd doublet of doublets, tq triplet of quartets, qt quartet of triplets, brd broad doublet.

b Coupling constants shown to the nearest integer were estimated graphically, others were taken from the numerical listing generated by the spectrometer.

c $\Delta\delta_{TFAD-0}$: $\delta(CF_3CO_2D)-\delta$[free base/$(CD_3)_2SO$], $\Delta\delta_{TFAD-1}$: $\delta(CF_3CO_2D)-\delta$[1 x $EtSO_3H$ salt/$(CD_3)_2SO$].

d Assignment uncertain between the resonances indicated.

e J <10 Hz but poorly resolved.

indicated that the amino substituent in the aromatic ring was fully protonated in deuterated trifluoroacetic acid (TFA). The pattern of the chemical shift changes was consistent with those observed by Faure et al (160). In TFA, a change in chemical shift was also observed for the pyrimidine carbons when compared to those of monoprotonated MAP. Particularly significant was the change observed for C-2, 4 and 6. In monoprotonated MAP an upfield shift of 7 and 12 units was observed for C-2 and 6 respectively whereas in TFA these values changed to upfield shifts of $\sim$10 and $\sim$7 units. Monoprotonation caused a downfield shift of 2 units on C-4 whereas in TFA an upfield shift of 2 units was observed. All these changes in chemical shift patterns pointed to the fact that protonation not only occurred on N-1 but also on N-3 as the chemical shift of carbons ortho and para to N-3 were affected, whereas, that of meta carbon was not significantly affected. Should protonation have occurred on either of the amino-substituents in the pyrimidine ring, the chemical shift of C-5 would have been significantly affected. The chemical shift changes on C-2 and 4, however, indicated that there was only partial protonation on N-3 because a full protonation on N-3 would have resulted in a much larger change in chemical shifts on C-2 and 4 being registered. Therefore, it can be concluded that protonation of MAP in solution occurred in the sequence of N-1, aromatic amine and N-3 as the acidity of the medium is increased. The pKa values of these three centres would, therefore, be wide apart. This is believed to be true for compounds which are structurally related to MAP. ^{1}H proton NMR spectra of MAP were also obtained under the same experimental conditions (Tables 12 and 13). Similar chemical shift patterns were observed with protons as with carbons in ^{13}C proton-coupled NMR spectra. This confirmed the correct assignment of

Table 11 continued .....

1''	25.57	tq	127.2, 4.1	23.70	tq	133.1, e	- 3.87
2''	13.17	qt	126.8, 4.8	12.62	qt	128.4, 5.3	- 0.55
1'''				45.31	tq	133.3, 4.7	
2'''				9.70	qt	127.4, 3.9	

---

a s singlet, d doublet, t triplet, q quartet, m multiplet, dd doublet of doublets, tq triplet of quartets, qt quartet of triplets.

b Coupling constants shown to the nearest integer were estimated graphically, others were taken from the numerical listing generated by the spectrometer.

c δ(1 x $EtSO_3H$) - δ(Free Base).

d Assignment uncertain between the resonances indicated.

e $\underline{J}$ <10 Hz but poorly resolved.

f Estimated graphically.

Table 12. ^1H NMR spectra of MAP ethanesulphonic acid salts in $(CD_3)_2SO$.

	Salt (1 x $EtSO_3H$)			Salt (2 x $EtSO_3H$)		
Proton	δ(ppm)	Mult.[a]	J(Hz)	δ(ppm)	Mult.[a]	J(Hz)
2'-H	6.65	d	2.0	6.69	d	2.0
5'-H	7.27	d	8.1	7.32	d	8.1
6'-H	6.40	dd	8.1, 2.0	6.47	dd	8.1, 2.0
1''-H	2.23	brq	7.6	2.22	brq	7.6
2''-H	1.05	brt	7.6	1.04	t	7.6
1'''-H	2.57	q	7.4	2.53	q	7.4
2'''-H	1.12	q	7.4	1.09	t	7.4

[a] d doublet, t triplet, q quartet, dd doublet of doublets, brt broad triplet, brq broad quartet.

Table 13. Proton NMR spectra of MAP in $[(CD_3)_2]SO$ and in CF_3CO_2D

	Free Base $[(CD_3)_2SO]$			CF_3CO_2D		
Proton	δ(ppm)	Mult.[a]	J(Hz)	δ(ppm)	Mult.[a]	J(Hz)
2'-H	6.65	d	1.5	7.84	brd	1.5[b]
5'-H	7.25	d	8.0	7.93	d	8.3
6'-H	6.38	dd	8.0, 1.5	7.61	dd	8.3, 1.5
1''-H	2.18	q	7.5	2.60	q	7.5
2''-H	1.00	t	7.5	1.29	t	7.5

[a] d doublet, t triplet, q quartet, dd doublet of doublets, brd broad doublet.

[b] estimated graphically.

chemical shifts to the carbons.

When MZP was protonated with one equivalent of ethanesulphonic acid an upfield shift of 7, 12 and 4 units was observed for C-2, 6 and 1" respectively. No significant change in chemical shifts was detected in the aromatic ring. Therefore, mono-protonation of MZP in solution occurred predominantly on N-1. X-ray crystallography studies also revelaed that MZPES was protonated on N-1 in the crystalline state (161).

The spectrophotometer method (153) used in the present study to determine the pKa of MZP and its analogues would, therefore, only be a measure of the basicity of N-1 nitrogen since the pH conditions employed would not be sufficient to protonate other centres. The change of the UV absorption spectrum of MZP in phosphate buffer with pH is depicted in Fig. 12. The single isobestic point at 278 nm indicated the presence of two species, the protonated and unprotonated form of MZP, in the solutions under investigation. This is typical of the UV absorption spectra of other related compounds in the series. A wavelength at which the protonated and unprotonated species had appreciably different extinction coefficients was then chosen and the optical densities were recorded for each of the pure species and for the mixture of species at each intermediate pH value. The pKa of the compound was calculated by using the Henderson Hasselbalch equation as described by Clark and Cunliffee (162) For a weak base, $pKa = pH + \log_{10}\left[\frac{d-dm}{d_I-d}\right]$ Eq.(10)

where d_I and dm are the optical densities (absorbance) of the protonated species and neutral molecules respectively and d is the optical density at the appropriate intermediate pH value. A specimen calculation of pKa value from the absorption spectra is described in Table 14.

Fig. 12. UV absorption spectra of MZP in Phosphate buffer 0.02M of different pHs

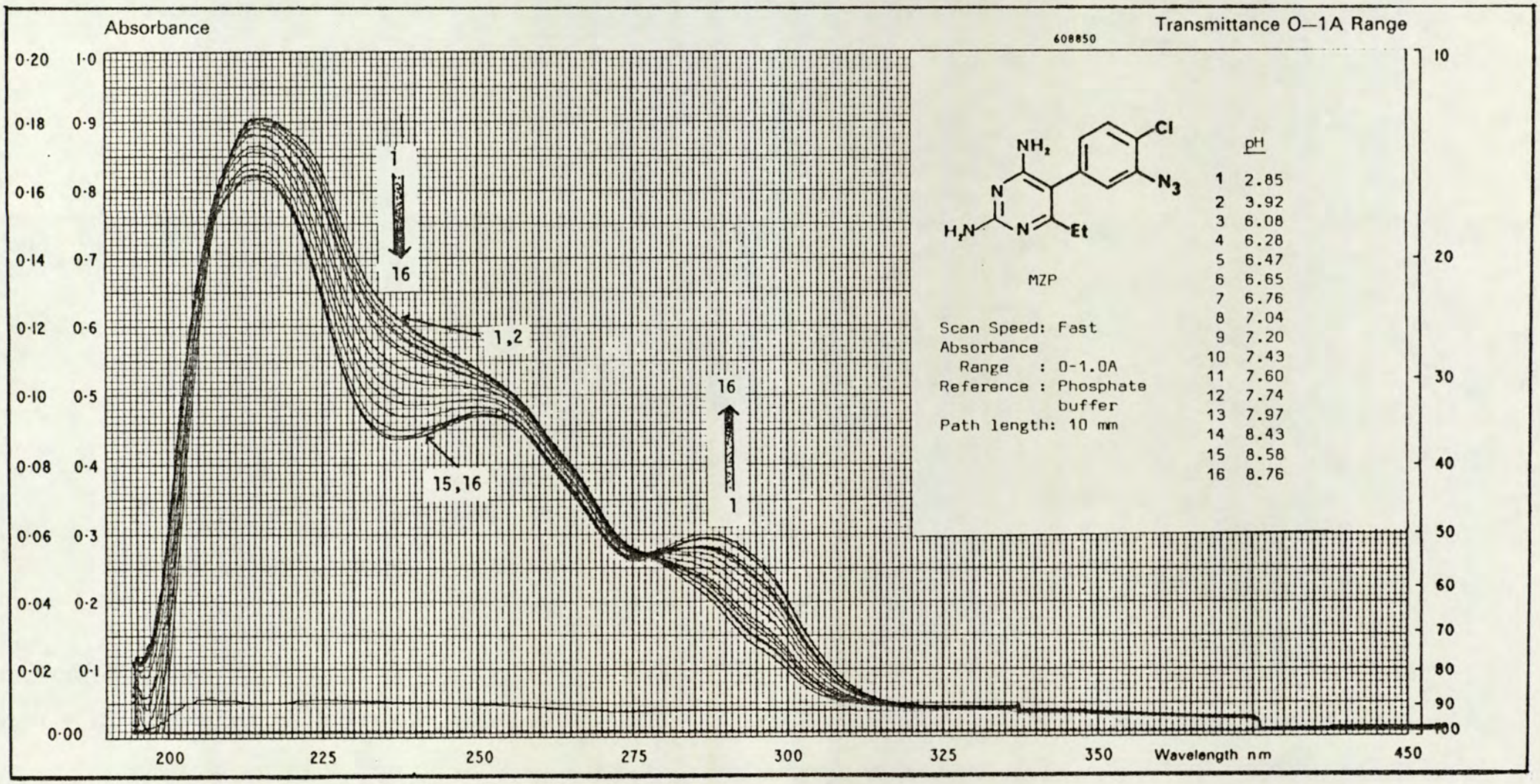


Table 14. Calculation of pKa value of MZP from the absorption spectra

Curve	pH	Absorbance (d) at 236 nm	$\text{Log}_{10} \frac{d-dm}{d_I-d}$	pKa
1	2.85	0.620 (d_I)		
2	3.92	0.620		
3	6.08	0.610	+1.2380	7.3180
4	6.28	0.600	+0.91116	7.1912
5	6.47	0.596	+0.8212	7.2912
6	6.65	0.580	+0.5533	7.2033
7	6.76	0.573	+0.4614	7.2214
8	7.04	0.548	+0.1880	7.2280
9	7.20	0.531	+0.02374	7.2237
10	7.43	0.509	-0.1880	7.2420
11	7.60	0.492	-0.3668	7.2332
12	7.74	0.473	-0.6110	7.1290
13	7.97	0.451	-1.0818	6.8882
14	8.43	0.437		
15	8.58	0.437		
16	8.76	0.437 (dm)		

The experiment was repeated and an average pKa value of MZP was determined - 7.19 ± 0.10. This implied that at physiological pH of 7.4, ~60% of MZP would be present as neutral molecules and ~40% as the protonated species. This would ensure that there are sufficient neutral molecules present to penetrate cells by passive diffusion and sufficient protonated species present to exert biological activity on

DHFR (116).

The pKa values of structurally related compounds were similarly determined and the results are presented in Table 15.

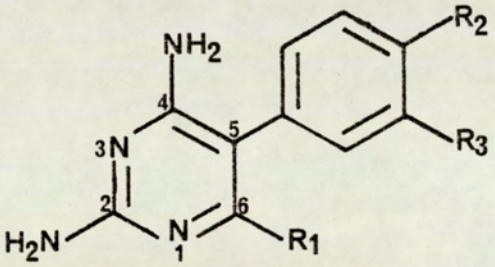


Table 15. pKa of MZP and its analogues at 20°C

Compound	R_1	R_2	R_3	pKa	Literature value (ref 129)
Pyrimethamine	Et	Cl	H	7.30 ± 0.16	7.34
Metoprine	Me	Cl	Cl	7.05 ± 0.03	7.15
MZP	Et	Cl	N_3	7.19 ± 0.10	-
ISOMZP	Et	N_3	Cl	7.11 ± 0.11	-
MAP	Et	Cl	NH_2	7.48 ± 0.08	-
ISOMAP	Et	NH_2	Cl	7.41 ± 0.14	-
MNP	Et	Cl	NO_2	6.84 ± 0.09	-
ISOMNP	Et	NO_2	Cl	6.81 ± 0.09	-

The pKa values determined for pyrimethamine and metoprine are in

close agreement with those obtained by Cavallito et al (129). Change of substituents in the aromatic ring did not significantly alter the pKa value of the compound, indicating that the basicity of N-1 nitrogen was not greatly influenced by the substituents of the aromatic ring. Only a slight increase in the basicity of N-1 nitrogen was observed when pyrimethamine was substituted at R_3 with an electron releasing group. Similarly a slight decrease in basicity of the compound was observed when pyrimethamine was substituted at R_3 with the electron withdrawing nitro and azido groups. The more powerful electron withdrawing nitro group produced a greater decrease in the basicity of N-1 nitrogen than the azido group. Interchanging the substituents R_2 and R_3 to produce compounds of the iso-series did not at all alter significantly the pKa values of the compounds. This suggests that the substituents do not exert their influence on the basicity of N-1 via the conjugation effect.

2.4. Partition Coefficients

As an indication of the lipophilicity of MZP and its analogues, octanol/phosphate buffer 5mM (pH 7.4) partition coefficients (P) were measured. The relative lipophilicities of this series of compounds are: MZP > pyrimethamine > MNP > MAP (Table 16). The lipid solubility of MZP is in the same range as that of metoprine(log P = 2.82) and the nitrosourea CCNU (log P = 2.83) (129). Metoprine was reported to penetrate significantly into normal brain tissue, brain tumours and lymph node tumours (129, 135). Nitrosoureas are the major class of antineoplastic compounds thought to be clinically useful for the treatment of brain tumours (129). MZP would, therefore, have potential applications in the treatment of brain or solid tumours because of its high lipid solubility.

Table 16. Lipophilicity index of MZP and its analogues at 20°C

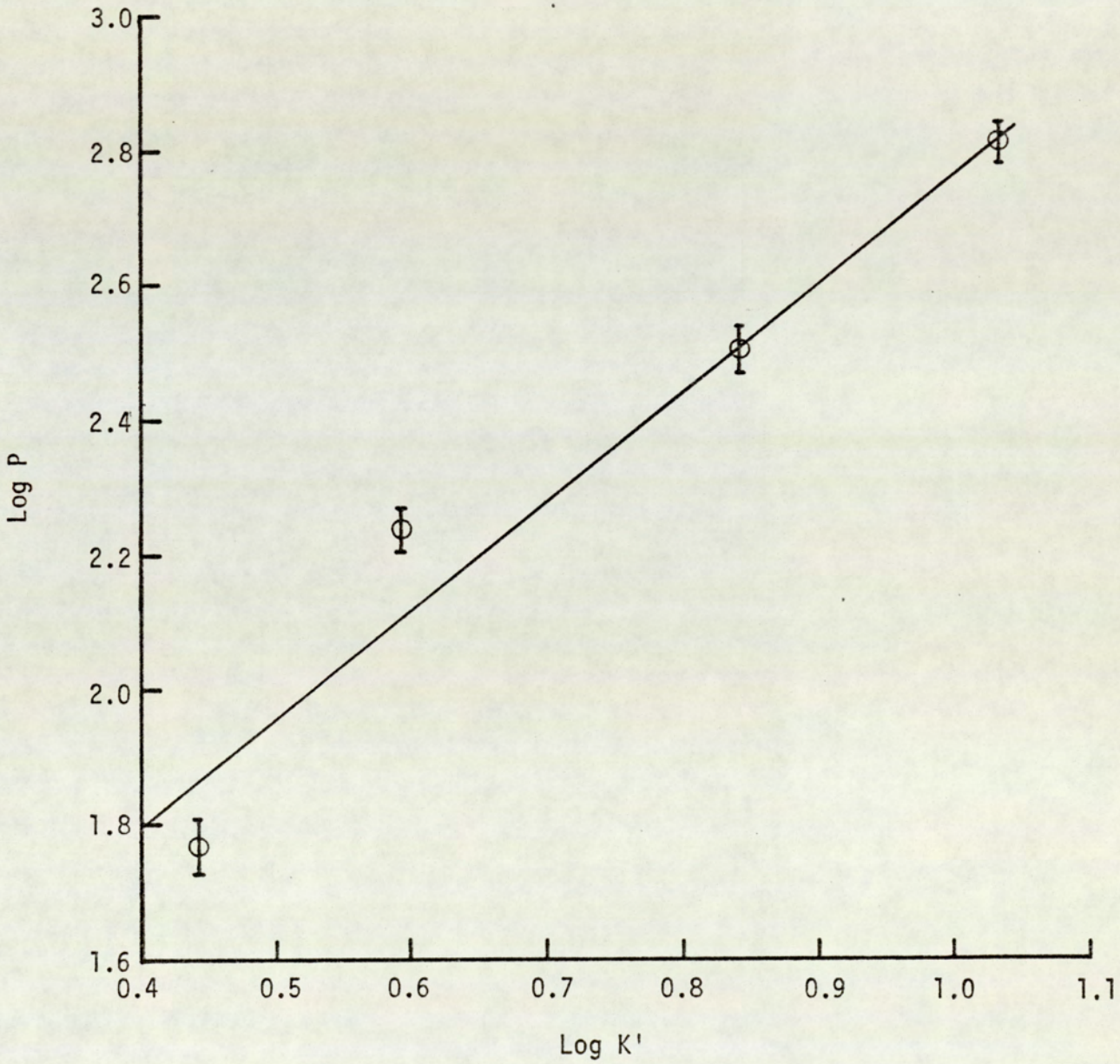
Compound	Log P[a]
MAP	1.768 ± 0.043
MNP	2.239 ± 0.021
Pyrimethamine	2.508 ± 0.025
MZP	2.811 ± 0.032
ISOMAP	1.677 ± 0.008
ISOMZP	2.787 ± 0.009

[a] Octanol/Phosphate buffer 5mM pH 7.4 partition coefficient

High performance liquid chromatography has been used to establish the relationship between capacity factor (log K') and partition coefficient (log P) in a series of compounds. This relationship can then be used to predict the partition coefficients of other compounds within the same series. This method is rapid, versatile, precise, reliable and is particularly useful for predicting log P of compounds which are unstable in the system employed by shake-flask method (164). The advantages of this method have been demonstrated by authors who applied it in various series such as: vasodilators (165), 1,4-benzodiazepines (166), penicillins and cephalosporins (164), phenolic derivatives (167), chlorinated benzenes, toluenes and anilines (168), various simple aromatic molecules (169) and benzamides (170). The capacity factor is calculated from the formula:

$$K' = \frac{t_R - t_o}{t_o}$$

Fig. 13. A plot of log P Vs log K' for the diaminopyrimidine analogues



where t_R is the retention time of the compound under investigation and t_o is the retention time of a non-retained product e.g. methanol. The capacity factors determined from HPLC analyses for the diaminopyrimidine analogues are depicted in Table 17.

Table 17. Capacity factors of MZP and analogues determined by HPLC

Compound	K'	Log K'
MAP	2.7671	0.4420
MNP	3.9128	0.5925
Pyrimethamine	6.9397	0.8413
MZP	10.8107	1.0339

An approximately linear relationship was found between log K' and the partition coefficient (log P octanol/phosphate buffer 5 mM pH 7.4) in this series of diaminopyrimidine analogues (Fig. 13). The correlation coefficient between the two parameters was found to be 0.9773. Therefore, HPLC could be employed as an alternative method for the determination of the partition coefficient of compounds in this particular series.

2.5. Perfluorocarbons

Perfluorocarbons (PFC) are cyclic or strain chain hydrocarbons in which the hydrogen atoms have been replaced with fluorine. The

carbon-fluorine bond is one of the most stable, so the PFC are considered to be chemically and biologically inert. PFC are excellent gas solvents, they dissolve ~40-50 vol % of oxygen and 100-150 vol % of carbon dioxide (171,172,173). Pure liquids of PFC are immiscible with water and hence incompatible with biological fluids. Microemulsions of PFC (particle size < 0.1 um diameter) dispersed in an isotonic electrolyte solution have been evaluated as potential blood replacement preparations, to replace the gas transporting properties of erythrocytes, in several species including rat, rabbit, dog, monkey and man (171,172). Unlike haemoglobin, the oxygen delivered by PFC is not bound to a chelate and hence the loading and unloading of the oxygen is directly related to its environmental partial pressure (pO_2). The small size of PFC particles also facilitate the transport of oxygen to areas where passage of blood through tissues is restricted, e.g. in cerebral and cardiac ischaemia (172). Because PFC are used in the emulsified form, the oxygen that can be dissolved is considerably less, ~10-16 vol %, than when the pure liquids are used. The preparation of PFC emulsions has been described by Meinert et al (173).

After intravenous administration, perfluorocarbon particles are cleared from the blood by the phagocytic cells of the reticulo-endothelial system (RES), particularly the liver and spleen, and the macrophages (171,174). There is no convincing evidence to suggest that PFC are degraded biologically. Urinary and faecal excretion has proven to be insignificant. PFC were found to leave the body by transpiration through the lungs and to a small extent through the skin (171). The rate at which different PFC left the body varied greatly. The molecular structure and size of the PFC, their relative lipid solubilities and the vapour pressure were considered to be

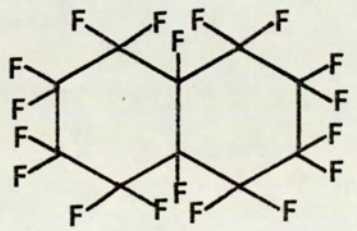
important factors affecting the transpiration rate (171).

The administration of PFC emulsions was reported to cause a transient depression of the phagocytic function of the RES in rats (174). The degree of RES blockade induced by PFC emulsion was only moderately greater than that obtained with intralipid. Since intralipid has been widely used as a nutritional source in the clinic without any report of adverse effect as a result of RES blockade, this depression of phagocytic activity may not be of clinical significance. Morphological changes in the ultrastructure of liver, spleen, lung and kidney cells as a result of PFC emulsion administration were observed (175). Haematological effects of neutropenia, thrombocytopenia and pulmonary leucostasis were also reported (175). The blood toxicity, however, was due to the emulsifier, Pluronic F68, and not the PFC itself (180). The mechanisms of the adverse reactions to emulsified PFC are not fully understood and whether or not these reactions are of clinical significance remains to be elucidated.

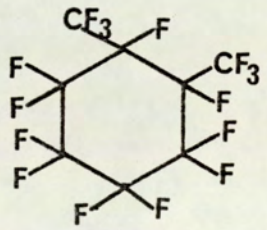
Besides being used as blood substitutes, perfluorocarbon emulsions may have other potential clinical applications. Tumours often have inadequate vasculature and areas of intermittent and irregular blood flow because the blood vessels in tumours can constrict and collapse. This leads to zones of necrosis and areas of hypoxia (176, 177, 178, 179). Radio- and chemotherapy efficacy may be limited by the resistance of these hypoxic cells. An intravenously administered PFC emulsion was reported to enhance the effectiveness of radiation therapy when an oxygen rich atmosphere (hyperbaric oxygen or 95% oxygen, 5% carbon dioxide, i.e. carbogen) was breathed before and during irradiation (176,177,178). Perfluorocarbon emulsions and oxygen alone did not produce any

antitumour effect. However, when PFC emulsion with oxygen was administered concomitantly with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), a significant synergistic effect on BCNU chemotherapy in an experimental rat brain tumour was observed (179). Therefore, PFC with oxygen seems to be effective as an adjuvant to cancer chemotherapy.

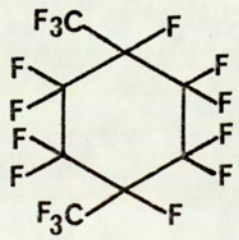
The present study was designed to evaluate the potential of perfluorocarbons as a vehicle for the delivery of lipid soluble antineoplastic agents. To be of any significant use, a high solubility of the drug in PFC is desired so that when a 10 or 20% emulsion in water is produced, precipitation of the drug does not occur. The compounds used in this study were MNP and MZP. The perfluorocarbons employed for investigation were perfluorodecalin (XXXI), 1,2-bis(trifluoromethyl)-1,2,3,3,4,4,5,5,6,6-decafluorocyclohexane (XXXII), 1,4-bis(trifluoromethyl)-1,2,2,3,3,4,5,5,6,6-decafluorocyclohexane (XXXIII) and tetrafluoroethylene pentamer epoxide (XXXIV).



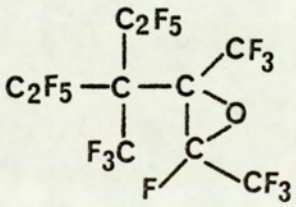
XXXI



XXXII



XXXIII



XXXIV

It was not possible to determine the solubility of MNP and MZP in the perfluorocarbons because the PFC interfered with both the UV and HPLC analytical methods used for assaying the diaminopyrimidine analogues. Consequently, the partition coefficients of the compounds between perfluorocarbon and phosphate buffer (5mM; pH 7.4) were determined by assaying the drug content of the aqueous phase before and after shaking with the perfluorocarbons.

The perfluorocarbons under investigation were found to be very poor solvents for both MNP and MZP (Table 18). The compounds were more soluble in the aqueous phase than in the perfluorocarbons as indicated by the log P values. Octanol was found to be a better solvent than the perfluorocarbons in dissolving MNP and MZP (Table 16). Therefore, the perfluorocarbons are not suitable solvents for the delivery of the compounds in the diaminopyrimidine series. This, however, does not rule out the possibility that the perfluorocarbons could be a suitable solvent for the delivery of other groups of compounds. Further research would need to be carried out with compounds of a wider range of lipophilicity than the two employed in this study.

Table 18. Partition coefficients of MNP and MZP between perfluorocarbons and phosphate buffer 5mM pH 7.4 at 20°C

Perfluorocarbons	Log P	
	MNP	MZP
XXXI	-1.751 ± 0.129	-1.005 ± 0.095
XXXII	-1.753 ± 0.13	-1.170 ± 0.121
XXXIII	-1.836 ± 0.323	-1.440 ± 0.247
XXXIV	-0.840 ± 0.376	-0.007 ± 0.033

3. STABILITY EVALUATIONS

The immediate objective in stability evaluations is to identify those factors which may influence the stability of the compound under investigation. Such factors may include temperature, pH, radiation, catalysts, moisture, oxygen, excipients, etc. In the majority of cases, the degradation of pharmaceuticals can be treated as zero, first or pseudo-first order reactions, even though many of the pharmaceutical compounds degrade by complicated mechanisms (110).

Arylazides are known to be thermal and photo-labile (6,7,8). Since MZPES was the first arylazide to be introduced into the clinic, it was desirable that its stability profiles were thoroughly investigated.

3.1. Thermal stability

Evaluation of the thermal stability of a compound not only enables the prediction of its shelf life when stored under a prescribed temperature but it also provides information on the suitability of the compound for heat sterilization. Sterility is an important requirement for all parenteral dosage forms. The British Pharmacopeia (82) recognizes six methods of sterilisation: autoclaving, dry heat, heating with a bactericide, filtration, exposure to ionising radiation and exposure to ethylene oxide. Autoclaving has been advocated as the ideal method for sterilising aqueous preparations. Sterilisation by other methods is only to be considered if an aqueous solution of the compound is not sufficiently stable to heat to allow sterilization by autoclaving or heating with a bactericide.

3.11. Effect of autoclaving MZPES aqueous solution

Autoclaving an aqueous MZPES solution at 115°C for 30 minutes or 121°C for 20 minutes induced a degradation of 2 and 5% of the compound respectively.

Table 19. Degradation of aqueous MZPES solutions induced by autoclaving at 115 or 121°C

Treatment	Concentration of MZPES ($mgml^{-1}$)	% MZPES remaining
115°C, 30 minutes	1	97.8 ± 1.0
121°C, 20 minutes	1	96.0 ± 0.2
121°C, 20 minutes	5	94.4 ± 1.3

Other methods of sterilising aqueous MZPES solution had to be found. Heating with a bactericide was not favoured because it involved introducing into the formulation an additional compound which had the potential to influence the efficacy and/or toxicity of MZPES. Therefore, additional anti-tumour and toxicological studies would have to be performed involving MZPES in combination with the bactericide. Moreover, heating aqueous solutions of MZPES at 100°C for 30 minutes also produced approximately 1% degradation of the compound. However, the degree of degradation produced was too small to be estimated accurately since the experimental error of the analytical method was ± 1.7%. Nevertheless, even traces of degradation products could pose a potential risk if these degradation products were more toxic than the parent compound or if they had the

ability to induce further decomposition. Filtration by aseptic techniques was, therefore considered a suitable means of sterilizing aqueous MZPES solution.

3.12 pH-Stability Studies

To investigate the effect of pH on the thermal stability of aqueous MZPES solutions, a 2 $mgml^{-1}$ solution was prepared. Citrate and phosphate buffers (0.1M) of different pHs were prepared. Equal volume of MZPES and buffer solutions were mixed together at room temperature and the pH of the resulting solution measured. It was found that when MZPES solution was added to the buffer solutions with pH > 5.0, spontaneous precipitation of the free base occurred. The precipitate was characterised by IR and the spectra obtained were identical to that of the authentic sample MZP. The precipitation occurred as a result of the extremely low aqueous solubility of the free base (22 ug ml^{-1}). This indicated the potential risk of drug precipitation in the physiological medium upon bolus intravenous injection. Therefore, MZPES solution must be diluted in an infusion fluid to a suitably low concentration prior to intravenous administration.

pH was found to have an insignificant influence on the stability of MZPES solutions between pH 3.0 and 4.5 (Table 20).

Table 20 pH-stability studies on aqueous MZPES solutions ($1 mgml^{-1}$)

pH	% MZPES remaining	
	100°C 30 minutes	121°C 20 minutes
3.07	99.47 ± 0.05	93.50 ± 0.95
3.58	100.70 ± 0.20	92.35 ± 0.22
4.09	99.50 ± 0.06	95.38 ± 0.04
4.56	100.41 ± 0.02	93.75 ± 0.08
5.00 and above	Precipitation of MZP free base	

3.13. Thermal Stability of MZPES in buffer solutions

The degree of degradation of MZPES in buffer solutions was similar to that in distilled water (Table 21). Increasing the concentration of the buffer solutions did not alter the extent of degradation. There was no significant difference in the extent of MZPES degradation between citrate and lactate buffers. In addition, it was found that the percentage degradation of MZPES was the same in both the 1 and 5 $mgml^{-1}$ solutions. Since the buffer solutions did not retard the degradative process, there would be little justification in buffering the aqueous MZPES solution except as a means of preventing the precipitation of the free base in the event of a pH change upon direct intravenous injection. In the latter case, a buffer with a strong buffering capacity would be required if a solution of MZPES were to be formulated at pH 3.0 - 4.0. However, the administration of large quantities of buffer chemicals was not considered to be highly desirable since it could affect the acid-base balance of the physiological system.

Table 21 Effect of autoclaving MZPES in buffer solutions at 121°C for 50 minutes

Solvent	MZPES concentration ($mgml^{-1}$)	△pH	% MZPES remaining
Water	1.0	-0.80	80.1 ± 0.2
Water	5.0	-0.48	81.6 ± 0.6
Citrate buffer 0.01M[a]	1.0	-0.03	81.4 ± 0.2
Citrate buffer 0.01M	5.0	0.00	80.9 ± 0.03
Citrate buffer 0.05M	1.0	-0.02	82.5 ± 1.1
Citrate buffer 0.05M	5.0	-0.01	81.5 ± 0.04
Lactate buffer 0.01M	1.0	-0.14	81.4 ± 0.6
Lactate buffer 0.01M	5.0	-0.10	81.2 ± 0.2
Lactate buffer 0.05M	1.0	-0.10	80.6 ± 0.7
Lactate buffer 0.05M	5.0	+0.05	83.1 ± 0.6
Water	1.0	-0.39	96.0 ± 0.2[b]
Water	5.0	-0.29	94.4 ± 1.3[b]
Citrate buffer 0.1M	1.0	0.00	93.0 ± 0.2[b]
Citrate buffer 0.1M	5.0	0.00	93.2 ± 0.6[b]

[a] All buffer solutions were at pH 4.0

[b] Autoclaving was carried out at 121°C for 20 minutes instead of 50 minutes

The degradation of MZPES solutions in water was accompanied by a colour change, from pale straw to yellow, and a decrease in the pH of the solutions. The yellow colour was due to the presence of a degradation product with a strong chromophore, possibly an azo compound. The decrease in pH could be the result of the formation of hydrazoic acid or the dissociation of the proton from the pyrimidine ring in the degradation process.

3.14. Effect of concentration, oxygen and buffer on the rate of thermolysis of aqueous MZPES solutions

The oxygenated solutions of MZPES in water were prepared by passing 95% O_2, 5% CO_2 gas through the solutions for 5-10 minutes just before the ampoules were sealed. There was no significant difference in the rate of thermolysis of aqueous MZPES solutions at 140°C between oxygenated solutions and solutions equilibrated with atmospheric air (Figs. 14,15). A small difference was observed in the thermolytic rate of the 1 and 10 $mgml^{-1}$ solutions of MZPES (Fig. 14). The degradation of 10 $mgml^{-1}$ MZPES solution occurred at a slightly faster rate, with a rate constant of 0.0190 min^{-1}, than the 1 $mgml^{-1}$ solution which had a rate constant of 0.0161 min^{-1} (Table 22). Thermolysis of MZPES in buffer solutions at pH 4.0 and in water occurred at virtually the same rate (Fig. 15). Therefore, oxygen and buffer solutions had an insignificant effect on the rate of thermolysis of aqueous MZPES solutions, whereas increasing the concentration of the solution caused a slight increase in the thermolytic rate. The rate constants and half-lives of these reactions are tabulated in Table 22.

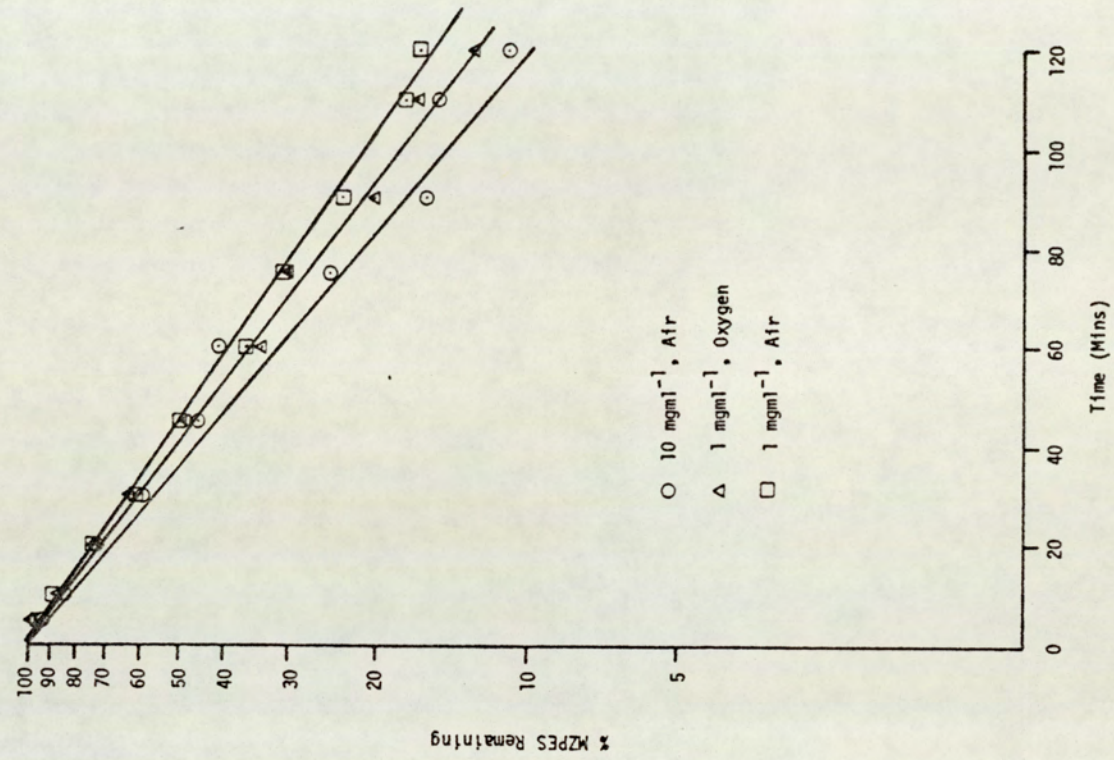


Fig. 14. Thermolysis of aqueous MZPES solutions at 140°C

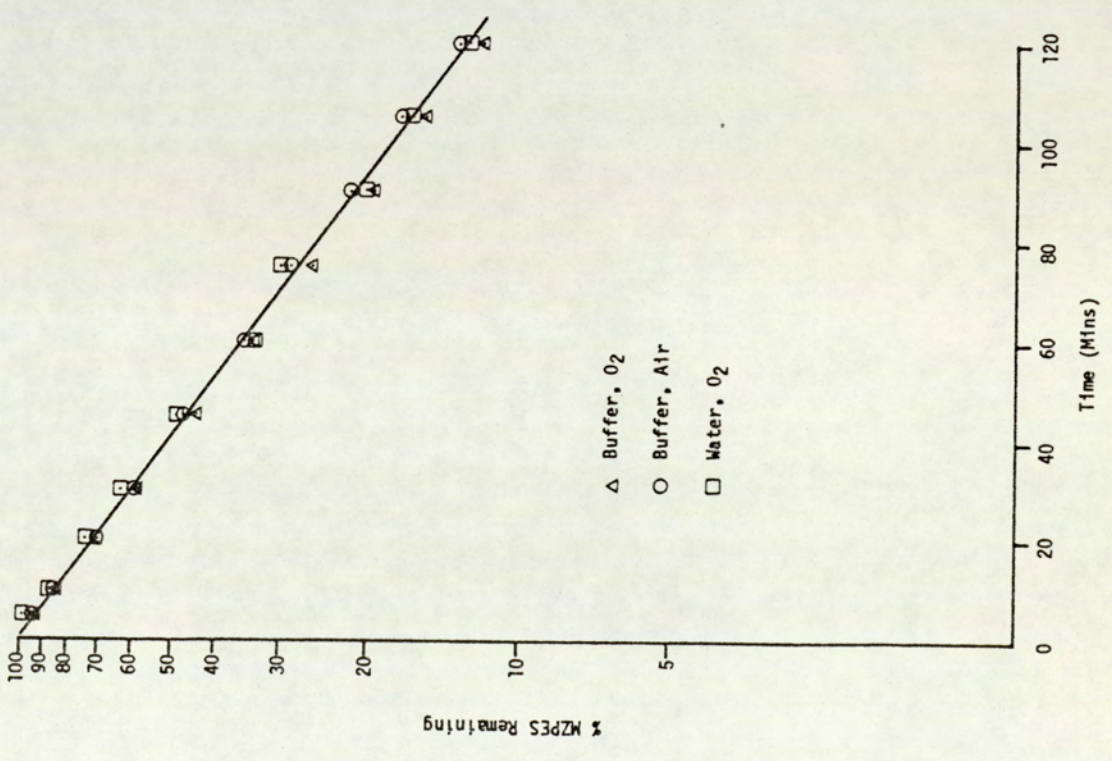


Fig. 15. Thermolysis of MZPES solutions in water and 0.02M phosphate buffer pH 4.0 at 140°C

Table 22. The rate of thermolysis of aqueous MZPES solutions under the influence of various factors at 140°C

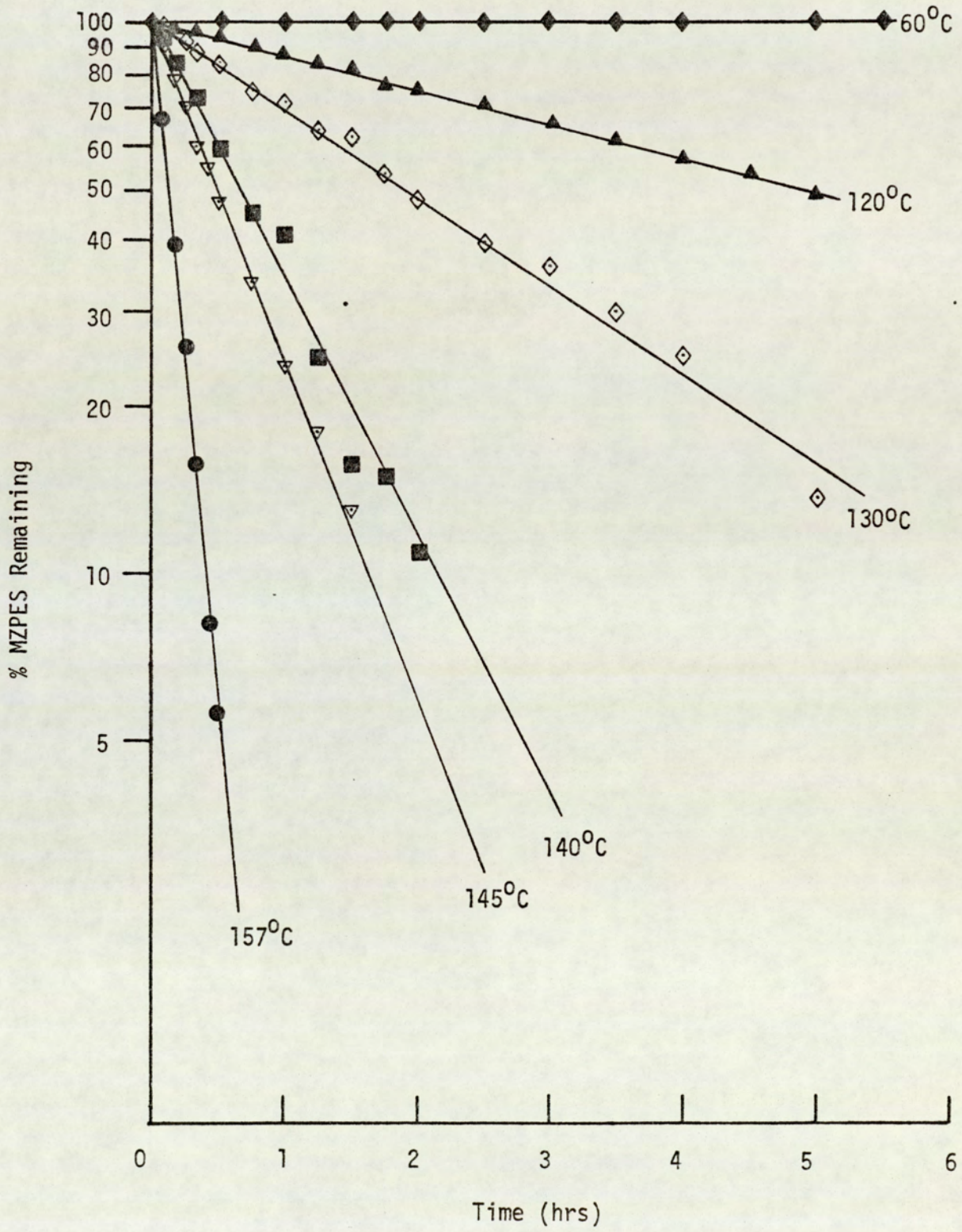
Gas treatment	Solvent	Concentration (mg ml^{-1})	K (min^{-1})	$t_{\frac{1}{2}}$ (min)
Air	Water	10	0.0190	36.56
Oxygen	Water	1	0.0177	39.19
Air	Water	1	0.0161	43.00
Oxygen	Phosphate buffer[a]	1	0.0180	38.60
Air	Phosphate buffer	1	0.0168	41.14

[a] Phosphate buffer 0.02M, pH 4.0.

3.15. Accelerated stability studies

The stability of aqueous MZPES solutions at elevated temperatures was evaluated so that the stability of the solution at normal storage temperatures could be predicted. At temperatures below 100°C, no significant degradation was observed over an eight hours' heating period. Degradation of MZPES solutions occurred more readily at temperatures above 100°C and followed first order kinetics (Fig. 16). Similar behaviour was observed with the thermal degradation of other aromatic azides (9,10,11,62). The concentration of MZPES

Fig. 16. Thermolysis of aqueous MZPES solutions (10 $mgml^{-1}$)



solution used in the present study was 0.025M and this was also the concentration of the formulated product. At this concentration, no induced decomposition of MZPES was observed. This result differed from that reported by Dyall (11) where induced decomposition of arylazides was observed at azide concentration > 0.01M, resulting in a deviation of first order decomposition kinetics. The discrepancy could be a result of different solvents used and/or it could be due to the fact that MZPES consisted of a larger molecular fragment than phenylazide and hence was more effective in stabilising the nitrene species.

The rate constants (K) and half-lives ($t_{\frac{1}{2}}$) of the thermal stability of MZPES solutions at different temperatures are tabulated in Table 23. An Arrhenius plot (Fig. 17) was constructed to predict the stability of MZPES solution at other temperatures. The activation energy was calculated to be 139.3 $KJmol^{-1}$. This value is close to that of phenylazide, 2,4-dichloro,4-chloro or 4-nitrophenylazide but is 10 KJ higher than that of 2-chlorophenylazide (11). The elevation of the activation energy in MZPES is probably the result of the presence of an additional 2,4-diaminopyrimidine substituent. Nevertheless, the activation energy of MZPES is typical of an arylazide that decomposes thermally without an anchimeric assistance from an α,β-unsaturated ortho substituent (11,63).

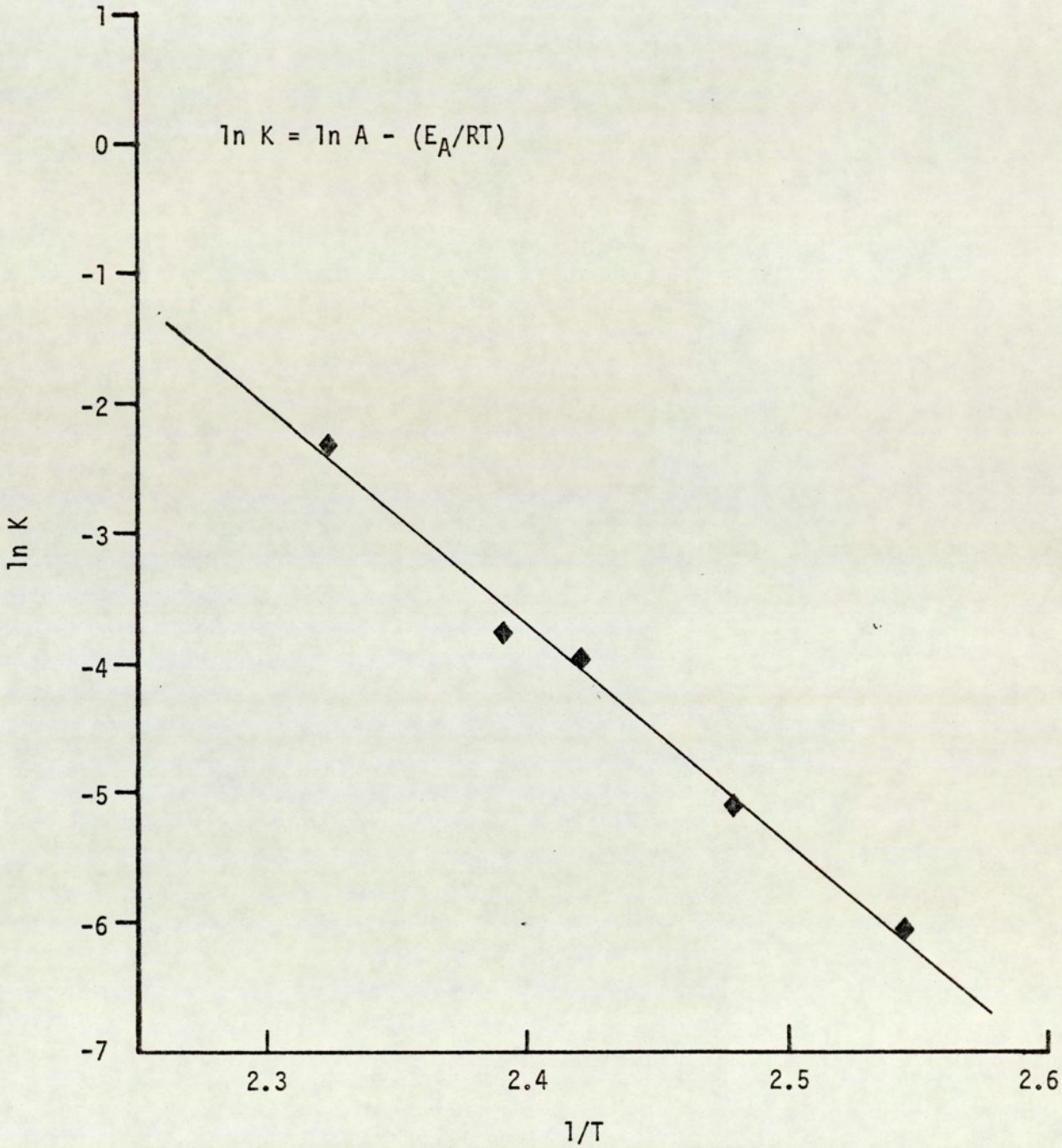
MZPES solution was predicted to be extremely stable at temperatures below 25°C (Table 23). The formulated injections could, therefore, be stored at room or refrigerated temperatures without any risk of thermal decomposition.

Table 23 Thermal stability of aqueous MZPES solutions

Temperature (^{0}C)	K (min^{-1}) x 10^{-3}	$t_{\frac{1}{2}}$(mins)	$t_{10\%}$(shelf life; mins)
157	98.8694	7.0	1.1
145	22.8902	30.3	4.5
140	18.9558	36.6	5.5
130	6.2376	111.1	16.8
120	2.3761	291.7	43.8
100[a]	-	2.11 days	0.32 days
90	-	1.04 weeks	1.10 days
60	-	1.27 years	2.3 months
55	-	2.73 years	4.9 months
50	-	6.01 years	10.9 months
25	-	4.64×10^{2} years	70.5 years
20	-	1.21×10^{3} years	1.84×10^{2} years
4	-	3.27×10^{4} years	4.97×10^{3} years
0	-	7.93×10^{4} years	1.20×10^{4} years
-10	-	1.02×10^{6} years	1.53×10^{5} years

[a] The stability data of MZPES solution below $100^{0}C$ were predicted from the Arrhenius plot.

Fig. 17. Arrhenius Plot of thermal stability for aqueous MZPES solutions



3.16. Shelf-life surveillance on MZPES injections

It is essential to evaluate the stability of aqueous MZPES solution under normal shelf storage conditions in order to eliminate errors in the predicted stability data obtained from elevated temperatures studies. Errors can occur as a result of abnormal degradative processes that only exist under accelerated conditions (110).

Samples from two batches of MZPES injections were employed in the present study. The first batch UA 84J03 was prepared specifically for stability evaluations, while the second batch UA 84M07 consisted of injections manufactured for the phase I clinical trials. No degradation was detected when the first batch of injections was stored for a period of 69 weeks at 4°C or at room temperature, 20°C (Fig. 18). Similarly, the results of the second batch of injections indicated that the injections were stable over a period of 21 months when stored at -10, 4 or 20°C (Fig. 19). However, storage at 50°C revealed that degradation of MZPES injections was evident after a 3 months' period.

The pH and colour of the injections of UA 84M07 were also monitored. No significant change was observed for injections stored at -10, 4 or 20°C. For injections stored at 50°C, a decrease in pH of the solution was detected after 3 months and a colour change from pale straw to yellow was observed. Qualitative observation revealed that the intensity of the yellow colour increased with storage time.

It can, therefore, be concluded that the injections of aqueous MZPES solution, 10 $mgml^{-1}$, are stable for at least 21 months when stored at -10, 4 or 20°C. The expiry date of the injections may be

Fig. 18. Shelf-life studies on MZPES injection 10 $mgml^{-1}$ BN: UA84J03

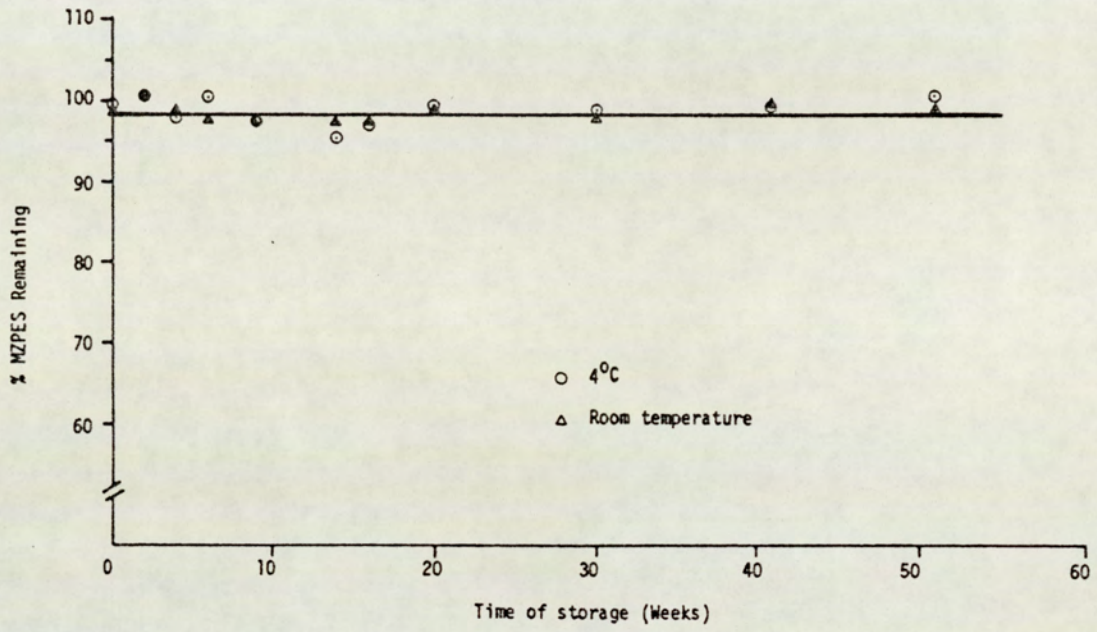
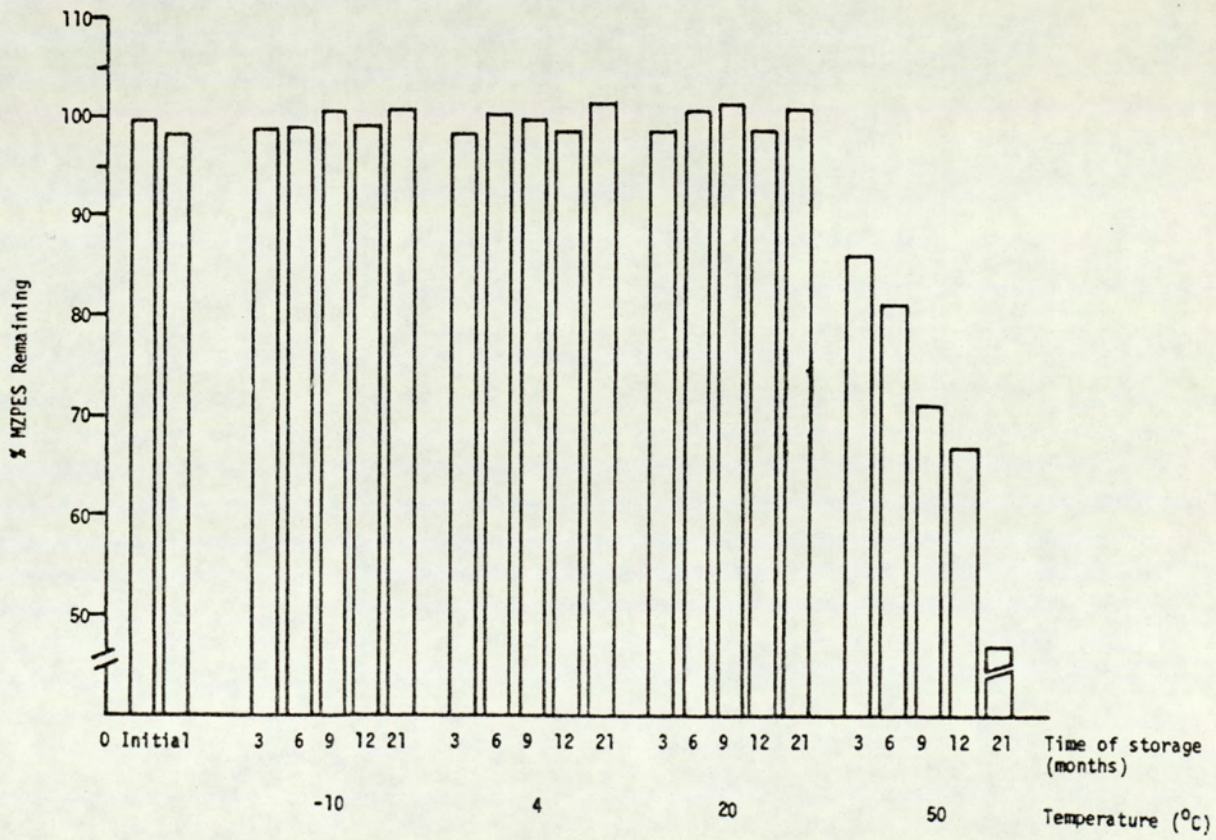


Fig. 19. Shelf-life studies on MZPES injection 10 $mgml^{-1}$ BN: UA84M07



extended subject to subsequent shelf-life stability data to be obtained for MZPES injections UA 84M07.

3.2. Photo-stability

The effect of radiation on degradation is more difficult to quantify because of the complicated nature of photochemical reactions. The intensity and wavelength of light, and many other factors such as the size, shape and material of the container have the potential to affect the rate of photochemical reactions (110). Moreover, secondary free-radical or thermal reactions can occur and complicate the kinetics of degradation (71,72,110). Therefore, it is difficult to predict quantitatively the photo-stability of a compound from accelerated conditions but an indication of stability can be obtained from the qualitative studies.

3.21. Effect of daylight on stability of aqueous MZPES solution

Preliminary photo-stability of MZPES was investigated by exposing ampoules of aqueous MZPES solution to daylight by placing the clear glass ampoules near a window. Degradation of the compound was evident after 48 hours, indicated by a colour change in the solution; and less than 5% of the MZPES in a 1 $mgml^{-1}$ solution was left after 126 days of exposure (Table 24). However, no degradation was detected after 126 days for the same solution stored in the dark. When a 0.5 $mgml^{-1}$ aqueous MZPES solution was exposed to daylight, only 44.95 ± 0.15% of the compound remained after 14 days. Therefore, photo-degradation of MZPES occurred more rapidly in dilute solutions.

Table 24. Photo-degradation of aqueous MZPES solution (1 $mgml^{-1}$) in daylight at 20°C

Day of exposure	% MZPES Remaining
10	76.69 ± 0.88
21	66.07 ± 0.71
28	56.88 ± 0.15
126	<5.00

3.22. Photo-degradation of aqueous MZPES solution in an Hanovia photo-reactor.

Photo-degradation of MZPES solutions proceeded via a first order reaction (Fig. 20). The concentration of MZPES solutions employed in this study ranged from 2.50×10^{-4} to 2.5×10^{-3}M. The half-lives of these solutions, when irradiated at wavelengths 254-366 nm, are recorded in Table 25.

Table 25. Photo-stability of aqueous MZPES solutions at ambient temperature

Concentration ($mgml^{-1}$)	K (min^{-1})	$t_{\frac{1}{2}}$ (mins)
0.1	0.2880	2.41
0.5	0.0624	11.10
0.8	0.0469	14.79
1.0	0.0331	20.96

Fig. 20. Photolysis of aqueous MZPES solutions at 20°C

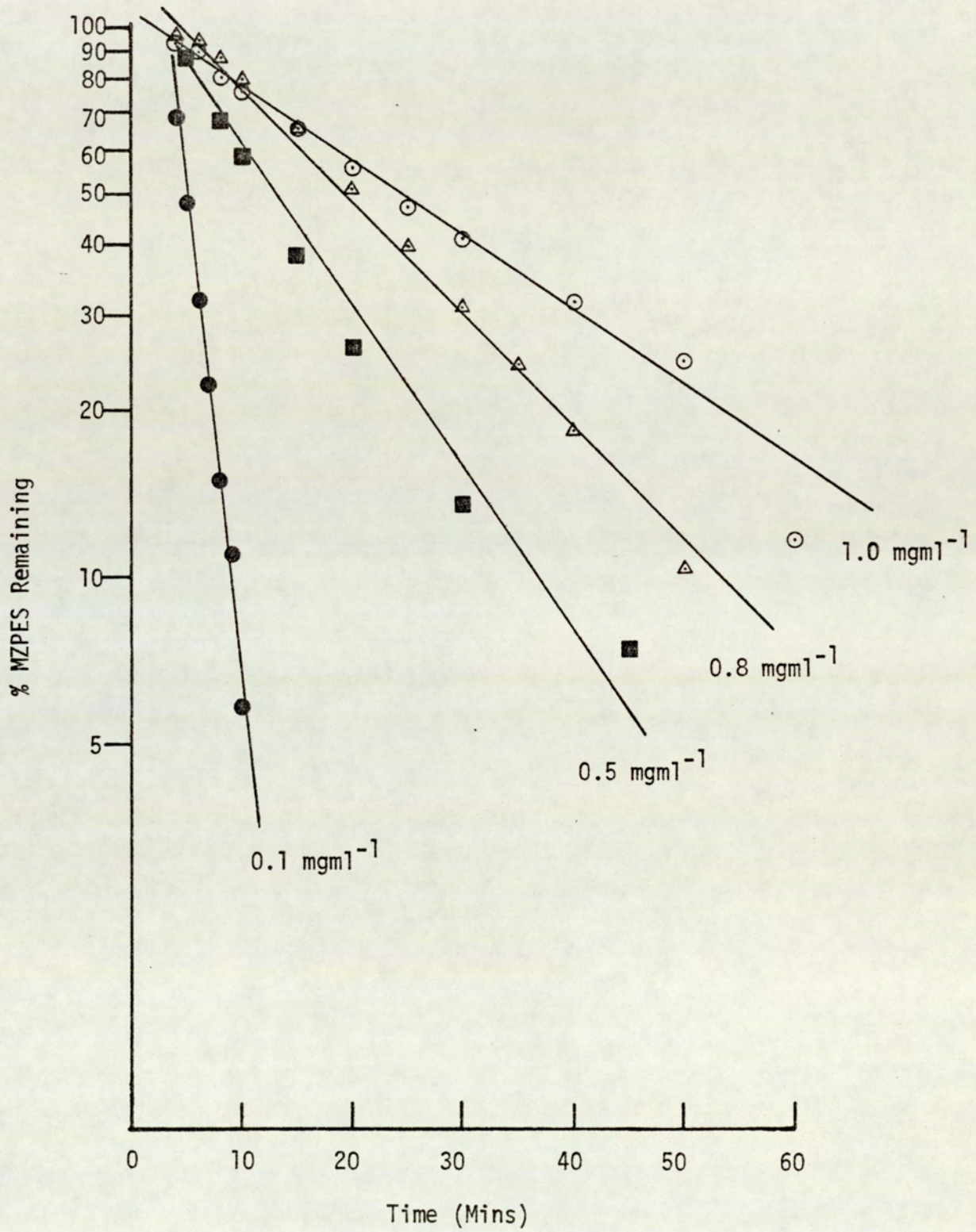
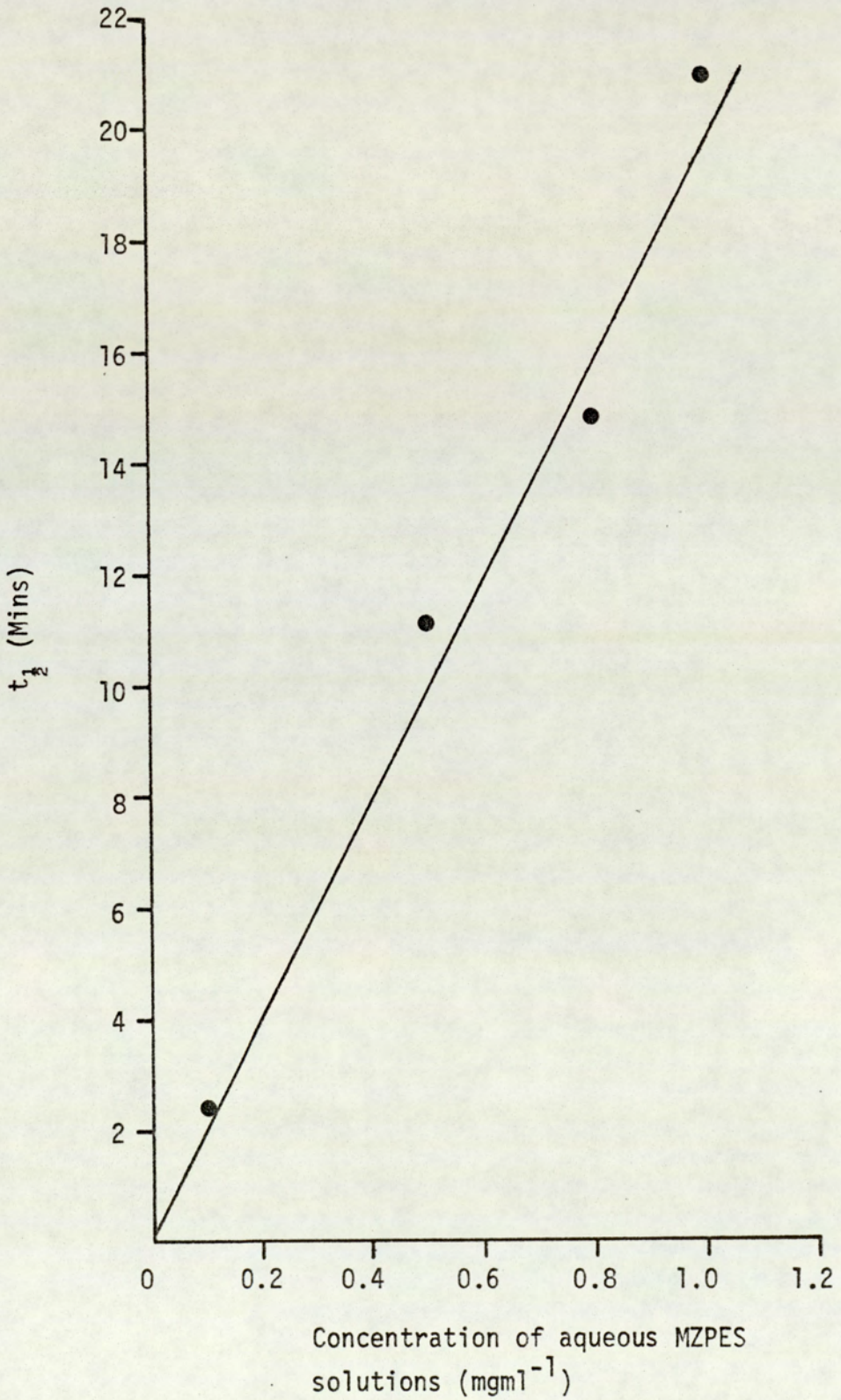


Fig. 21. Correlation between concentration of aqueous MZPES solutions and the photolytic half-life



A linear relationship (correlation coefficient = 0.9908) was found between the concentration and the photolytic half-life of the aqueous MZPES solutions (Fig. 21). This indicated that at the concentrations employed in this study, the photo-degradation of MZPES solutions proceeded according to Einstein's law of photochemical equivalence. The number of MZPES molecules undergoing reaction at each concentration at a given time was the same and was directly dependent on the energy of the irradiating source. Consequently, the rate of degradation was faster in the more dilute solutions.

The present results showed that photolysis of MZPES solutions at concentration $<2.5 \times 10^{-3}$M was not accompanied by a secondary reaction. This is consistent with the observation that free radical auto-catalytic chain decomposition only occurs if the concentration of phenylazide is greater than 3×10^{-3}M (71). Although the present study showed that the rate of photo-degradation of MZPES solutions was slower at higher concentrations, this relationship was only valid for concentration of MZPES solution <1.0 mgml^{-1}. At concentration much higher than 1.0 mgml^{-1}, secondary reaction could occur between the products and the substrate resulting in an enhancement of the degradation rate.

It can be concluded from this study that aqueous solutions of MZPES are photo-sensitive and therefore the formulated products must be protected from the influence of radiation/light.

3.23. Effect of oxygen, nitrogen and air on the rate of photolysis of aqueous MZPES solutions

Various gases were passed through 0.1 mgml^{-1} aqueous MZPES solutions prior to and during irradiation. The degradation of MZPES in air or oxygen-saturated water proceeded *via* first order kinetics

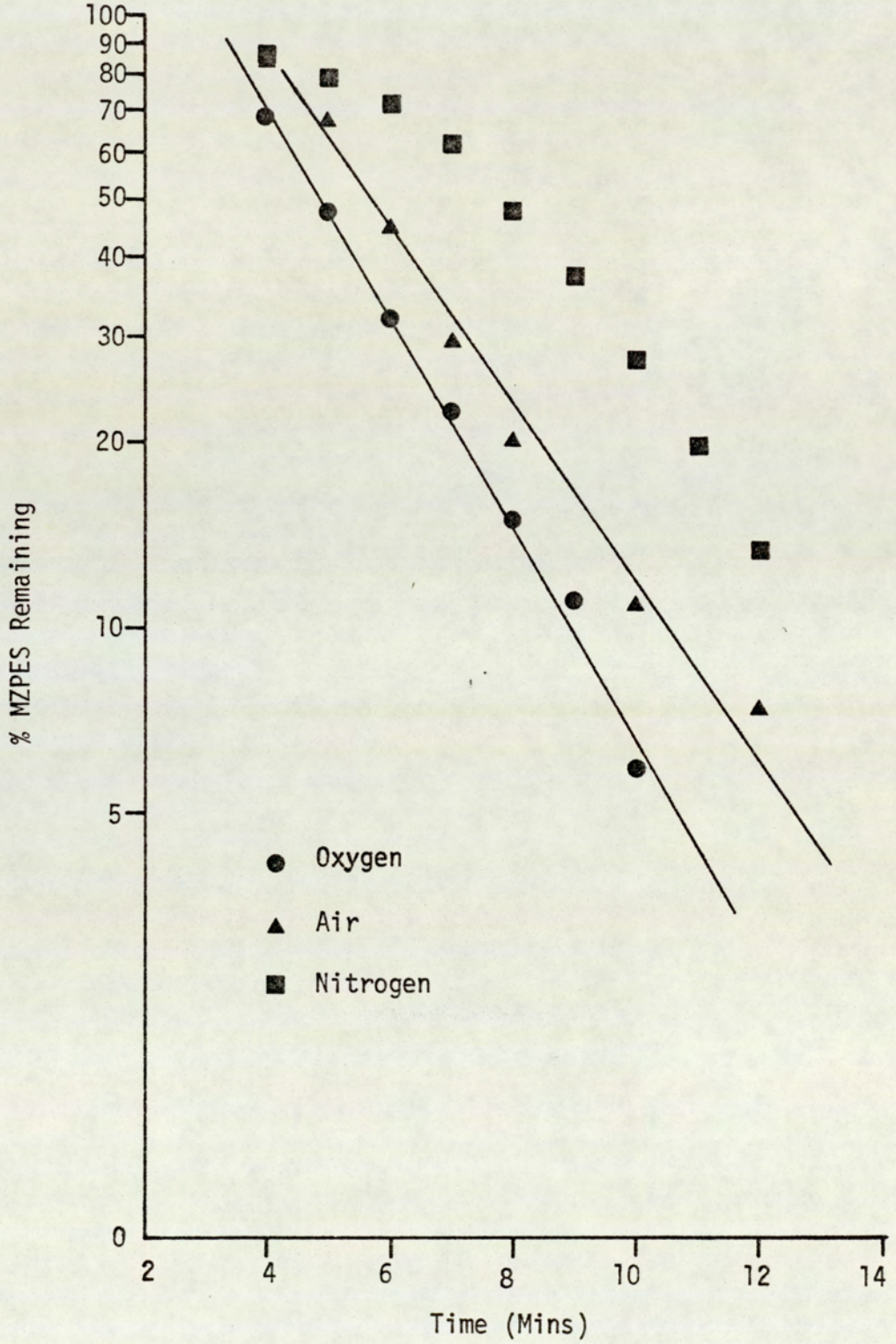
(Fig. 22). No significant difference was observed between the photolytic rate of MZPES in air and oxygen-saturated water. The degradation of MZPES in nitrogen-saturated water followed zero order kinetics. It was, however, included in the log % remaining vs time plot (Fig. 22) for comparison. The rate of photo-degradation of MZPES in nitrogen-saturated water was found to be slower than in air or oxygen-saturated water. The half-lives of these photochemical degradations are included in Table 26. These results indicated that bubbling nitrogen through the aqueous solution of MZPES, prior to sealing of the ampoules, would provide a means of retarding photo-degradation of the compound.

Table 26. Photo-degradation of 0.1 $mgml^{-1}$ aqueous MZPES solutions under the influence of different gases at ambient temperature

Gas used in study	K (min^{-1})	$t_{\frac{1}{2}}$ (mins)
Oxygen	0.2880	2.41
Air	0.2470	2.81
Nitrogen	8.0173[a]	6.24

[a] Zero-order rate constant

Fig. 22. Photolysis of aqueous MZPES solutions (0.1 $mgml^{-1}$) at $20^{o}C$ under the influence of various gases



3.3. Isolation and identification of the thermolytic and photolytic products

Aqueous solutions of MZPES where >90% degradation had occurred as result of thermolysis or photolysis were used in the present study. The degradation products were isolated by fraction collections of the eluate from HPLC columns. The methanol in the samples was evaporated using a rotary evaporator and aqueous solutions containing the different degradation products were then freeze dried.

The thermolysis of MZPES in aqueous solutions yielded one major product (Fig. 23). The mass spectrum of this product was characterized by a molecular ion at m/z 263 [265] and an m-1 ion at 262 [264]; this spectrum was identical to that of an authentic sample of m-aminopyrimethamine (MAP). Moreover, the retention time of the isolated thermolytic product on HPLC column was identical to that of MAP. These observations confirmed that MAP was the major thermolytic product when MZPES was thermolyzed in aqueous solutions. A yield of 28% MAP was obtained when a 10 $mgml^{-1}$ aqueous MZPES solution was thermolysed at 145^{o}C for 2 hours. This yield was not significantly altered when an oxygenated solution of MZPES was thermolyzed. m-Nitropyrimethamine (MNP) was not detected in the thermolytic products.

The photolysis of MZPES in aqueous solutions resulted in more varied products depending on the photolysis conditions (Figs. 24, 25 and 26). When MZPES was photolyzed in nitrogen-saturated water, three major products were isolated (Fig. 25). The first product had a mass spectrum and HPLC retention characteristics identical to those of the authentic sample MAP. The mass spectrum of this product and that of MAP both exhibited a molecular ion at m/z 263 [265] and an m-1 ion at m/z 262 [264]. The second product isolated had a mass

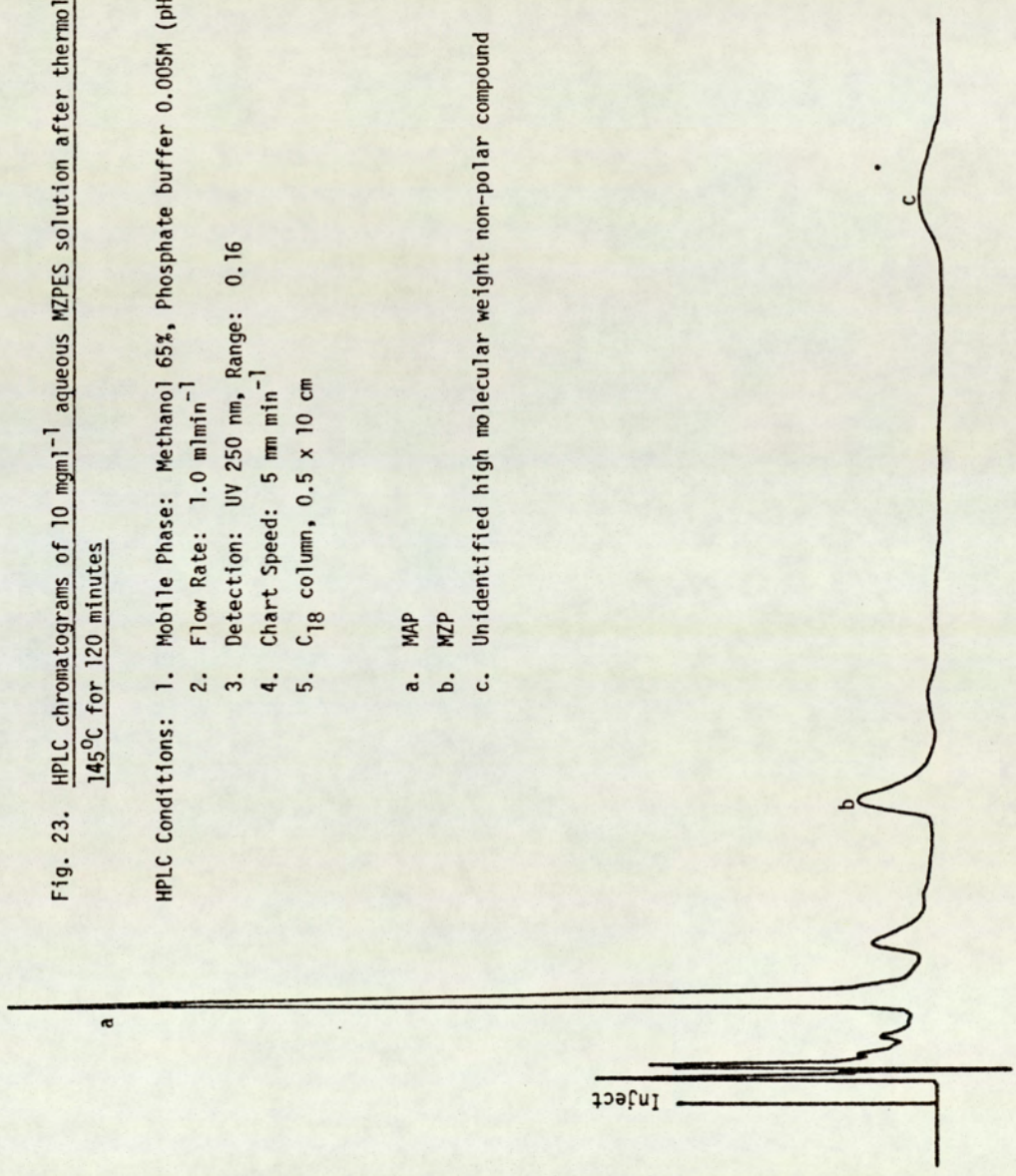


Fig. 23. HPLC chromatograms of 10 $mgml^{-1}$ aqueous MZPES solution after thermolysis at $145^{0}C$ for 120 minutes

HPLC Conditions:
1. Mobile Phase: Methanol 65%, Phosphate buffer 0.005M (pH 7.5) 35%
2. Flow Rate: 1.0 $mlmin^{-1}$
3. Detection: UV 250 nm, Range: 0.16
4. Chart Speed: 5 mm min^{-1}
5. C_{18} column, 0.5 x 10 cm

a. MAP
b. MZP
c. Unidentified high molecular weight non-polar compound

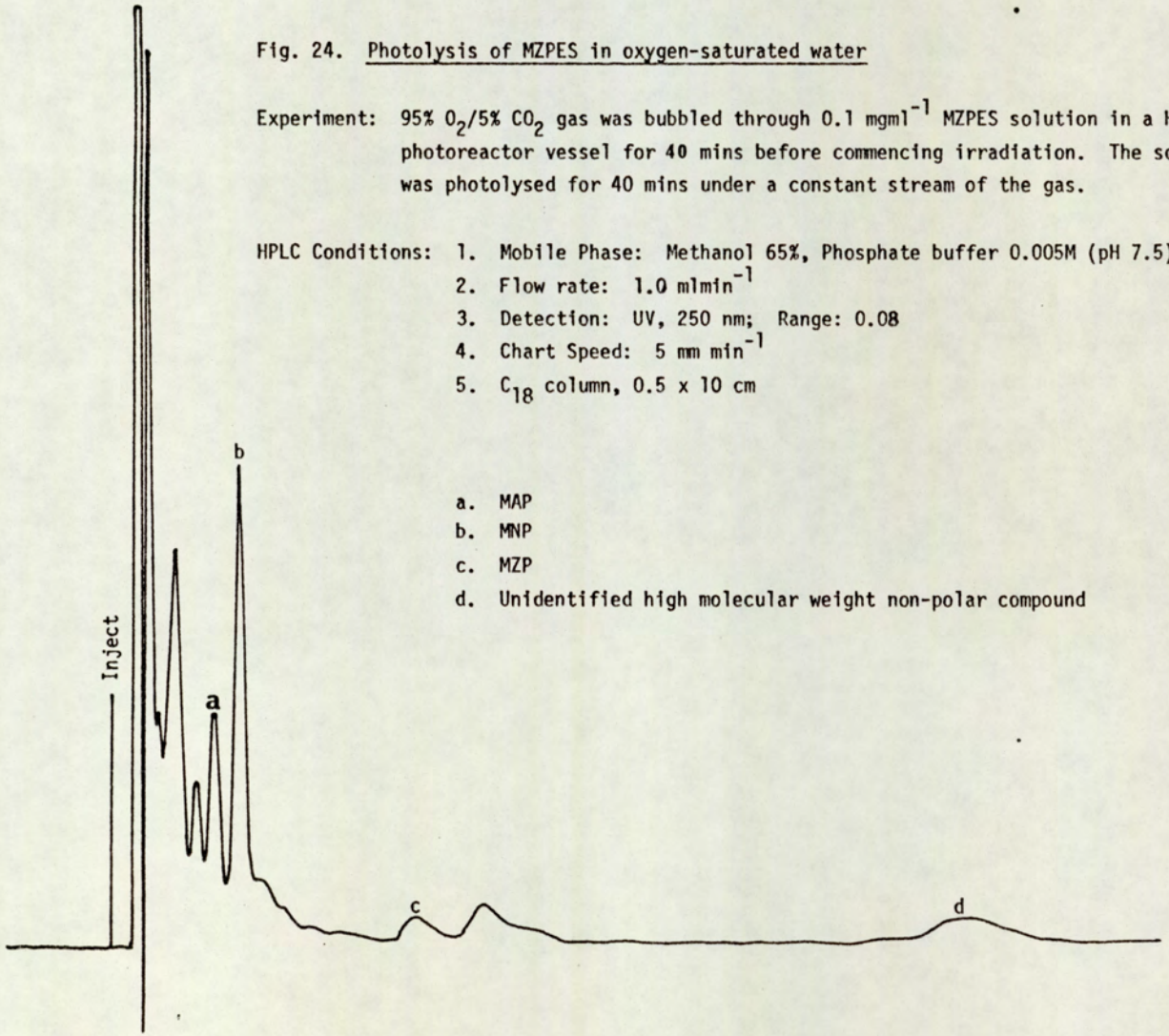


Fig. 24. Photolysis of MZPES in oxygen-saturated water

Experiment: 95% O_2/5% CO_2 gas was bubbled through 0.1 $mgml^{-1}$ MZPES solution in a Hanovia photoreactor vessel for 40 mins before commencing irradiation. The solution was photolysed for 40 mins under a constant stream of the gas.

HPLC Conditions:
1. Mobile Phase: Methanol 65%, Phosphate buffer 0.005M (pH 7.5) 35%
2. Flow rate: 1.0 $mlmin^{-1}$
3. Detection: UV, 250 nm; Range: 0.08
4. Chart Speed: 5 mm min^{-1}
5. C_{18} column, 0.5 x 10 cm

a. MAP
b. MNP
c. MZP
d. Unidentified high molecular weight non-polar compound

Fig. 25. Photolysis of MZPES in nitrogen-saturated water

Experiment: Nitrogen gas was bubbled through 0.1 $mgml^{-1}$ MZPES solution in a Hanovia photoreactor vessel for 40 minutes before commencing irradiation. The solution was photolysed for 40 minutes under a constant stream of nitrogen gas.

HPLC Conditions:
1. Mobile Phase: Methanol 65%, Phosphate buffer 0.005M (pH 7.5) 35%
2. Flow Rate: 1.0 $mlmin^{-1}$
3. Detection: UV 250 nm, Range: 0.08
4. Chart Speed: 5 mm min^{-1}
5. C_{18} column, 0.5 x 10 cm

a. MAP
b. 5,5'-Bis(2,4-diamino-6-ethylpyrimidin-5-yl)-2,2'-dichlorohydrazobenzene
c. MZP
d. Unidentified high molecular weight non-polar compound

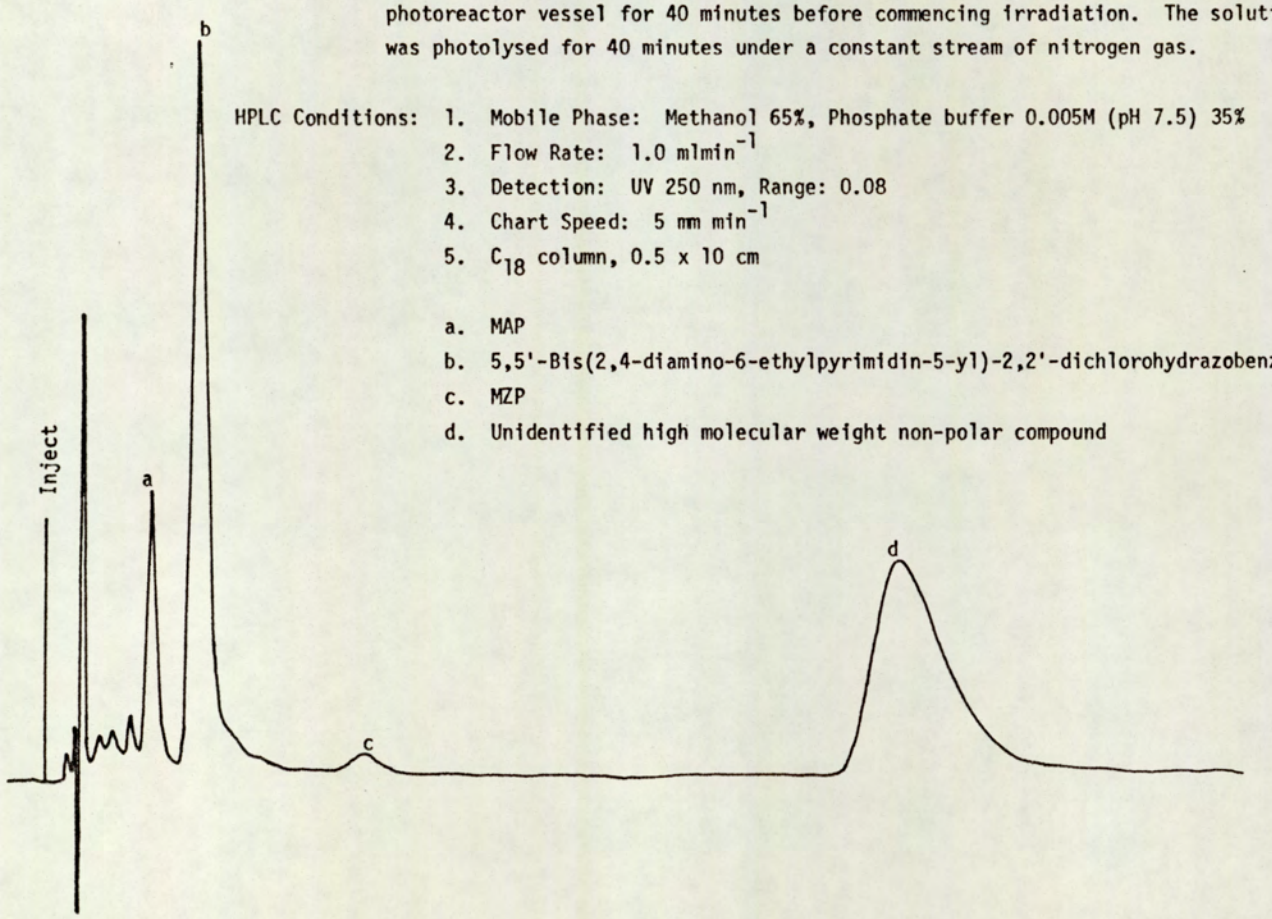
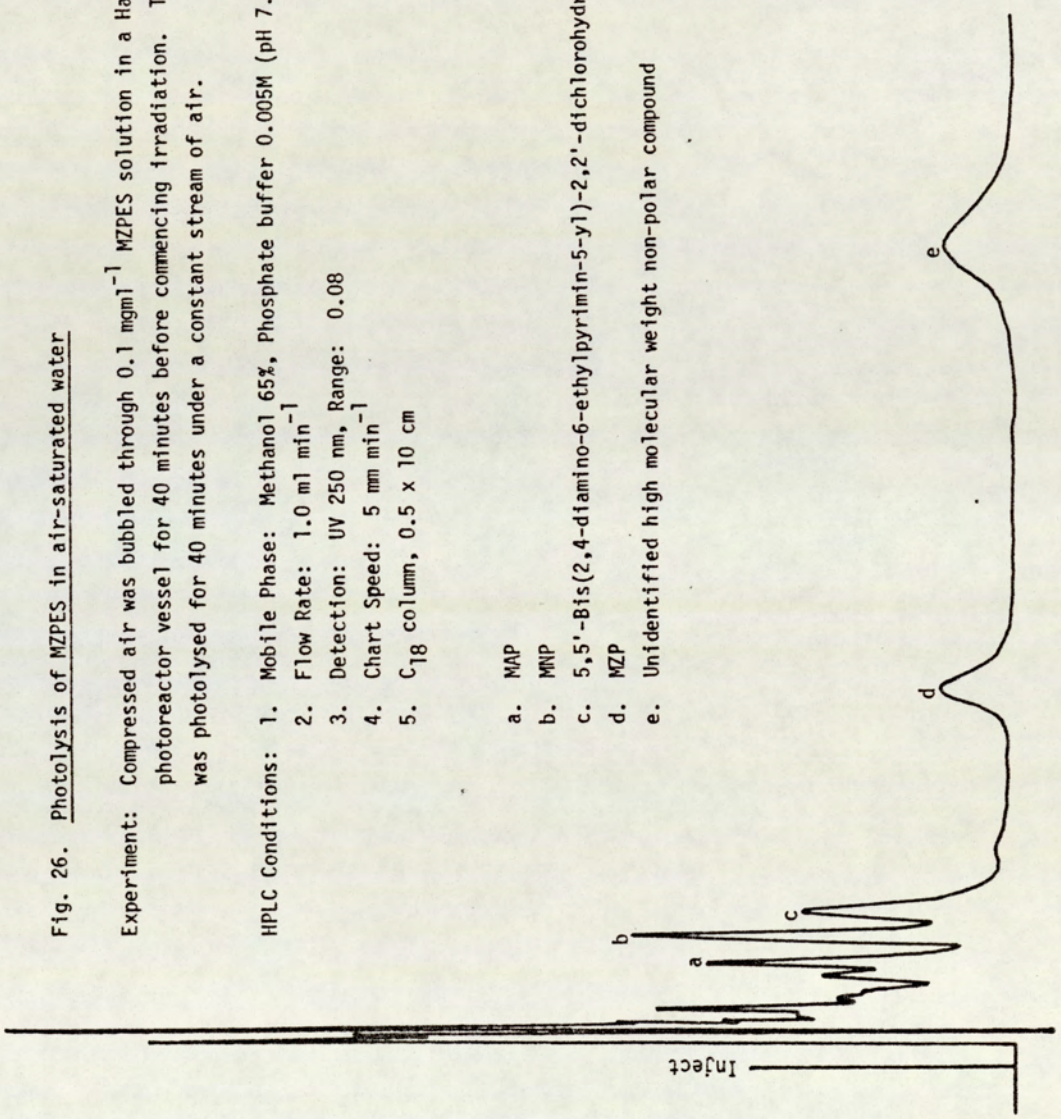


Fig. 26. Photolysis of MZPES in air-saturated water

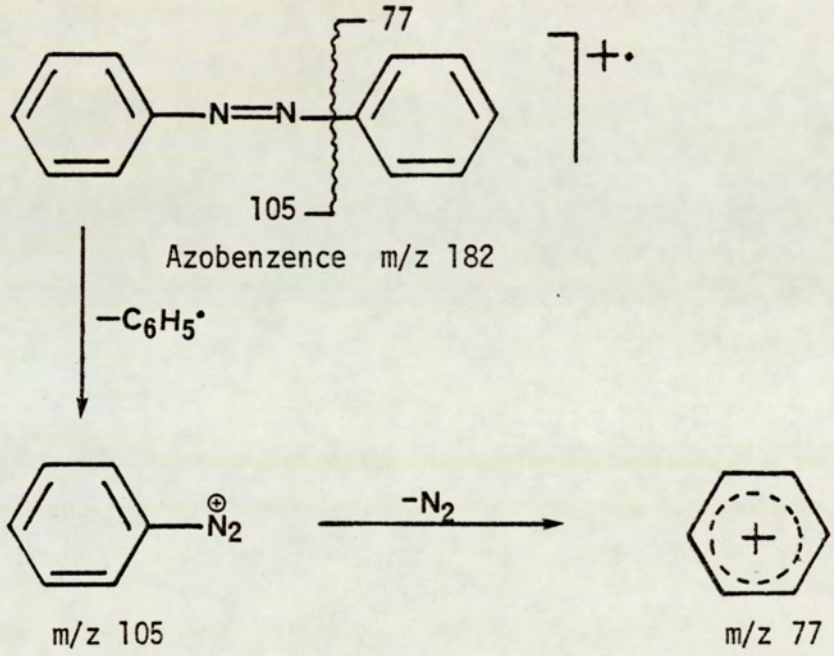
Experiment: Compressed air was bubbled through 0.1 $mgml^{-1}$ MZPES solution in a Hanovia photoreactor vessel for 40 minutes before commencing irradiation. The solution was photolysed for 40 minutes under a constant stream of air.

HPLC Conditions:
1. Mobile Phase: Methanol 65%, Phosphate buffer 0.005M (pH 7.5) 35%
2. Flow Rate: 1.0 ml min^{-1}
3. Detection: UV 250 nm, Range: 0.08
4. Chart Speed: 5 mm min^{-1}
5. C_{18} column, 0.5 x 10 cm

a. MAP
b. MNP
c. 5,5'-Bis(2,4-diamino-6-ethylpyrimin-5-yl)-2,2'-dichlorohydrazobenzene
d. MZP
e. Unidentified high molecular weight non-polar compound

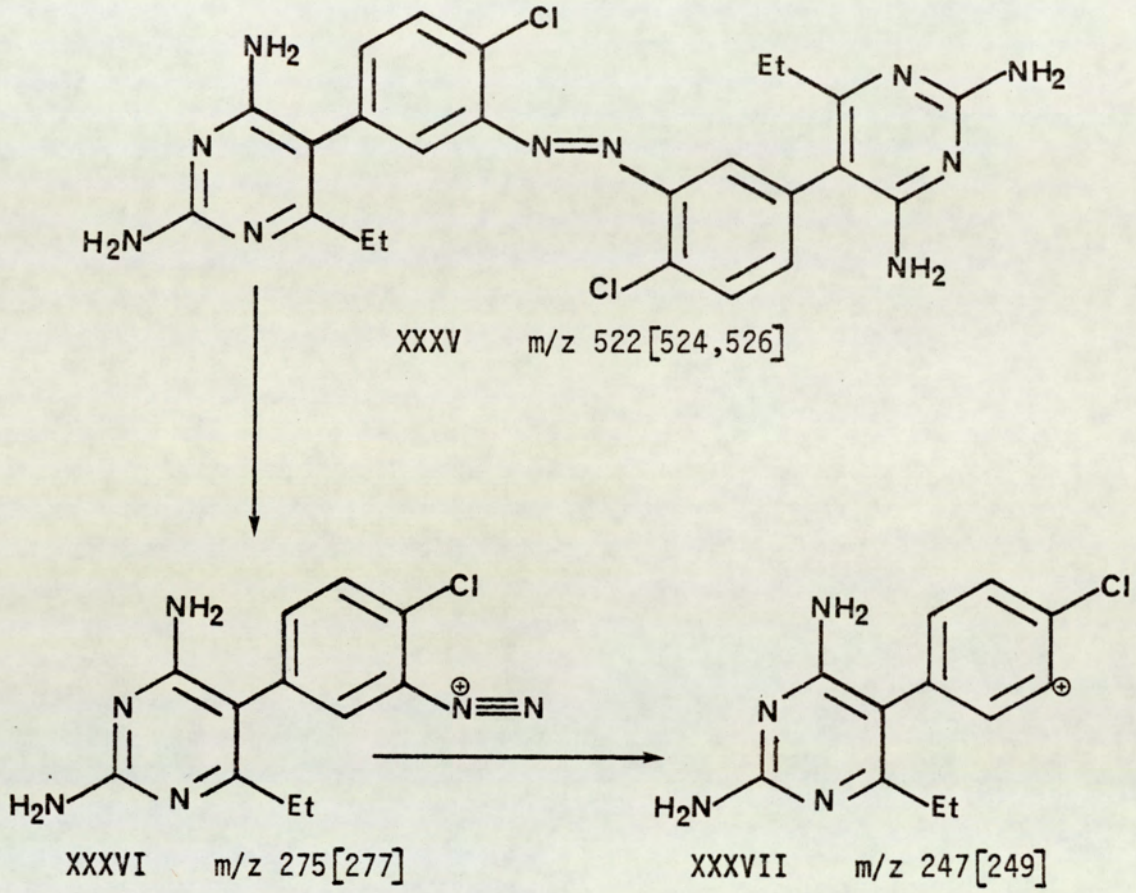


spectrum characterized by ions at m/z 522, 523, 524, 526, 527 and a significant fragmentation ion at m/z 262 [264]. The ion at m/z 522 corresponded to the mass of the azo compound, 5,5'-bis(2,4-diamino-6-ethylpyrimidin-5-yl)-2,2'-dichloroazobenzene (XXXV). However, the fragmentation did not fit the pattern for an azo compound. Tam (181) reported that the mass spectrum of azobenzene m/z 182 was characterised by the fragmentation ions at m/z 105 and 77 (scheme 11) and not by symmetrical fragmentation at -N=N- double bond.



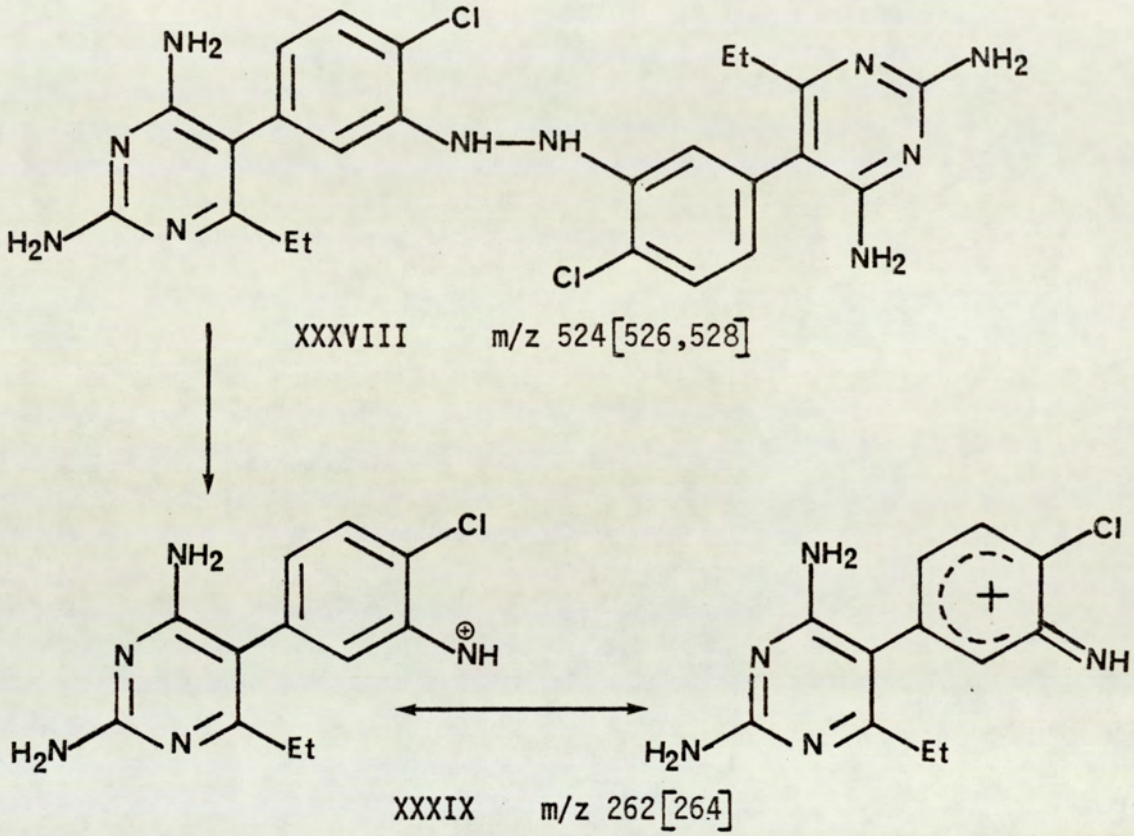
Scheme 11. Fragmentation of azobenzene in mass spectrometry

Therefore, since the framgmentation ions at m/z 275 (XXXVI) and 247 (XXXVII) were not observed in the mass spectrum of the second photolytic product isolated, it was concluded that this product was not the azo compound. The HPLC retention time of this compound also suggested that the photoproduct was more polar than would be expected of the azo compound.



Scheme 12 Expected fragmentation of 5,5'-bis(2,4-diamino-6-ethylpyrimidin-5-yl)-2,2'-dichloroazobenzene in mass spectrometry.

It is believed that the photoproduct isolated is the hydrazo compound, 5,5'-bis(2,4-diamino-6-ethylpyrimidin-5-yl)-2,2'-dichlorohydrazobenzene (XXXVIII). This compound would give a molecular ion at m/z 524 [526, 528] and a fragmentation ion at m/z 262 [264] (XXXIX).



Scheme 13. Expected fragmentation of 5,5'-bis(2,4-diamino-6-ethylpyrimidin-5-yl)-2,2'-dichlorohydrazobenzene in mass spectrometry.

Since the attempts to synthesize the azo or hydrazo compound by other routes had not been successful (Section B, 12), the identity of this second photoproduct could not be conclusively proven. The third product isolated from the photolysis of MZPES in nitrogen-saturated water had a mass spectrum characterized by fragmentation ions at m/z 669 and 419. The structure of this high molecular weight compound was not elucidated. However, unidentified amorphous polymeric materials are common degradation products isolated from the photolysis of aromatic azides (9, 39).

Only two products were isolated in sufficient quantities for characterisation from the photolysis of MZPES in oxygen-saturated water. The first product had a mass spectrum and HPLC retention characteristics identical to that of the authentic sample MAP. The second product isolated had a mass spectrum characterized by a molecular ion at m/z 293 [295], an m-1 ion at m/z 292 [294] and fragmentation ions at m/z 246 [248] and 211 [213]. This mass spectrum was identical to that of the authentic sample m-nitropyrimethamine (MNP). The HPLC retention time of this photoproduct was also identical to that of MNP. Therefore, photolysis of MZPES in oxygen-saturated water resulted in the formation of MAP, MNP and other unidentified polar compounds.

The photoproducts and the relative amounts of their formation under the influence of various gases are summarised in Table 27.

There was very little variation observed in the percentage yield of MAP under the influence of the different gases. The yield of MNP, however, was highly dependent on the amount of molecular oxygen present. This indicated that MNP must be formed via the reaction of an arylnitrene with molecular oxygen and not via the abstraction of oxygen atoms from water by an arylnitrene. The unidentified polar photoproducts were formed more readily in oxygen-saturated than in air-saturated solution. The unidentified high molecular weight non-polar compound was formed more readily in nitrogen-saturated water than in air or oxygen-saturated water.

Table 27. Products formed from the photolysis of aqueous MZPES solutions at room temperature

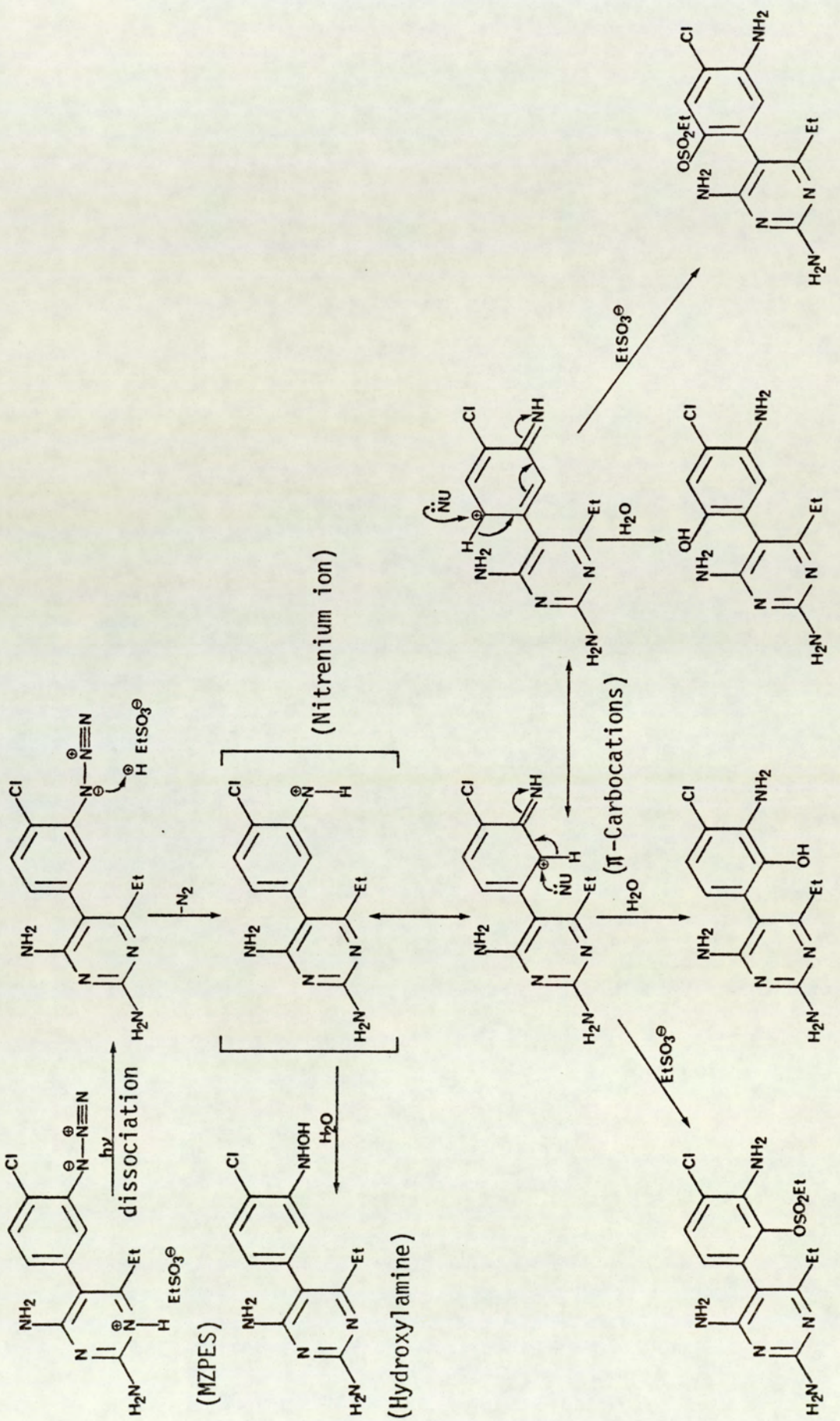
Conditions	Photoproducts	Yield (%) [if determined]
Oxygen-saturated water (Fig. 24)	MAP	4.05
	MNP	9.24
	Unidentified polar compounds (4)	-
	Unidentified high molecular weight non-polar compound	-
Nitrogen-saturated water (Fig. 25)	MAP	3.78
	Hydrazo compound[a]	-
	Unidentified high molecular weight non polar compound	-
Air-saturated water (Fig. 26)	MAP	3.32
	MNP	6.61
	Hydrazo compound[a]	-
	Unidentified polar compounds (4)	-
	Unidentified high molecular weight non-polar compound	-

a 5,5'-bis(2,4-diamino-6-ethylpyrimidin-5-yl)-2,2'-dichlorohydrazobenzene

Attempts to identify the more polar photoproducts, obtained when an MZPES solution was photolysed in the presence of molecular oxygen, were not successful. It was not possible to isolate any of these compounds in sufficient purity or quantity for analysis. However, since these compounds were more polar than MAP and they were formed predominantly in the presence of molecular oxygen, suggesting that they were probably hydroxy derivatives of MAP.

It has been reported that aminophenols are formed when arylazides are decomposed in protonic acids (60,61,182). The reaction proceeds *via* the intermediacy of a nitrenium ion following

Scheme 14. The possible routes to the formation of the more polar compounds following the photolysis of MZPES in solution



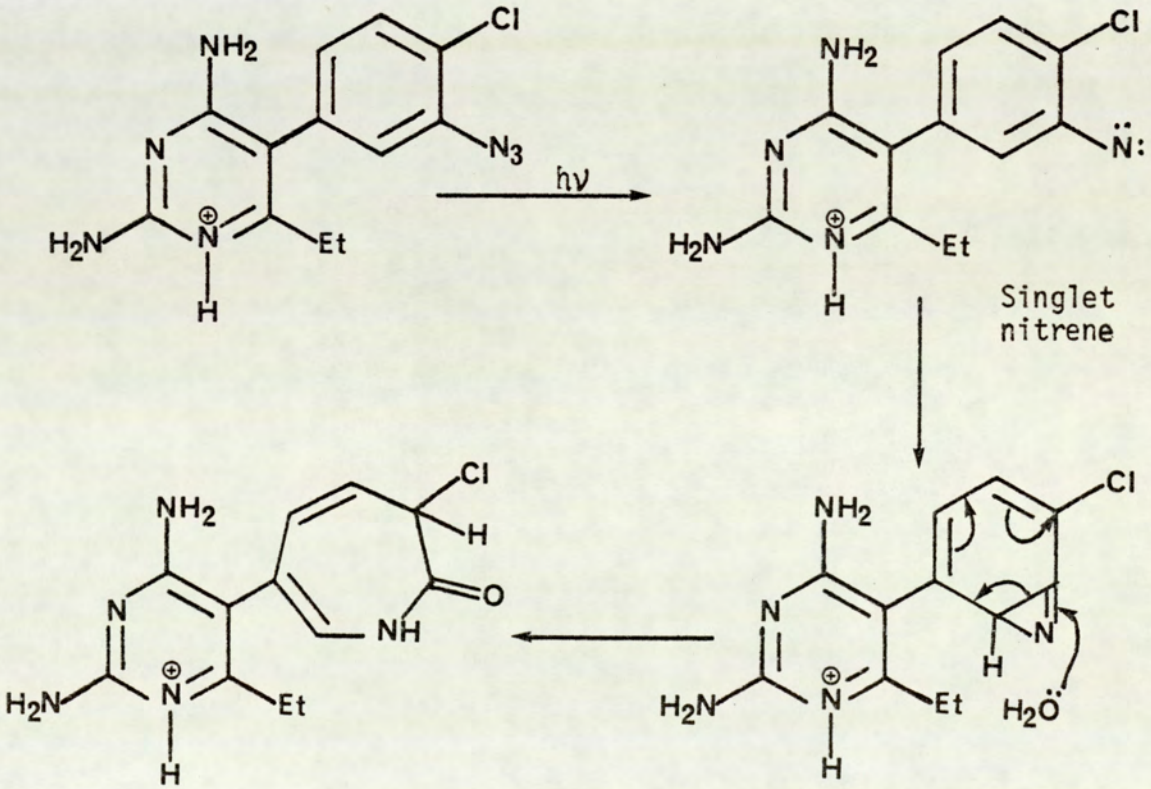
acid-catalysed loss of nitrogen and subsequent charge delocalization to form a π-carbocation which then reacts with the appropriate nucleophile to furnish a substituted aniline. The attacking nucleophile may be exogenous, the counterion of the acid or the reaction solvent, or it may be derived from an electron-rich site elsewhere on the molecule when cyclisation occurs. An aromatic trifluoromethanesulphonate was identified as one of the several products formed when arylazides were decomposed in trifluoroacetic anhydride at $0^{o}C$ with trifluoromethanesulphonic acid (triflic acid, TFSA) (161, 183).

It was possible, that, in the current study, irradiation of MZPES in solution induced a dissociation of the proton from the pyrimidine ring (observed by a decrease in the pH of the degraded solutions) and the free acid was then available to partially protonate the excited azide. Loss of nitrogen would generate a nitrenium species which would subsequently react with water to form aminophenols or it could react with the ethanesulphonate anion to give aromatic ethanesulphonate (Scheme 14). The reaction of the nitrenium ion, where the charge is localised on the nitrogen atom, with water could also lead to the formation of the hydroxylamine derivative.

3.31. Photolysis of MZPES in aqueous tetrahydrofuran

Photolysis of arylazides in aqueous tetrahydrofuran (THF) has been shown to promote ring expansion and azepinone formation (184). An expected route of azepinone formation from MZPES is shown in scheme 15. However, when MZPES was photolyzed in 30% THF/70% water, no azepinone was detected in the photoproducts as revealed by an examination of a mixture of the photoproducts using 400 MHz ^{1}H nmr

spectroscopy. The aromatic protons are distinguishable from the olefinic protons of the azepinone by their differences in chemical shifts. The NMR of the photoproducts showed only the aromatic protons at δ 7.00 - 7.70 and the olefinic protons characterized by δ 6.00-6.50 (184) were not observed, indicating the absence of azepinones. Instead, an increased yield of MAP was obtained, probably as a result of the powerful reducing properties of THF. The absence of azepinone formation could probably be attributed to the o-chloro substituent as o-chlorophenylnitrene has been reported to give no azepines under conditions where other nitrenes do (33). This may be due to the acceleration of intersystem crossing from the singlet to the triplet state caused by the o-chloro substituent.



Scheme 15 Possible route to azepinone formation

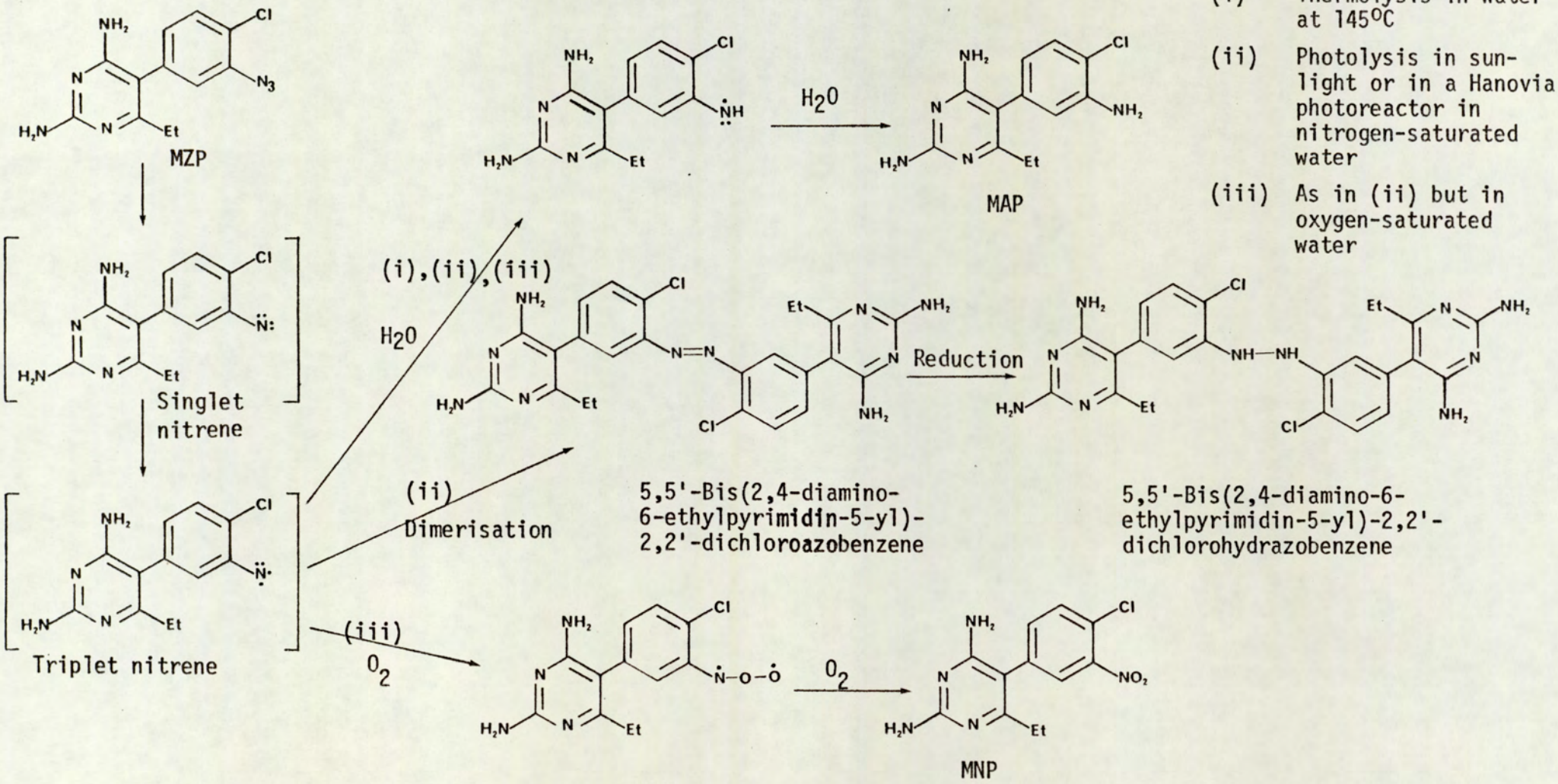
3.32. Photolysis of other 2,4-diaminopyrimidines related to MZPES

There was no significant change observed when aqueous solutions of MAPES, MNPES and pyrimethamine ethanesulphonate were photolyzed under conditions where > 90% decomposition of MZPES occurred. Similarly, when MAP, MNP and pyrimethamine were photolyzed in ethanol, no change was observed; whereas under identical conditions degradation of MZP was evident. These results indicated that MAP, MNP and pyrimethamine were not the source of the unidentified products formed during the photolysis of MZPES. This was further confirmed in experiments, when, after > 90% MZPES was degraded in a nitrogen-saturated solution by photolysis, subsequent saturation of the solution with oxygen and continued irradiation did not alter the nature or the ratio of the products formed. Similarly, no change in the nature and ratio of the products formed was observed if a solution of MZPES was saturated with nitrogen and irradiated after > 90% photo-degradation of the compound had previously occurred in an oxygen saturated solution. These results indicated that the formations of the final photoproducts were irreversible processes.

3.33. Mechanisms to the formation of MAP, MNP and the hydrazo compound

Hitherto, the decomposition of arylazides in aqueous solution has not been reported. However, the decomposition of arylazides in solution, in gneral, has been shown to proceed *via* the reactive nitrenes (6-23). The singlet nitrene is reported to be the first reactive species formed. This intermediate can then undergo rearrangements and further reactions to give the singlet-derived

Scheme 16. Thermal and Photo-degradation of MZPES



Conditions:

(i) Thermolysis in water at 145°C

(ii) Photolysis in sunlight or in a Hanovia photoreactor in nitrogen-saturated water

(iii) As in (ii) but in oxygen-saturated water

products, or it can intersystem cross to the triplet nitrene followed by further reactions resulting in the triplet-derived products.

The degradation products isolated from the thermolysis and photolysis of MZPES in a nitrogen-saturated solution were triplet-derived products. No singlet-derived products were isolated. This suggested that the rearrangement of the singlet nitrene was perhaps suppressed under the conditions used in these studies. There were more unidentified photoproducts obtained when MZPES was photolyzed in oxygen-saturated water and amongst the unidentified products some could well be derived from the singlet nitrenes.

The possible pathways by which MAP, MNP and the hydrazo compound might be formed are summarized in scheme 16. MAP was probably formed via the abstraction of hydrogen from the reaction solvent by the corresponding triplet nitrene species (9,41,42); whereas MNP was probably formed via the reaction of the corresponding triplet arylnitrene with triplet oxygen (46,49). Dimerization of the triplet nitrene would result in the formation of an azo compound and subsequent reduction under the experimental conditions could give rise to the hydrazo compound. Theoretically, the hydrazo compound could also be formed via the reduction of the nitro compound or the oxidation of the corresponding arylamine. However, since the photolysis of MAP and MNP under identical conditions did not produce any change in these compounds (Sectin C, 3.32), the hydrazo compound could not have been formed from either MAP or MNP.

3.4. Stability of MZPES in 5% Dextrose infusion

The stability of MZPES in 5% Dextrose solution, a diluent eventually chosen for the administration of the drug, was evaluated at 4^{o} and $20^{o}C$. 5 ml of the 10 $mgml^{-1}$ MZPES solution (batch No. UA

84M07) was introduced into 500 ml of the Dextrose infusion. A giving set was attached and the infusion was allowed to drip into a 1L beaker at a rate pre-determined to deliver the infusion in eight hours. The control infusions were wrapped in aluminium foil except for the part of the giving set, whereas the test infusions were exposed to daylight. The results indicated that there was no significant degradation of MZPES in Dextrose infusion over the 8 hours' period at both 4 and 20°C (Figs. 27,28). There was also no significant difference between the control and test samples. The fluctuations observed in the results were very likely a consequence of analytical errors. The deviation observed is within the generally pharmaceutically acceptable ± 10% variation in drug content of a preparation. The total drug delivered was calculated from the average of the drug concentrations over the 8 hours' period (Table 28). The calculation took into account the actual volume of the infusion fluid which was found to be 550 mls.

Table 28. The total dose of MZPES delivered in 5% Dextrose infusion over an 8 hours' in vitro study

Sample	Total MZPES delivered (mg)	% of given dose
4°C Control	47.3	94.6
Test	47.3	94.6
20°C Control	47.2	94.4
Test	46.9	93.8

The second set of experiments involved the evaluation of the stability of MZPES reconstituted in 5% Dextrose infusion and stored

Fig. 27. Stability of MZPES injection in 5% Dextrose Infusion at 4°C

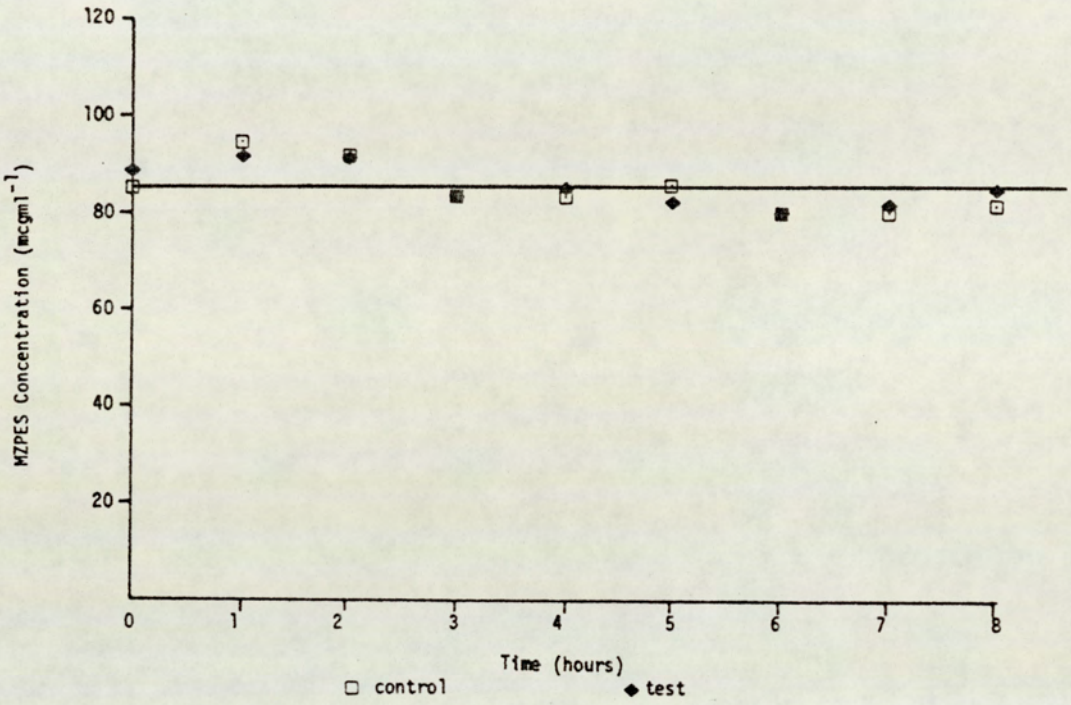


Fig. 28. Stability of MZPES injection in 5% Dextrose Infusion at 20°C

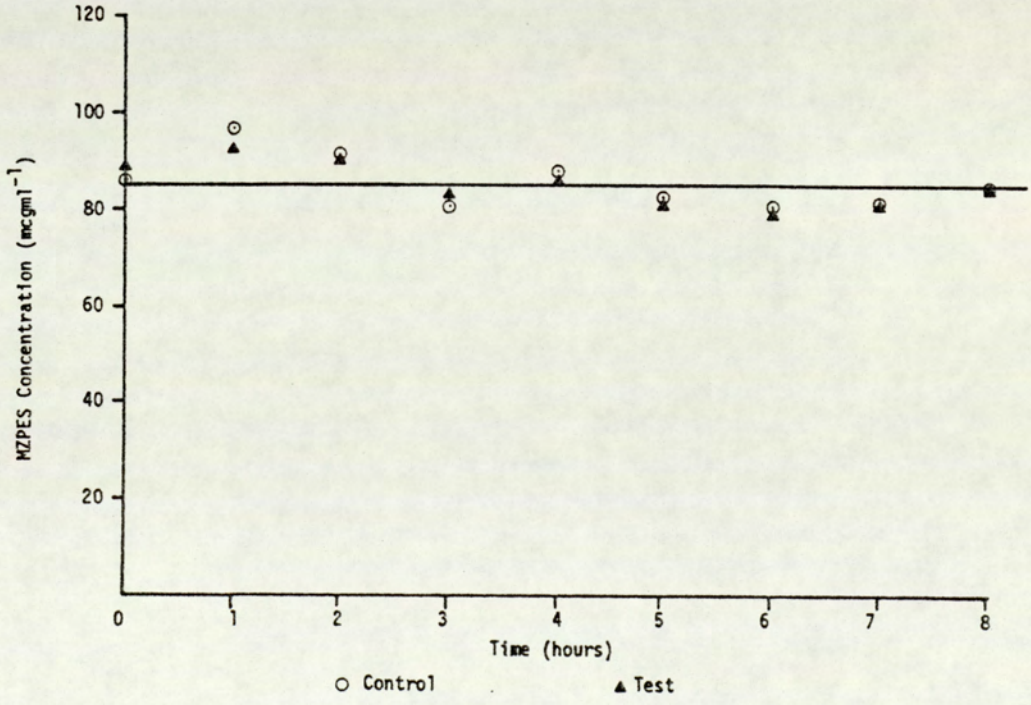
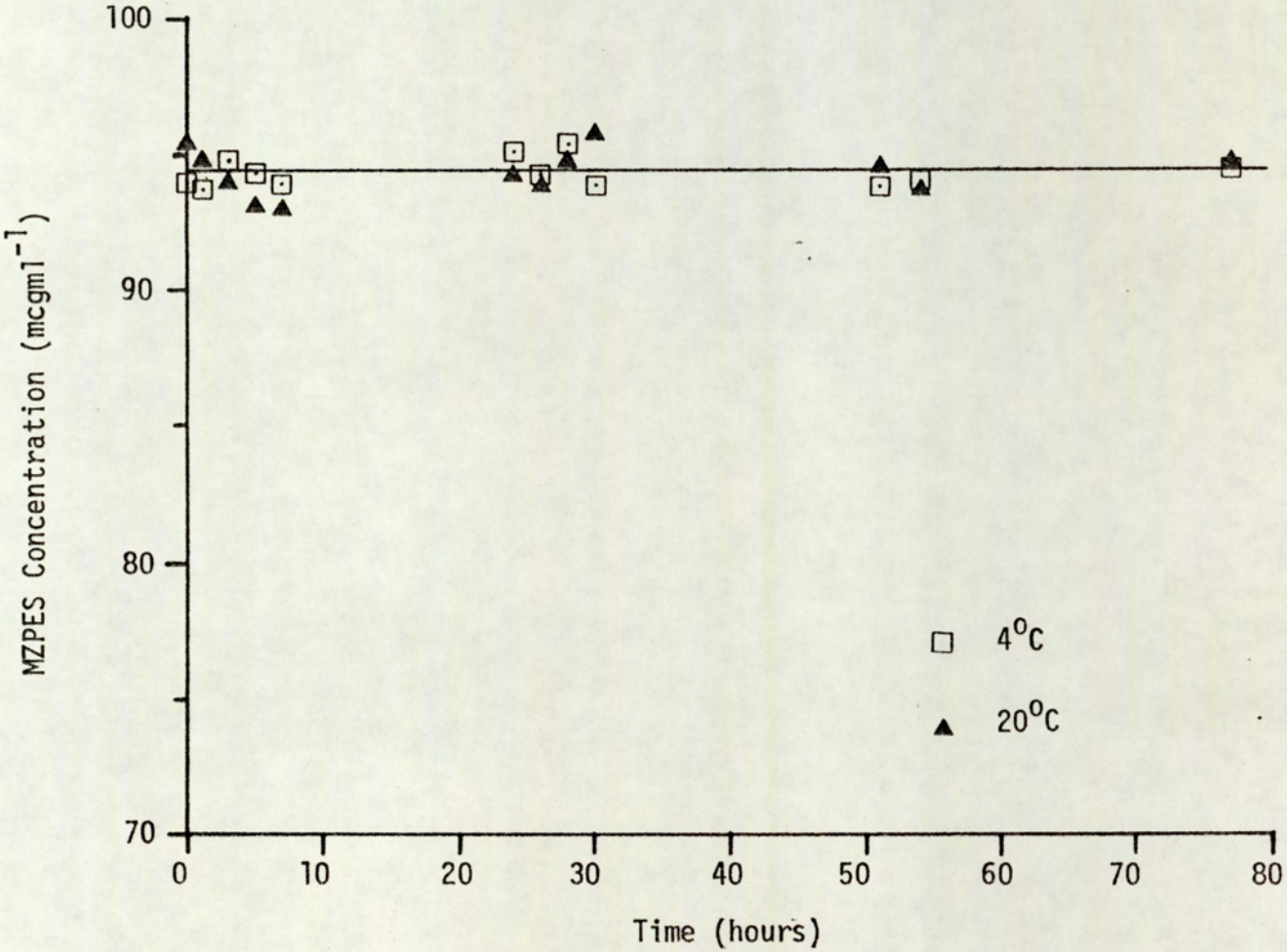


Fig. 29. Stability of MZPES injection in 5% Dextrose Infusion



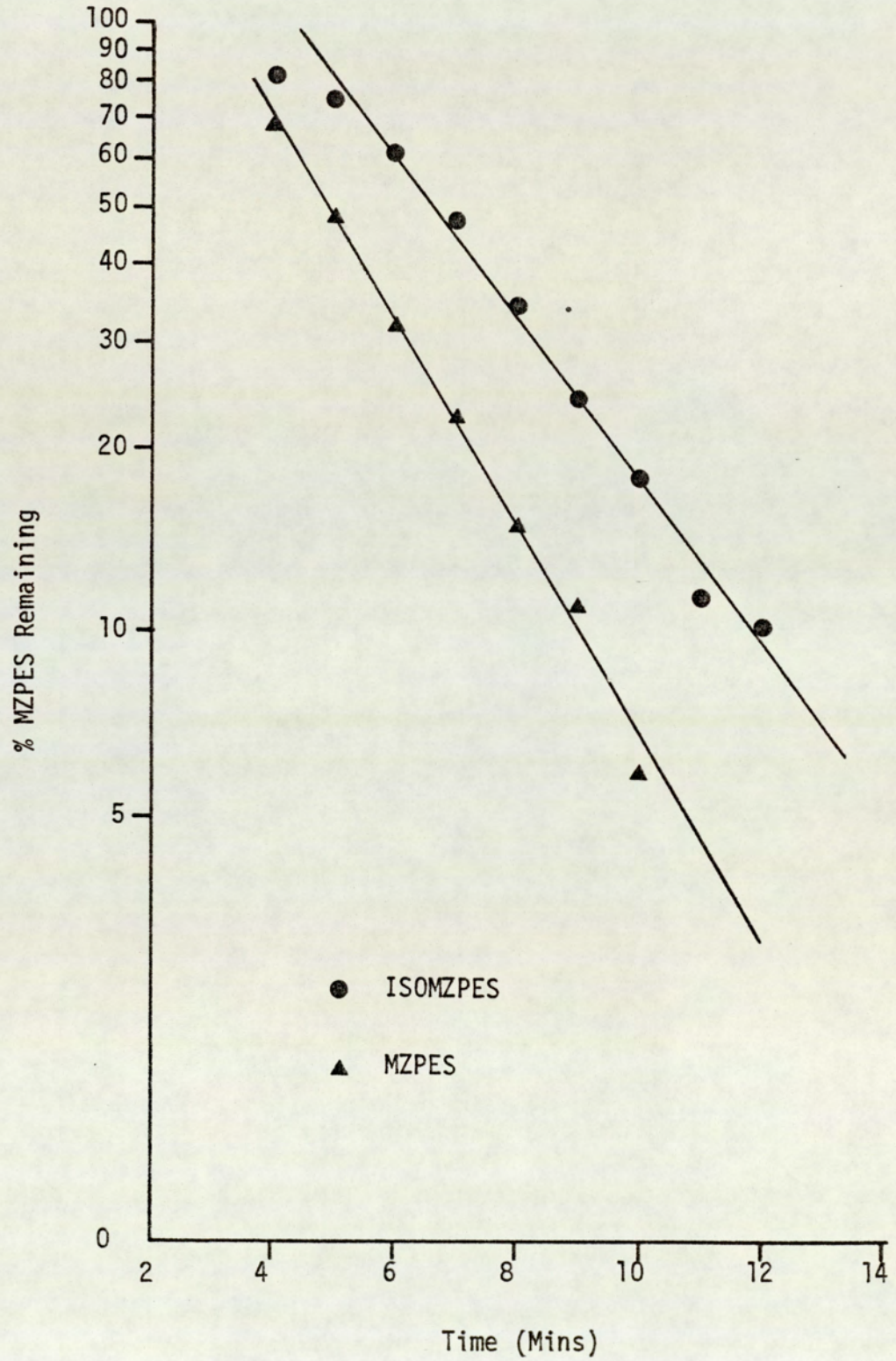
in the dark for 80 hours prior to administration. The results (Fig. 29) indicated that MZPES was stable in 5% Dextrose infusion at both 4 and 20°C for at least 80 hours when stored under the wrap of aluminium foil. Therefore, the infusion could be reconstituted at least 80 hours prior to its use.

3.5. Photo-degradation of ISOMZPES

The photo-degradation of ISOMZPES in the presence of molecular oxygen was studied and compared with that of MZPES. The rate of photolysis of an aqueous ISOMZPES solution was slower than that of MZPES (Fig. 30). The reaction half-life for ISOMZPES and MZPES was 3.21 and 2.41 mins respectively. Differences in the ratio of product distribution were also observed. A considerably higher yield of arylamine was obtained from the photolysis of ISOMZPES compared to MZPES (Figs. 24, 31).

These results indicate that interchanging the chloro and the azido substituent on MZPES can lead to an alteration in the degradation profile. Moreover, ISOMZPES was reported to be 5 times more active as a DHFR inhibitor than MZPES (161). These preliminary studies reveal that ISOMZPES is an interesting compound that warrants further investigation.

Fig. 30. Photolysis of 0.1 $mgml^{-1}$ aqueous solutions of MZPES and ISOMZPES under 95% O_2/5% CO_2



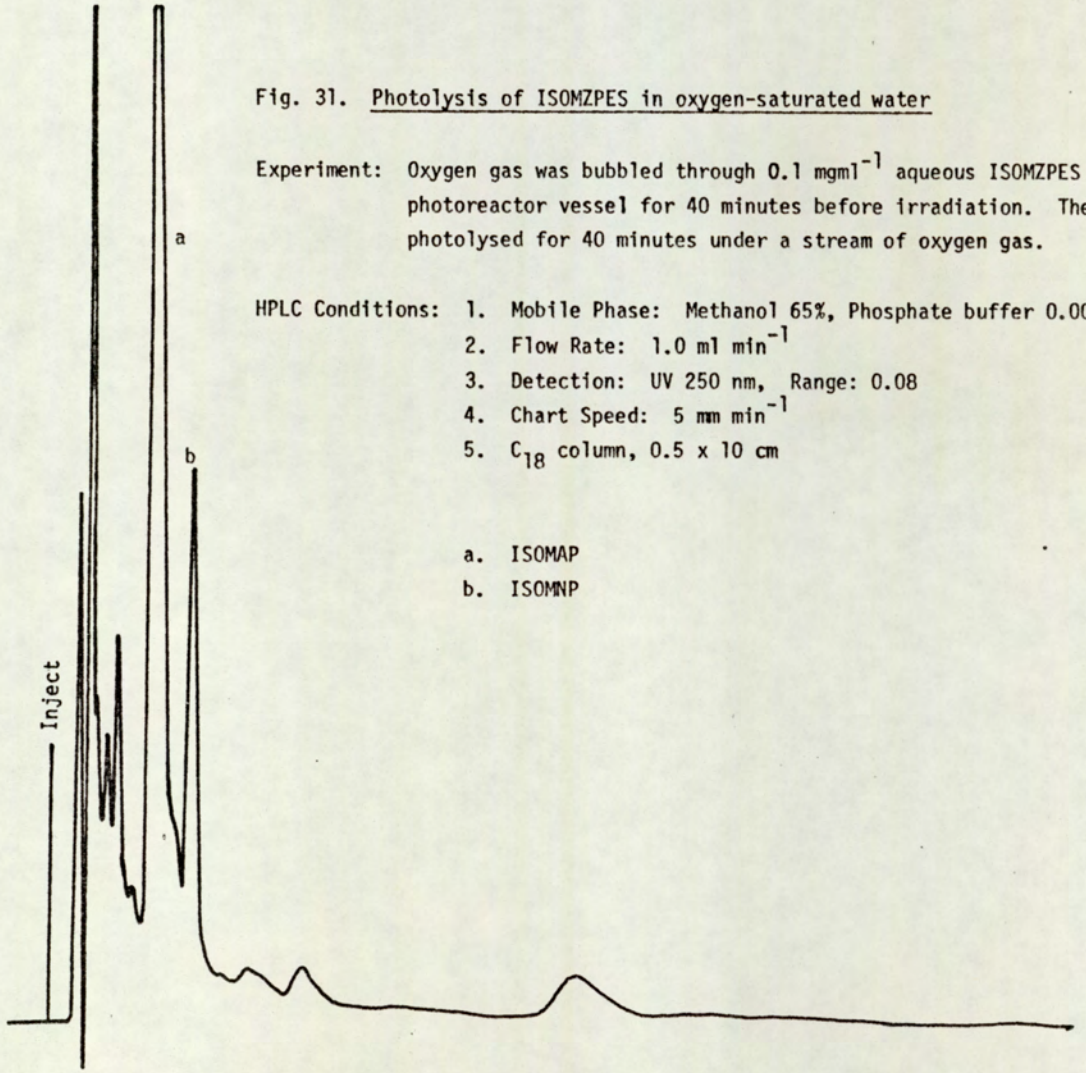


Fig. 31. Photolysis of ISOMZPES in oxygen-saturated water

Experiment: Oxygen gas was bubbled through 0.1 $mgml^{-1}$ aqueous ISOMZPES solution in a Hanovia photoreactor vessel for 40 minutes before irradiation. The solution was photolysed for 40 minutes under a stream of oxygen gas.

HPLC Conditions:
1. Mobile Phase: Methanol 65%, Phosphate buffer 0.005M (pH 7.5) 35%
2. Flow Rate: 1.0 ml min^{-1}
3. Detection: UV 250 nm, Range: 0.08
4. Chart Speed: 5 mm min^{-1}
5. C_{18} column, 0.5 x 10 cm

a. ISOMAP
b. ISOMNP

4. Formulation, manufacture and quality assurance of the MZPES injections for clinical trials

MZPES was formulated as a 10 $mgml^{-1}$ solution in water at pH 4.10, and recommended to be diluted in 500 mls 5% Dextrose Infusion prior to administration in order to avoid the potential risk of precipitation of the free base at physiological pH. The introduction of MZPES injection into saline infusion was not recommended because of the risk of precipitating the less soluble hydrochloride salt in saline. The aqueous solubility of MZPHCl and MZPES are 2.2 and 17.6 $mgml^{-1}$ respectively.

The two batches of MZPES injections, UA84M07 and UA85K29, used in clinical trials, were manufactured at Medisch Centrum Slotervaart, Amsterdam, under the supervision of Mrs T J Schoemaker. The injections were prepared under aseptic conditions and sterilized by filtration. The dissolution rate of the drug posed some difficulties during scale-up manufacture. Hot water for injection together with a high speed Turrax mixer were used to aid dissolution. Nitrogen gas was bubbled through the solution before the ampoules were being filled and sealed. When all the ampoules of MZPES were sealed, sterile nutrient broth was filtered through the same manufacturing system and sealed into ampoules under identical conditions. The ampoules containing the nutrient broth were then incubated at different temperatures and examined for contamination. This procedure enabled any sterility fault within the manufacturing system, e.g. the integrity of the filters, to be readily identified.

The quality control of the manufactured products was carried out at Aston University. A sample from the batch of MZPES injections was taken and assayed for drug content, identity, purity and sterility. The identity of the compound was established by precipitating MZP

free base under alkaline conditions. An IR spectrum of the precipitate was obtained and compared to that of an authentic MZP sample. The content and purity of MZPES in solution were determined using HPLC methodology (Section C, 1.3). The sterility tests were carried out as described in The British Pharmacopoeia (82). The bacteriostatic effect of the drug was inactivated by passing the sample of MZPES injections through a sterile filter and then washing the filter with sterile water several times to remove any traces of the drug. The filter was then cultured in the appropriate growth media and examined for sterility.

To avoid photo-degradation of the drug, MZPES injections were packed into opaque plastic containers. The injections were stored at $4^{o}C$.

5. CLINICAL PHARMACOKINETICS OF MZPES IN PHASE 1 TRIAL

The phase 1 clinical trial of MZPES commenced in January 1985 at two centres. The trial centre based in Birmingham was supervised by Dr G Blackledge and the other centre based at Charing Cross Hospital in London was under the supervision of Dr E S Newlands. Clinical pharmacokinetic studies were conducted on patients entering the trial. Pharmacokinetic studies on patients treated in Birmingham were carried out as part of the current work. Results from the Charing Cross Hospital are included in the summary Table 30 to enable conclusions concerning the pharmacokinetic parameters of MZPES to be reached.

The aims of a phase 1 trial are to determine the maximum tolerated dose (MTD) of MZPES, to establish the nature of the toxicities of the drug and to provide sufficient pharmacological and pharmacokinetic data upon which to base the dose of MZPES for subsequent phase 2 studies.

The patients included in the study were those who had histological evidence of advanced and progressive malignancy and in whom conventional therapy had failed. Each patient had a performance status >60 (Karnofsky scale) and an estimated survival of >8 weeks. No patient had received other therapy for at least 3 weeks (6 weeks for nitrosourea and mitomycin C) prior to receiving MZPES. All patients had acceptable haematological status - total leucocyte count of >4,000/mm^3 and platelet count of >100,000/mm^3 - and normal hepatic and renal function as defined by standard liver function tests, serum urea, electrolytes and creatinine level. Voluntary informed consent for the trial was obtained from all participants.

The recommended starting dose for phase 1 clinical trials of novel anti-neoplastic agents is 1/10th of the LD_{10} in mice (185).

The starting dose of MZPES was calculated to be 5.4 mgm^{-2}. The doses were escalated in a modified Fibonacci search (186). A minimum of 3 patients and 4 courses were evaluated at each non-toxic dose level and at least 6 courses were evaluated at doses where toxicity was evident. A minimum of 3 weeks was allowed between subsequent doses within the same patient.

MZPES was administered diluted in a 500 ml 5% Dextrose infusion which was infused over a period of 1 hour. Blood samples for pharmacokinetic analysis were taken before and immediately after the infusion and at 1.0, 1.33, 1.67, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12.0, 24.0 and 48.0 hours after the start of the infusion. Blood was sampled using an indwelling venous venflon with an attached 3-way tap. Clotting of blood in the line between sampling times was prevented by the injection of 0.5 mls of 1000 units ml^{-1} heparin after each sampling. At each sampling, 2 mls of blood was drawn off to waste and then an approximately 7-10 mls sample was drawn into a plastic syringe. The blood was immediately transferred to a heparinized blood sample tube (Sterilin Ltd) and mixed. It was centrifuged at 2000 r.p.m. in an Heraeus centrifuge at a temperature of $4^{o}C$ for 10 minutes. The supernatant plasma was pipetted into a sterilin sample tube and stored frozen at $-20^{o}C$ prior to analysis. The analysis of the plasma samples was carried out by Mr S Pashley (187).

5.1 Dose escalation study

The phase 1 trial of MZPES was still in progress at the end of July 1986. The summary of the dose escalation study on 31st July 1986 is reported in Table 29. By that time, 51 patients had received 102 doses at both trial centres, with 28 patients and 48 doses being conducted in Birmingham.

Table 29. MZPES Phase 1 dose escalation study

Dose (mgm^{-2})	No. of patients entered	No. of doses administered	Evaluable kinetics
5.4	4	7	0
11.0	5	8	1
18.0	6	8	2
27.0	5	6	4
38.0	3	7	2
50.0	4	7	3
67.0	5	7	5
83.0	7	13	7
105.0	7	9	4
125.0	4	6	1
150.0	3	5	1
180.0	4	5	0
210.0	8	11	2
250.0	3	3	0

Typical clinical pharmacokinetic profiles of MZPES are shown in Figs. 32 and 33 where graphs of plasma concentration Vs time and ln plasma concentration Vs time are plotted. Plots are shown from the end of infusion onwards for patients 26 and 37 treated at 83 and 150 mgm^{-2} respectively. MZPES exhibits a biphasic pharmacokinetic profile, with a rapid distribution phase followed by a prolonged elimination phase. The data obtained approximately fitted a two-compartment pharmacokinetic model.

Fig. 32. Pharmacokinetics of MZPES in Phase 1 Trial - Plasma Concentration Vs Time

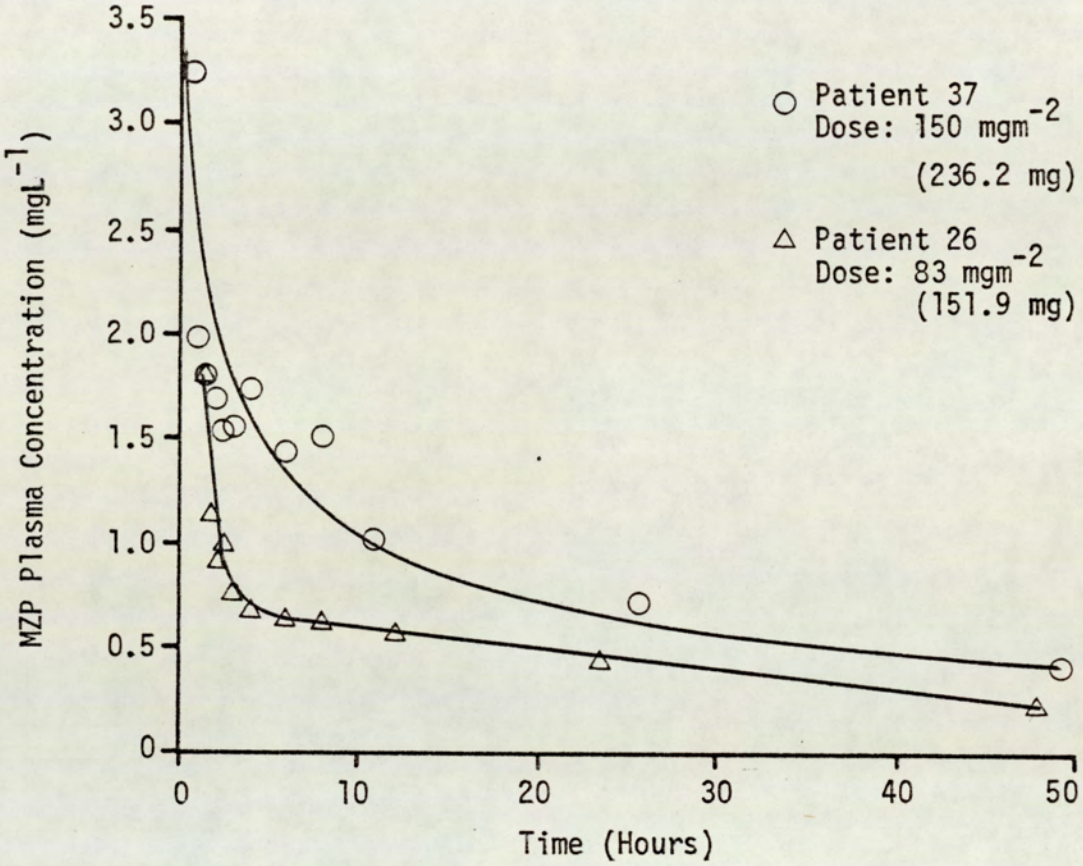
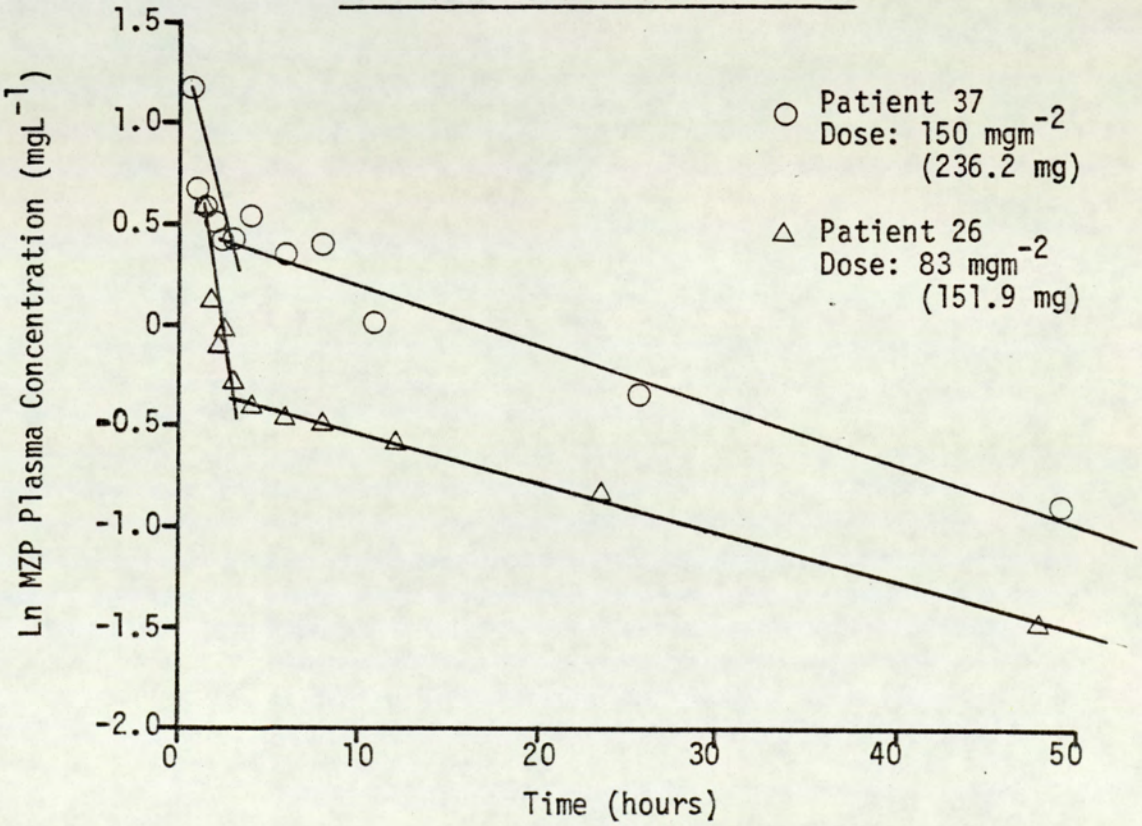


Fig. 33. Pharmacokinetics of MZPES in Phase 1 Trial - Ln Plasma Concentration Vs Time



5.2. Pharmacokinetic evaluation of MZPES

The pharmacokinetic behaviour of MZPES was characterized by the biexponential plasma disappearance of the drug (Figs. 32 and 33). The pharmacokinetic parameters of MZPES were calculated based on a two-compartment open model described in section B, 13 and a summary of these parameters at each dose escalation is reported in Table 30. The pharmacokinetic parameters of individual patient treated in Birmingham are included in Appendix 2.

The distribution half-life of MZPES was found to be 0.35 ± 0.22 hours (CV 62.3%) and the elimination half life of the drug was found to be 37.42 ± 17.11 hours (CV 45.72%). MZPES, therefore, has much shorter elimination half-life compared to other lipophilic 2,4-diaminopyrimidines such as pyrimethamine ($t_{\frac{1}{2}}$ = 85 hours, range 79-93 hours), etoprine ($t_{\frac{1}{2}}$ = 176 hours, range 167-189 hours) and metoprine ($t_{\frac{1}{2}}$ = 216 hours, range 105-268 hours) (129). The relatively shorter half-life of MZPES may be attributable to the introduction of the biotransformation susceptible azido group into the molecule. This relatively shorter half-life of MZPES enables flexibility in the dosing schedule.

The apparent volume of the central compartment (V_C) and the apparent volume of distribution(V_B) of MZPES were found to be 34.41 ± 24.48 L (CV 71.13%) and 145.60 ± 34.79 L (CV 23.89%) respectively. The total body clearance of MZPES was found to be 3.22 ± 1.37 Lhr^{-1} (CV 42.69%).

The trapezoidal area under the curve (Trap. AUC) and the total area under the curve (AUC_t) of MZPES each correlated well with the administered dose (Figs 34 and 35). The correlation coefficients of the two parameters with the dose are 0.9932 and 0.9683 respectively. The correlation between the peak plasma concentration of MZPES with

Table 30 continued.....

105	0.205 ±0.130	42.364 ±12.474	123.783 ± 34.058	36.372 ± 8.375	68.609 ±29.077	2.202 ±0.669	2.193 ±1.047	3
125	0.0985	75.793	201.50	36.731	99.849	1.858	1.842	1
150	0.315	23.016	99.79	42.668	56.955	3.248	3.005	1
Mean	0.354	37.420	145.600				3.217	
	±0.220	±17.108	± 34.790				±1.373	
(CV%)	(62.29%)	(45.72%)	(23.89%)				(42.69%)	

Fig. 34. Pharmacokinetics of MZPES in Phase 1 Trial - Trap. AUC Vs Dose

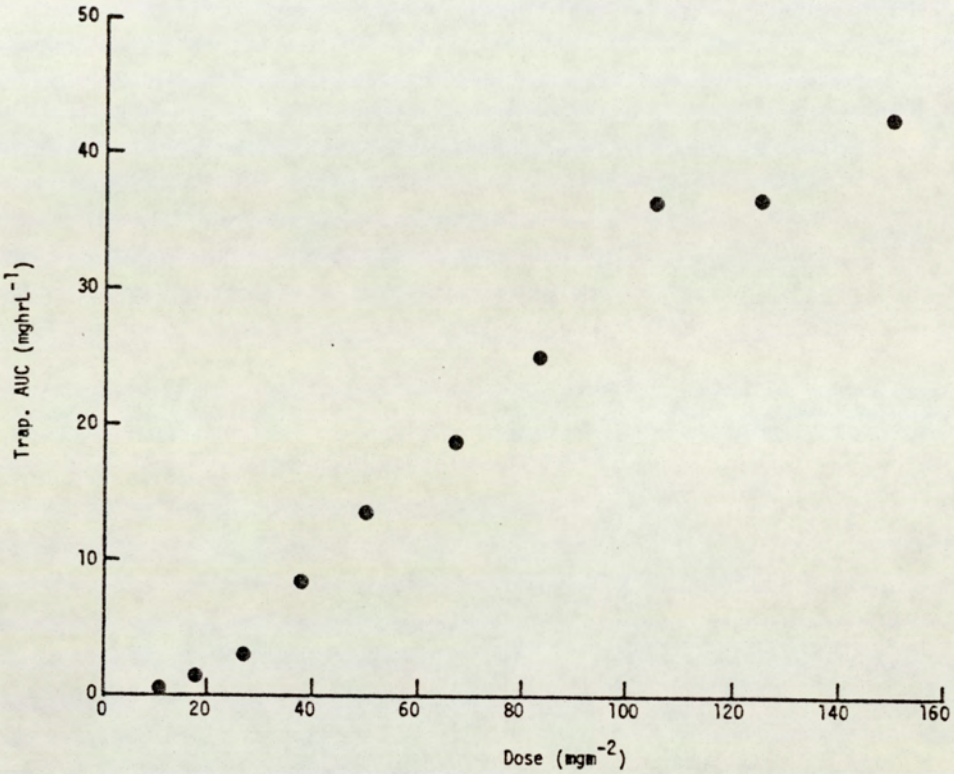
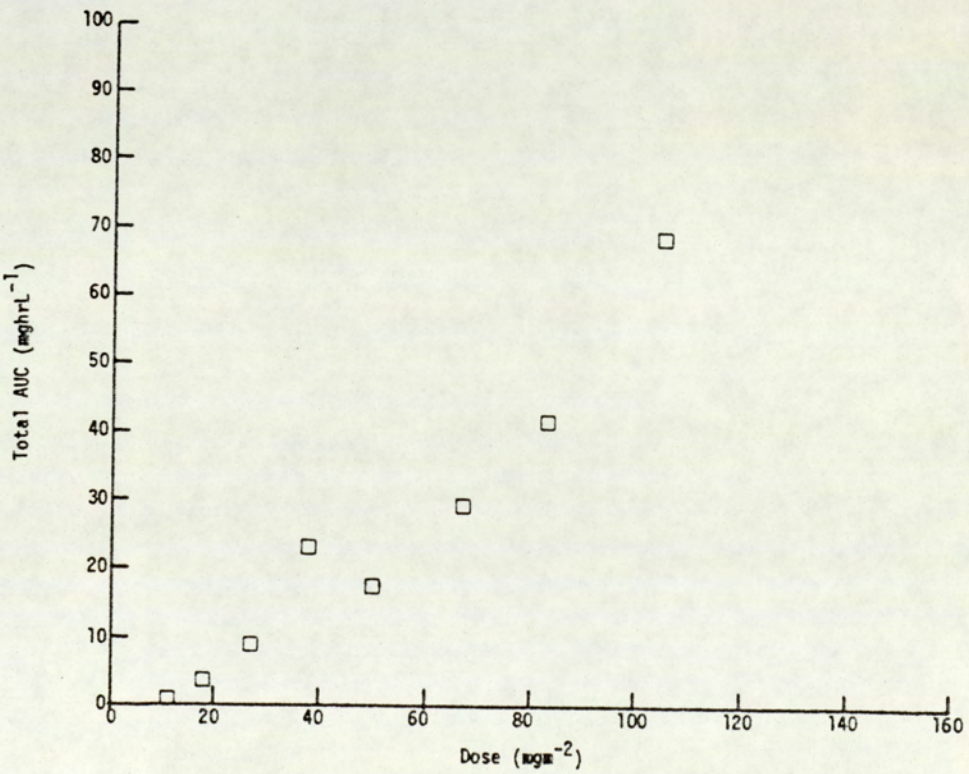


Fig. 35. Pharmacokinetics of MZPES in Phase 1 Trial - Total AUC Vs Dose



the administered dose was found to be poor (correlation coefficient = 0.4743). This poor linear relationship could be a result of fluctuation in the rate of infusion of the drug in different patients. The rate of infusion could vary between 0.38 to 0.75 Lhr^{-1} at the two extremes.

Unlike metoprine, no haematological toxicity was observed in patients treated with MZPES, even at 250 mgm^{-2}. With metoprine, thrombocytopenia and more rarely leucopenia was evident at doses as low as 65 mgm^{-2} (137). Above this dose, administration of metoprine was only possible if leucovorin (folinic acid) was included in the schedule. Central nervous system toxicities (hallucinations, focal seizures, hyperactivity and insomnia) were observed in patients receiving metoprine >225 mgm^{-2} but these symptoms abated within 6 hours after i.v. leucovorin was given (137). Only one isolated case of convulsion was observed in a patient treated with 210 mgm^{-2} MZPES. Subsequently, electro-encephalograms of patients receiving MZPES were monitored before, during and after administrations of MZPES but no change in the electrical activity of the brain waves was detected.

All patients receiving MZPES at doses >50 mgm^{-2} experienced mild sedation. Patients receiving MZPES at doses >180 mgm^{-2} complained of dizziness and headache ranging from mild to severe. Nausea and vomiting was observed in 3 of the 6 patients treated in Birmingham with MZPES >180 mgm^{-2}. Below this dose, nausea and vomiting was not observed.

No leucovorin rescue had been necessary in patients receiving MZPES. The results obtained so far indicate that MZPES is less toxic than metoprine. The dose limiting toxicities remain to be elucidated.

6. CONCLUSIONS

m-Azidopyrimethamine [MZP] is the first arylazide to be introduced into the clinic. It is a novel lipophilic antifolate designed to overcome some of the problems encountered by both the hydrophilic antifolate methotrexate (131-139) and the prototype lipophilic antifolate metoprine (123, 141-146). The novelty of introducing an arylazide into Man necessitated an extensive study of the physico-chemical characteristics and the stability profiles of this compound in a pharmaceutical system. This would ensure the development of a safe, effective and stable dosage form for clinical use. The physico-chemical properties of MZP and its salts investigated in the current study included solubility, ionisation constant and partition coefficient. The stability profiles of MZP were determined by studying the effect of heat and light on the degradation of the chosen salt, MZPES, in solution.

To facilitate quantitative and stability evaluations of MZP and its salts, high performance liquid chromatography (HPLC) methods were developed. Quantitative studies were also carried out using UV absorption spectroscopy when the purity of the compound was known. Attempts were made to develop a thin layer chromatography (TLC) method for the analysis of MZP, in the presence of its degradation products, but were not successful.

Initial investigations revealed that MZP free base was extremely insoluble in water, ~ 20 ugml^{-1}. From the knowledge of the starting dose in Man, predicted from the toxicological data obtained in mice, it was decided that a 500-fold increase in solubility would be required to achieve a suitable solution formulation. This appeared to rule out the possibility of solubilizing the drug by complexation with ligands since this method was reported to produce only a

moderate enhancement of solubility (102, 103). Salt formation was found to be an effective way of enhancing the aqueous solubility of MZP but the degree of enhancement depended on the acid used. The hydrochloric, methanesulphonic and lactic acid salts only produced moderate increases (90 to 200-fold) in the aqueous solubility of MZP, whereas the solubility of MZP was greatly enhanced when present in the form of the phosphoric, citric and ethanesulphonic acid salts. m-Azidopyrimethamine ethanesulphonate [MZPES] was the most water soluble salt formed, with a molar dissolution of 43.8×10^{-3}M [17.6 $mgml^{-1}$]. The solubility of MZPES was found to be further increased in organic solvents such as dimethylsulphoxide [381.0 $mgml^{-1}$] and dimethylacetamide [194.7 $mgml^{-1}$]. Despite this large enhancement in solubility, the use of such organic solvents was not encouraged because of the associated toxicities (85). The use of cosolvents or surfactants to solubilize MZP was also rejected because of the inherent toxicities of these agents (88-89, 90-93) in intravenous administration and the potential risk of drug precipitation when these formulations were diluted (87). Buffer solutions were found to cause a decrease in the solubility of MZPES; the more concentrated the buffer solutions, the greater the decrease in solubility.

^{13}C NMR and ^{1}H NMR spectroscopy were employed to study the protonation of MZP, MAP and pyrimethamine in dimethylsulphoxide solution. It was found that at physiological pH protonation of these compounds in solution occurred predominantly on the N-1 nitrogen of the pyrimidine ring (Fig. 11). The basicity of this nitrogen in MZP was found to have a pKa value of 7.19. This indicates that at physiological pH, $\sim$ 60% of MZP would be present as neutral molecules and $\sim$ 40% as the protonated species. The presence of both species in near equal proportion is essential to ensure that sufficient neutral

molecules are present to penetrate cells by passive diffusion and sufficient protonated species are present to exert biological activity on DHFR (116).

The ability of a compound to penetrate cells by passive diffusion is also highly dependent on its lipophilicity. The log P value of MZP partitioning between octanol and phosphate buffer 5mM (pH 7.4) was found to be 2.81. This log P value is in the same range as that of the antitumour drugs metoprine and the nitrosourea CCNU (129). Both metoprine and the nitrosoureas have been shown to penetrate significantly into lipid-rich tissues such as brain tissues, brain tumours and lymph node tumours (129, 135). Therefore, MZP would be expected to penetrate the blood-brain barrier and other lipid-rich tissues and hopefully would have potential applications in the treatment of brain or solid tumours.

The stability studies on MZPES concentrated on the effect of heat and radiation because reports on other arylazides revealed that this class of compounds was susceptible to thermal and photo-degradation (6,7,8). Aqueous solutions of MZPES were found to be unstable at autoclaving temperatures. Therefore, filtration by aseptic techniques was chosen as a means of sterilizing MZPES solutions. Thermal degradation of MZPES in solution followed first order kinetics. The rate of thermolysis of MZPES in water was not significantly affected when the solution was saturated with air or oxygen but a slight enhancement in thermolytic rate was observed when the concentration of the solution was increased from 1.0 to 10.0 $mgml^{-1}$. The use of buffer solutions did not affect the rate of thermolysis of MZPES solution. However, aqueous MZPES solutions were found to be extremely unstable above pH 5.0 where precipitation of the free base occurred.

Accelerated stability studies revealed that MZPES solution was extremely stable at low temperatures, below 20°C, but at temperatures > 100°C degradation occurred rapidly. The half-life of a 10 $mgml^{-1}$ aqueous MZPES solution at 4°C, predicted from Arrhenius plot, is 3.27 x 10^4 years, whereas at 140°C the half-life of the same solution is determined to be 36.6 mins. The activation energy for thermal degradation of a 10 $mgml^{-1}$ aqueous MZPES solution was calculated to be 139.3 $KJmol^{-1}$. Shelf-life stability studies confirmed that aqueous solutions of MZPES stored at 20, 4 and -10°C were stable for at least 21 months.

Aqueous solutions of MZPES were found to be extremely sensitive to radiation. The half-life of a 0.1 and 1.0 $mgml^{-1}$ aqueous solutions of MZPES exposed to radiation of wavelength 254-366 nm were 2.41 and 20.96 mins respectively. The photo-degradation of MZPES solution at concentrations between 0.1 and 1.0 $mgml^{-1}$ was found to proceed according to Einstein's law of photochemical equivalence; a linear relationship was found between the photolytic half-life and the concentration of the solutions. The rate of photolysis of MZPES solution was not significantly different whether the solution was saturated with air or oxygen. Saturation of the solution with nitrogen, however, caused a slight decrease in photolytic rate.

Thermal degradation of MZPES in aqueous solutions resulted in the formation of MAP as the major product. A yield of 28% MAP was obtained when a 10 $mgml^{-1}$ aqueous MZPES solution was thermolyzed at 140°C for 2 hours. Photolysis of MZPES in aqueous solutions afforded more varied products. The nature and the ratio of the products formed were influenced by whether MZPES was photolyzed in solution saturated with oxygen, air or nitrogen. In oxygen-saturated solution, MAP, MNP, four unidentified polar compounds and an

unidentified high molecular weight non-polar compound were obtained. In nitrogen-saturated solution, MAP, a hydrazo compound and an unidentified high molecular weight non-polar compound were obtained. The photo-products of MZPES in air-saturated solution included all the products obtainable from oxygen and nitrogen-saturated solution. The formation of MNP was shown to be dependent on the presence of molecular oxygen. MAP, MNP and the hydrazo compound were postulated to be derived from the reactions of triplet nitrenes (6-23). The identity of the polar compounds was postulated to be the hydroxy derivatives of MAP, formed *via* the nitrenium species (60,61,161, 182,183). Irradiation of MZPES in aqueous THF did not produce the expected azepinone (184). Instead, an increased yield of MAP was obtained. This may be due to the *O*-chloro substituent accelerating the intersystem crossing from singlet to triplet nitrene (33). Photolysis of other 2,4-diaminopyrimidines, such as MAP, MNP and pyrimethamine, did not result in any degradation of these compounds. Therefore, MAP, MNP and pyrimethamine are not the source of the degradation products of MZPES.

The physico-chemical characteristics and the stability profiles of MZPES provided an important basis for the eventual clinical formulation of this compound. MZPES was formulated as a 10 $mgml^{-1}$ aqueous solution at pH 4.10 and recommended to be diluted in 500 mls 5% Dextrose Infusion prior to administration in order to avoid the potential risk of precipitation of the free base at physiological pH. The injections were prepared under aseptic conditions and sterilized by filtration. Prior to filling and sealing of the ampoules, the solution was saturated with nitrogen. This was carried out because saturation of the solution with nitrogen was shown to retard the photo-degradation process and it was also desirable to

prevent the formation of the photoproduct MNP, obtainable only in oxic conditions, which had been shown previously to be highly toxic (189). The injections were then packed into opaque plastic containers and stored at 4°C to maximise the stability of the drug. Each batch of injections was tested for drug content, identity, purity and sterility before the injections were released for clinical use. Stability studies of MZPES in 5% Dextrose Infusion showed that reconstitution of the drug in infusion fluid could be carried out at least 80 hours prior to administration. In vitro evaluation of drug delivery in the infusion fluid over an eight hours' study showed that on average 94% of total MZPES dose was delivered.

The clinical pharmacokinetics of MZPES exhibited a biphasic profile. The data obtained fitted approximately to a two-compartment open model. The distribution and elimination half-lives of MZPES in man were found to be 0.35 ± 0.22 and 37.42 ± 17.11 hours respectively. The apparent volume of the central compartment and the apparent volume of distribution of MZPES were calculated to be 34.4 ± 24.5 and 145.6 ± 34.8 L whereas the total body clearance was calculated to be 3.2 ± 1.4 L hr^{-1}. Good correlations were obtained between the trapezoidal area under the curve with dose and between the total area under the curve with dose. The correlation between peak plasma concentration of MZPES (measured as the base MZP) and dose was, however found to be poor; probably as a result of variation in the rate of infusion.

All patients receiving MZPES at doses >50 mgm^{-2} experienced mild sedation. Patients receiving MZPES at doses >180 mgm^{-2} complained of dizziness and headache ranging from mild to severe. Nausea and vomiting was observed in 3 of the 6 patients treated in Birmingham with MZPES >180 mgm^{-2}. An isolated case of convulsion was observed

in a patient treated with 210 mgm^{-2} MZPES. Haematological toxicity was not observed in patients receiving MZPES therapy and no leucovorin rescue has been necessary. These results contrast greatly with the side effects observed with metoprine. Thrombocytopenia was evident in metoprine therapy at doses as low as 65 mgm^{-2} (137) and above this dose administration of metoprine was only possible if leucovorin rescue was included in the schedule. Central nervous system toxicities (hallucinations, focal seizures, hyperactivity and insomnia) were reported in patients receiving metoprine >225 mgm^{-2} (137). MZPES is also different from metoprine in that it has much shorter elimination half life of 37.4 hours, compared to that of 216 hours in metoprine. The results, therefore, indicate that MZPES is less toxic than metoprine. The dose limiting toxicities of MZPES, however, remain to be elucidated as the Phase 1 clinical trial has not been completed at the time of writing.

The present work also uncovers several interesting subjects which require further investigation. Perfluorocarbons have been reported as effective adjuvants to cancer chemotherapy when they are administered concomitantly with oxygen (179). The current study attempted to exploit perfluorocarbons as drug delivery agents to achieve the synergistic effects. Preliminary investigations showed that perfluorocarbons were not suitable solvents for dissolving compounds in the 2,4-diaminopyrimidine series. However, a more extensive study with compounds of a wider range of lipophilicity would be required to reach a definite conclusion regarding the possible use of perfluorocarbons as drug delivery vehicles.

Preliminary investigations of MZP and its analogues and their counterparts in the iso-series showed that compounds in these two series exhibited very similar physico-chemical characteristics.

However, the photolysis of ISOMZPES under identical conditions to that of MZPES gave rise to different degradation profiles which would require further investigations.

To understand fully the photochemistry of MZPES, further studies would be required to isolate and identify the structure of the polar compounds obtained when MZPES solution was photolyzed in oxic conditions and the structure of the high molecular weight compound obtained when MZPES solution was photolyzed in hypoxic conditions.

In vitro studies revealed that photolysis of MZPES resulted in the generation of highly reactive nitrene species. This suggests that patients receiving MZPES could have a problem of photo-sensitivity if these reactive species were generated in the skin on exposure to sunlight. Reactions of photo-generated nitrenes with bionucleophiles, such as the physiological thiols, could have clinical significance in affecting drug action and toxicity and hence would warrant further investigation.

Table 31 continued.....

10	Ethylacetate(70), ethanol(30)	NH_4OH	A	0.381	0.415	0.338	0.415	0.432	0.423, 0.390, 0.339
11	Ethylacetate(70), ethanol(70), ether(30)	NH_4OH	A	0.346	0.350	0.288	0.346	0.350	0.346, 0.317, 0.267
12	Ethylacetate(25), ethanol(70), water(5)	Py	A	0.391	0.450	0.355	0.427	0.464	0.446, 0.391, 0.336
13	Toluene(50), methanol(80), acetone(20)	Py	A	0.517	0.565	0.448	0.548	0.556	0.548, 0.430
14	Chloroform(50), propan-2-ol(40)	NH_4OH	B	0.624	0.597	0.564	0.617	0.624	Not run

Key: Py = Pyridine
TEA = Triethylamine
NH_4OH = Ammonium hydroxide

A = Silica plates
B = Freshly prepared silica gel plates
C = Freshly prepared alumina plates

a = Pyrimethamine
b = MNP
c = MAP
d = MZP
e = MZPES
f = mixture a to e

Appendix 2

Table 32. Pharmacokinetic parameters of individual patient treated with MZPES in Birmingham during Phase 1 clinical trial

Patient No.	Patient details					Time (hr) / Plasma concentration of MZPES (mg L^{-1})											
	Sex	Wt (Kg)	Ht (m)	SA (m^2)	Dose (mg m^{-2})												
3	M	86.8	1.93	2.174	11	0	0.870	1.320	1.650	1.980	2.500	3.000	4.000	5.980	7.980		
						0	0.149	0.063	0.060	0.144	0.109	0.063	0.055	0.057	0.039		
3	M	86.8	1.93	2.174	18	0	1.050	1.400	1.680	1.980	2.500	3.050	4.000	6.020	7.980	12.020	
						0	0.200	0.144	0.134	0.124	0.127	0.120	0.101	0.115	0.146	0.100	
5	M	64.8	1.68	1.736	18	0	1.17	1.530	1.730	2.030	2.480	3.050	4.000	6.050	7.970		
						0	0.403	0.057	0.186	0.260	0.195	0.214	0.136	0.165	0.183		
5	M	60.5	1.68	1.690	27	0	0.88	1.280	1.650	2.020	2.520	3.000	4.050	6.150	8.000	12.180	
						0	0.504	0.337	0.302	0.258	0.253	0.282	0.249	0.288	0.311	0.257	
6	M	56.6	1.70	1.650	27	0	1.080	1.350	1.670	2.000	2.480	2.980	3.980	5.980	7.980		
						0	0.269	0.159	0.164	0.142	0.144	0.129	0.172	0.122	0.113		
7	F	58.0	1.70	1.67	27	0	1.150	1.480	1.830	2.180	2.530	3.030	4.080	8.000	12.180		
						0	0.572	0.284	0.297	0.267	0.243	0.240	0.233	0.235	0.226		
7	F	55.7	1.70	1.642	27	0	1.050	1.420	1.680	2.050	2.550	3.120	6.050	7.970	12.080		
						0	0.571	0.313	0.248	0.239	0.220	0.272	0.196	0.183	0.236		
20	F	49.6	1.56	1.469	50	0	0.950	1.330	1.670	1.980	2.530	3.000	4.020	6.030	8.000	11.750	24.420	48.250
						0	1.061	0.280	0.275	0.294	0.304	0.364	0.376	0.293	0.287	0.413	0.288	0.148
21	M	77	1.727	1.906	67	0	0.870	1.270	1.670	2.020	2.520	2.980	3.970	6.030	7.950	11.950	24.080	48.170
						0	3.956	0.726	0.505	0.509	0.472	0.405	0.413	0.424	0.383	0.324	0.299	0.170
23	M	55.2	1.626	1.584	67	0	1.020	1.330	1.680	2.120	2.550	3.070	4.000	6.000	8.030	12.050	23.920	48.000
						0	1.700	0.792	0.866	0.963	1.078	1.056	0.899	0.904	0.799	0.777	0.430	0.211
24	M	90.72	1.727	2.044	67	0	1.000	1.380	1.750	2.050	2.530	2.970	4.130	6.000	7.700	11.700	23.570	48.000
						0	1.925	1.019	0.938	0.967	0.918	0.842	0.672	0.678	0.654	0.574	0.475	0.298

Pharmacokinetic parameters

Patient No.	Cp max (mg L^{-1})	K_{α} (hr^{-1})	$t_{\frac{1}{2}\alpha}$ (hr)	k_{β} (hr^{-1})	$t_{\frac{1}{2}\beta}$ (hr)	A	B	R	S	V_c (L)	V_b (L)	K_{12} (hr^{-1})	K_{21} (hr^{-1})	K_{10} (hr^{-1})	Trap AUC (mg hr L^{-1})	AUC_t (mg hr L^{-1})	CL (L hr^{-1})
3	0.1485			-0.1360	5.0958						218.98				0.5396	0.8032	29.78
3	0.200			-0.0293	23.6583						274.12				1.4498	4.8743	8.0296
5	0.4025			-0.1182	5.8635						75.09				1.4348	2.5518	8.8751
5	0.5040			-0.0219	31.7122						102.32				3.4487	14.7558	2.2361
6	0.2685			-0.0465	14.9087						274.24				1.1198	3.5021	12.7476
7	0.5720			-0.0185	37.4214						166.75				3.0082	14.6081	3.0879
7	0.5710	-5.1323	0.1350	-0.0410	16.8900	2.7046	0.2469	0.5342	0.2420	10.8861	119.6501	4.2554	0.4669	0.4510	2.7899	6.5448	4.9093
20	1.0610	-1.0830	0.6399	-0.0172	40.4040	0.8172	0.3608	0.5221	0.3581	44.9045	141.53	0.7025	0.3436	0.0541	13.8289	21.7919	2.4274
21	3.9560	-3.4082	0.2033	-0.0200	34.6792	7.3465	0.4478	2.5181	0.4442	11.8736	188.5316	2.8962	0.2146	0.3173	16.4026	24.5645	3.7675
23	1.6995	-1.0449	0.6632	-0.0345	20.1100	1.0153	1.0555	0.6299	1.0375	37.1389	70.6229	0.4643	0.5495	0.0655	25.4202	31.5997	2.4337
24	1.9245	-0.8423	0.8228	-0.0190	36.4158	0.9636	0.7338	0.6560	0.7270	58.4567	131.328	0.4436	0.3749	0.0428	24.3874	39.7026	2.4992

Patient No.	Patient details					Time (hr) / Plasma concentration of MZPES (mg L^{-1})											
	Sex	Wt (Kg)	Ht (m)	SA (m^2)	Dose (mg m^{-2})												
24	M	91.7	1.727	2.053	83	0	1.250	1.720	2.120	2.500	2.850	3.230	4.100	6.130	8.050	12.050	21.470	47.800
						0	1.235	1.134	0.939	1.019	1.021	0.983	0.865	0.743	0.594	0.782	0.336	0.323
25	M	90.5	1.74	2.053	83	0.770	1.020	1.330	1.670	2.020	2.500	3.000	4.100	6.050	7.970	11.000	24.150	48.230
						2.908	1.163	1.005	1.008	0.989	1.107	1.121	0.867	0.897	0.904	0.725	0.583	0.338
25	M	91.5	1.74	2.062	83	0	0.930	1.470	1.830	2.230	2.550	3.050	4.030	6.020	8.050	12.100	23.800	48.050
						0	1.742	1.008	1.004	0.952	0.932	0.860	0.847	0.814	0.747	0.655	0.521	0.277
26	M	67.3	1.803	1.857	83	0	1.200	1.450	1.680	2.030	2.520	3.020	3.980	6.000	8.000	11.950	24.000	48.080
						0	4.794	1.103	1.016	0.734	0.915	0.907	0.848	0.738	0.717	0.698	0.484	0.331
26	M	65.0	1.803	1.830	83	0	1.400	1.780	2.150	2.520	3.030	4.020	6.000	8.000	12.100	23.670	47.920	
						0	1.808	1.129	0.905	0.981	0.751	0.672	0.629	0.616	0.565	0.441	0.230	
27	F	65.9	1.59	1.68	83	0	1.120	1.500	1.950	2.250	2.630	3.030	3.900	5.920	7.850	11.880	21.000	47.250
						0	1.729	0.871	0.767	0.692	0.695	0.638	0.637	0.628	0.597	0.491	0.321	0.309
29	M	47.6	1.72	1.55	105	0	1 150	1.500	1.750	2.100	2.530	3.030	4.050	6.030	8.030	12.050	27.030	48.870
						0	1.676	1.052	0.948	1.048	0.969	1.087	1.088	1.163	0.994	0.930	0.639	0.465
30	M	65.5	1.63	1.71	105	0	1.200	1.430	1.720	2.070	2.500	3.030	4.020	6.000	8.000	12.000	26.830	48.650
						0	1.974	1.267	1.329	1.295	0.990	1.257	1.236	0.966	1.220	0.960	0.928	0.670
36	M	79.2	1.85	2.031	125	0	1.080	1.380	1.680	2.020	2.520	3.000	4.000	6.000	8.020	12.250	24.030	48.530
						0	1.858	1.011	0.976	0.935	0.818	0.891	0.827	0.857	0.959	0.705	0.825	0.557
37	F	58.4	1.56	1.574	150	0.720	1.000	1.330	1.670	2.000	2.500	3.000	4.020	6.020	8.020	10.900	25.780	49.120
						3.248	1.976	1.805	1.799	1.678	1.528	1.544	1.728	1.432	1.506	1.012	0.715	0.413

Pharmacokinetic parameters

Patient No.	Cp max (mg L^{-1})	K_α (hr^{-1})	$t_{1/2\alpha}$ (hr)	k_β (hr^{-1})	$t_{1/2\beta}$ (hr)	A	B	R	S	V_c (L)	V_b (L)	K_{12} (hr^{-1})	K_{21} (hr^{-1})	K_{10} (hr^{-1})	Trap AUC (mg hr L^{-1})	Total AUC (mg hr L^{-1})	CL (Lhr^{-1})
24	1.2350	-0.2974	2.3304	-0.0222	31.2643	0.4594	0.8078	0.4060	0.8002	97.446	146.65	0.0886	0.1976	0.0334	23.2552	37.987	3.2507
25	2.9080	-6.9071	0.1003	-0.0224	30.9338	10.4561	0.9874	2.0603	0.9794	10.789	120.89	6.0621	0.6164	0.2510	30.4001	45.588	2.7082
25	1.7415	-1.7443	0.3973	-0.0251	27.6566	1.1584	0.9142	0.5731	0.9036	59.848	133.26	0.9302	0.7834	0.0558	26.507	37.148	3.3391
26	4.7935	-6.7313	0.1030	-0.0215	32.2558	24.9882	0.8761	3.1449	0.8651	4.3188	116.86	5.9227	0.2488	0.5813	28.634	44.491	2.5107
26	1.8075	-1.3077	0.5299	-0.0243	28.5101	1.9067	0.7381	0.9080	0.7264	41.616	142.29	0.8664	0.3825	0.0831	22.583	31.824	3.459
27	1.7285	-1.7233	0.4021	-0.0183	37.8953	1.6509	0.6391	0.7491	0.6328	44.134	153.92	1.1837	0.4941	0.0638	19.744	35.906	2.8148
29	1.6760	-2.1581	0.3211	-0.0190	36.5627	1.7142	1.1290	0.6700	1.1176	41.458	103.03	1.2616	0.8684	0.0471	36.145	60.359	1.9529
30	1.9740	-10.784	0.0643	-0.0122	56.6824	16.3288	1.2153	0.3167	1.2068	7.4009	105.23	9.8640	0.7584	0.1738	44.858	100.920	1.2866
36	1.8575	-7.0331	0.0985	-0.0091	75.7926	8.7647	0.9016	1.1861	0.8972	19.0316	201.50	6.2812	0.6643	0.0968	36.731	99.849	1.8424
37	3.2480	-2.1989	0.3152	-0.0301	23.0162	2.1318	1.6857	1.1343	1.6693	44.827	99.79	1.1742	0.9878	0.0670	42.668	56.955	3.0046

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