Dihydrofolate Reductase Inhibitors as Potential Antitumour Radioaffinity Reagents

By

Edward Anthony Bliss

A thesis presented for the degree of

Doctor of Philosophy

at the

University of Aston in Birmingham.

June 1980

Department of Pharmacy

University of Aston in Birmingham.

Acknowledgements

The author wishes to express his sincere gratitude to Professor M.F.G. Stevens for his energetic example, challenging and tireless nature both at work and at games; to Dr. R.A. Paterson who became a close friend and mentor in his rôle as industrial supervisor; to Dr. P.J. Goodford for his encouragement and material support in providing the necessary facilities at Beckenham; to Mr. L. Lee for expert technical assistance and much profitable discussion in enzyme kinetics; to Mr. R. Dunbar for carrying out the experiments with the myeloma flank-implants in mice; to the Wellcome Foundation Limited for the award of a Wellcome Research Studentship.

Special thanks are also extended to: Miss P. Bugby, Dr. F. Cottee, Mr. M. Gunn, Professor K. Hellman (of the Imperial Cancer Research Fund), Dr. H. Hodson, Dr. R. Hyde, Dr. J. Lindon, Miss Y. Lucking, Dr. C. Moreno, Mr. D. Sawyer and Dr. R. Wrigglesworth.

Finally, the author would like to leave on record his appreciation of the contributions of his friends and colleagues who have provided useful discussion, advice and support, in the Department of Pharmacy at Aston University; in the Departments of Biophysics/Biochemistry, Chemistry, Library Services, Precision Engineering and Security at the Wellcome Research Laboratories, Beckenham, Kent; and at the Westminster Hospital, London.

SUMMARY

The principle of this research was to incorporate the radiolabile azido group into the structures of reversible inhibitors of dihydrofolate reductase (DHFR) which on administration would interact with this enzyme in normal and neoplastic cells. Subsequent selective X- or γ-irradiation of the tumour causes the formation of nitrene intermediates which covalently bond with their immediate surroundings.

Various azido, chloro, nitro, amino, acetylamino, diazonium salt and *p*-substituted-benzenesulphonamido derivatives have been prepared in the following four chemical series:- (i) 2,4-diamino-6-substituted-quinazoline, (ii) 4,6-diamino-1,2(2H)-2,2-dimethyl-1-(substituted-phenyl)-*s*-triazine, (iii) 2,4-diamino-6-(substituted-phenyl)-*s*-triazine and (iv) 2,4-diamino-5-(3-substituted-4-chlorophenyl)-6-ethylpyrimidines.

The hitherto unreported nucleophilic displacement by hydrazine of the amino groups of 2,4-diaminoquinazoline is described: this compound when refluxed in hydrazine hydrate for 6 h yielded 2,4-dihydrazino-quinazoline *via* 2-amino-4-hydrazinoquinazoline.

The activities of sixty compounds were tested without photoactivation against *E.coli* and L1210 DHFR. The inhibitor constants of ten of the compounds, most interesting in terms of structure, activity and the aims of the project, were then determined using the method of Dixon, and also by fitting the raw kinetic data to the curve function for linear competitive enzyme inhibition (Michaelis-Menten). Inhibitor constants in the range 0.6-87nM were obtained for these compounds.

Twelve of the compounds tested *in vivo* showed significant activity as anti-tumour agents; five of these were azido derivatives.

The hypothetical principle was tested indirectly *in vivo* in mice bearing myeloma flank-implants. An exploratory experiment with 2,4-diamino-5-[3-azido-4-chlorophenyl]-6-ethylpyrimidine gave results consistent with the proposed radiopotentiation mechanism and further experiments are warranted.

The strongly lipophilic analogues of the diaminopyrimidine series (iv) might be suitable for treating, say, CNS and testicular tumours which are inaccessible to Methotrexate, and psoriasis since they may well be percutaneously absorbed.

Submitted by Edward Anthony Bliss for the degree of Doctorate of Philosophy, 1980.

Key words:- Dihydrofolate reductase
2,4-Diaminoheterocycles
Enzyme inhibitors
Radioaffinity reagents
Anti-cancer

CONTENTS

List of Schemes, Tables and Figures

General Abbreviations and Terms

ASTON	refers to University of Aston
ATRAR	Anti-tumour Radioaffinity Reagents
CF	Citrovorum factor; N^5-formyl-FH_4; Folinic acid
CSF	Cerebrospinal fluid
CSSR	Crystal Structure Search and Retrieval (system)
DHFR	Dihydrofolate reductase
DHFRI	Dihydrofolate reductase inhibitor(s)
DMF	N,N'-Dimethylformamide
DMSO	Dimethylsulphoxide
FH_2	Dihydrofolate
FH_4	Tetrahydrofolate
Glu	Glutamate residue
i.p.	Intraperitoneal
i.r.	Infrared (spectroscopy)
6-MP	6-Mercaptopurine
m.s.	Mass spectrum
MTX	Methotrexate
n.m.r.	refers to ^1H n.m.r.
NADPH	Reduced form of Nicotinamide Adenine Dinucleotide Phosphate
ORTEP	Oak Ridge Thermal Ellipsoid Plot
RAR	Radioaffinity reagents
SAN	Sulphanilamide
S.D.	Standard Deviation
S.E.	Standard Error
SOD	Superoxide dismutase
targeted	refers to "active-site-directed"
TFA	Trifluoroacetic acid

t.l.c.	Thin layer chromatography
TMP	Trimethoprim
T.S.	Thymidylate synthetase
u.v.	Ultraviolet
water	refers to double distilled water
WELLCOME	The Wellcome Research Laboratories

Equations in the text are given capital Roman numerals.

Equations in the appendices are given small Roman numerals.

^{o} refers to degrees centigrade

PART I

The Hypothesis

About 17% of cancer patients die because of recurrence at the primary tumour site [1] . The two traditional approaches to therapy are radiotherapy and surgery, frequently together, and in both cases treatment is usually supplemented with cytotoxic drugs. However, conventional radiotherapy/chemotherapy combinations have evolved empirically, and the rôle of the chemical agent has been widely underestimated. Chemotherapeutic drugs alone cannot usually be targeted to localised regions of the body and, furthermore, not all cells in solid tumours may be accessible to cytotoxic drugs.

Radiosensitisers such as misonidazole and metronidazole have only limited use in sensitising malignant tissue to irradiation, suffering as they do from problems of neurotoxicity in the high doses that are required [2] , and it is probably fair to say that as yet the success of the current range of electron-affinic hypoxic cell sensitisers has yet to be clinically realised [3] .

As a logical extension of this, what is desirable now is a chemical agent rationally designed to possess both radiosensitising properties and intrinsic anticancer activity at relatively low levels. When used in conjunction with radiotherapy such molecules could be expected to produce a more than additive effect selectively on malignant tissue.

An arylnitrene was first used as a labelling reagent for a specific antibody by Fleet, Porter and Knowles in 1969 [4] , and the general photoaffinity principle was first reviewed by Knowles in 1972 [5] . This principle has since been used in many areas of molecular biology including the study of hormone receptors [6,7] and

enzyme reactions[8,9,10] . Photoaffinity reagents have affinity for a biological target site by virtue of their overall chemical structure but bear in the molecule the biologically inert[a] azido group. On photolysis at a non-destructive wavelength (350 nm) the azido group is degraded to the highly reactive nitrene intermediate which has a half life shorter than 10^{-3} sec. which must subsequently react with the nearest reactive group within the inhibitor-macromolecule complex to form a covalent bond. For this to occur in practice it is essential that the affinity between the reagent and biological receptor is high. The merit of generating nitrenes in this procedure lies in the reactivity of the transient species formed because both hydrophilic and hydrophobic targets are vulnerable to attack. Alkyl- and aryl-azides are well known to photolyse and thermolyse to nitrene intermediates but it was only recently that Stevens and Butchart[11] discovered that aryl-azides also radiolyse with X- or γ-irradiation to yield nitrenes.

From this Stevens proposed a novel approach to radiosensitisation of tumours and recently he and his co-workers have exploited the idea in developing potential photo- or radio-sensitisers capable of irreversibly intercalating DNA [12,13] . Nitrenes are potentially ideal agents for this purpose since their reactivity is not unduly influenced by the oxygen tension [5] . Since structure-activity relationships of "small molecule" ("non-classical") inhibitors of dihydrofolate reductase (DHFR: EC 1.5.1.3) have been well documented (for reviews see refs. 14-26) the hypothesis was that a reversible DHFR inhibitor (DHFRI), bearing a strategically placed azide group, might be rendered an irreversible inhibitor by interaction with

[a] i.e. in terms of chemical reactivity

accurately administered X- or γ-irradiation. The tetrahydrofolate (FH_4) biosynthesis, being blocked, would effect cell death, since the FH_4 cofactors are necessary for the cellular production of most importantly, purines[27,28] (hence RNA, proteins and DNA), thymidylate [29] (hence DNA), and methionine [30]. However, it has been estimated that as little as 5% of intracellular DHFR activity will sustain near-normal rates of FH_4 synthesis in L1210 cells [31]. Therefore it is essential that the reversible inhibitor is at saturating levels intracellularly, so that more than 96% DHFR is bound (competitively) by the compound.

Thus, were it to prove possible to localise the candidate DHFR-targeted radioaffinity reagent (RAR) within tumours, either by physico-chemical parameter modification, e.g. by the use of liposomes[32], or by direct injection, such compounds might afford a novel, alternative radiopotentiation mechanism.

The primary objective of this work was chemically to synthesise and biologically to test novel aryl azido analogues of known "small-molecule" DHFR inhibitors, both with and without u.v. or ionising irradiation, *in vitro* against DHFR and against tumour cells in culture, and *in vivo* against animal tumour models.

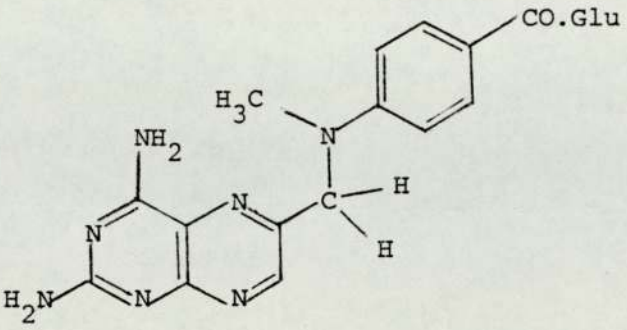
A secondary objective was to synthesise and test a range of potential DHFR inhibitors, incorporating other novel structural features. This secondary objective is discussed further in Part II. The intention was to combine in single molecules these secondary novel features with the aryl azide function.

A third objective was to assess the toxicity and biological stability *in vivo* of such aryl azides in order to weigh the clinical feasibility of the hypothetical anti-tumour radioaffinity-reagent (ATRAR) principle.

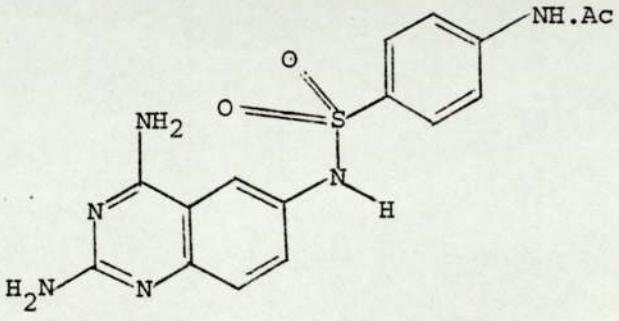
PART II

CHAPTER 1

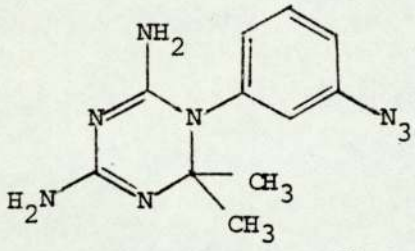
Chemical Strategy and Rationale



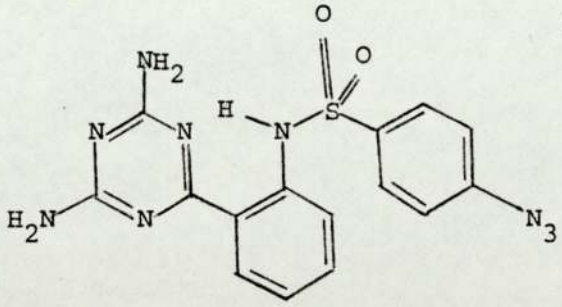
MTX



Compound (12)
of the *Quinazoline series*

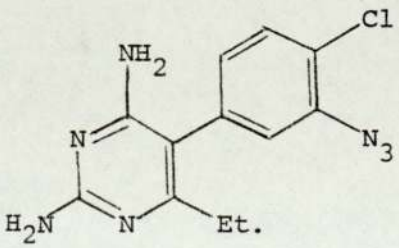


Compound (39)
of the
Dihydrotriazine series (Guanils)



Compound (68)
of the *Aryl-s-triazine series*

Compound (93)
of the *Pyrimethamine series*



Four series of small-molecule DHFRI were synthesised:

(i) 2,4-diamino-6-substituted-quinazolines; the *quinazolines*, e.g. (12)

(ii) 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(substituted-phenyl)-*s*-triazines; the *dihydrotriazines* or *guanils*, e.g. (39)

(iii) 2,4-diamino-6-(substituted-phenyl)-1,3,5-triazines; the *aryl-s-triazines*, e.g. (68)

(iv) 2,4-diamino-5-(3-substituted-4-chlorophenyl)-6-ethylpyrimidine; the *pyrimethamine series*, e.g. (93).

The antifolate activity of compounds of the types (i), (ii) and (iv) above has been extensively documented (e.g. see refs:**14-26**).

The interest in the benzenesulphonamido compounds such as those synthesised in the quinazoline, e.g. (12), and aryl-*s*-triazine, e.g. (68), series has centred around the following rationale:

1. In MTX the bridging between the pyrazine and the benzene moieties is $-CH_2-NCH_3-$, i.e. tetrahedral-carbon plus pyramidal-nitrogen. The possibility exists that in the sulphonamide derivatives reported herein the novel bridging with $-NH-SO_2-$ (i.e. pyramidal-nitrogen plus approximately tetrahedral-sulphur) might cause a different orientation of the two aryl rings relative to each other, thus possibly effecting a novel binding mode.

2. Kendrew models of the sulphonamides were constructed with torsion angles along the C·C-N-S-C·C bridge being made similar to those of four crystallographically solved analogues of N-phenylbenzene-sulphonamides: N-(4-bromo-2-methylphenyl)-benzenesulphonamide[33], N-(4-bromophenyl)-benzenesulphonamide[33], N'-2-chlorophenyl-sulphanilamide[34], and sulphadimethoxine[35]. Such crystallographic data was obtained initially for 8 compounds in the form of a visual 3-D

display on a cathode-ray oscilloscope screen using a Tektronix 4012 interphase linked directly to the CSSR database in Edinburgh. From the Kendrew models the superimposability of the two benzene rings of, for example, the quinazoline sulphonamides over the corresponding benzene and pyrazine rings of MTX was assessed, and the binding modes of these sulphonamides to *E.coli* DHFR were postulated.

3. Several research groups have tested various other analogous two- and three-atom bridges (e.g. see Refs **36-43**) with interesting positive results.

4. Little has been published on the cellular transport in mammalian cells on 2,4-diaminoheterocycle/N-phenylbenzene-sulphonamide combinations.

The activity of the dihydrotriazines (guanils) and pyrimethamine analogues against mammalian (tumour) DHFR and as anticancer agents is well known (e.g. see Refs **15, 18, 44-52**). Pyrimethamine analogues were of special interest since they pass the blood brain barrier by virtue (mainly) of their lipophilicity. These and additional aspects are further discussed in Part V.

It has been established that compounds in the aryl-*s*-triazine series are unable to bind reversibly to any significant extent[53] . Despite this, compounds in this series were prepared in order to explore firstly, the effect on DHFRI activity of extending 2'- and 4'-substitution on the phenyl ring via a sulphonamido bridge, secondly

any potential increase in binding to DHFR caused by the azido substituent (as opposed to other non-azido functions), and thirdly the relative potency as DHFRI of several interesting compounds first synthesised by Mackenzie and Stevens [54].

PART II CHAPTER 2

Synthesis of the quinazolines

The methods of Davoll and Johnson[55] were followed in the synthesis of 2,4-diaminoquinazoline, the 6-nitro and 6-amino analogues, (scheme II.2-1).

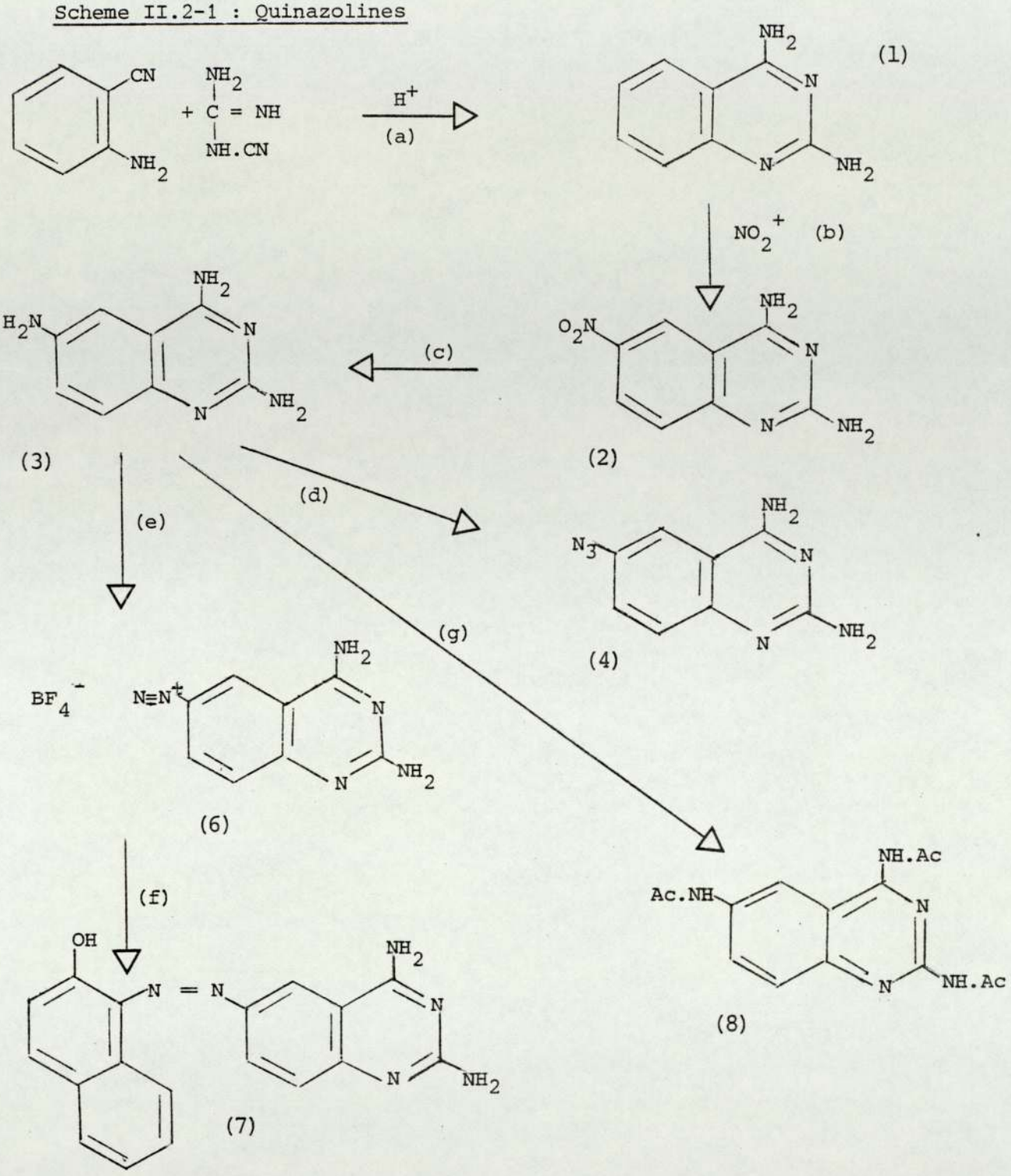
The mechanism of the acid catalysed reaction between cyanoguanidine and anthranilonitrile involves the nucleophilic attack by the amino group of cyanoguanidine on the aryl cyano group to give a guanidine intermediate (9), followed by nucleophilic substitution by the anilino group to give 2,4-diaminoquinazoline with the elimination of cyanamide (scheme II.2-2).

In contrast, in alcoholic base the same guanidine intermediate is formed and the alkoxide catalyses the ring closure of the guanidine by an addition mechanism, to give 2,4-diamino-6-[2-aminophenyl]-*s*-triazine, (scheme II.2-3).

In the reduction of 2,4-diamino-6-nitroquinazoline (2) the relative proportions of reduced stannicomplex, water and sodium hydroxide used in the final step are critical.

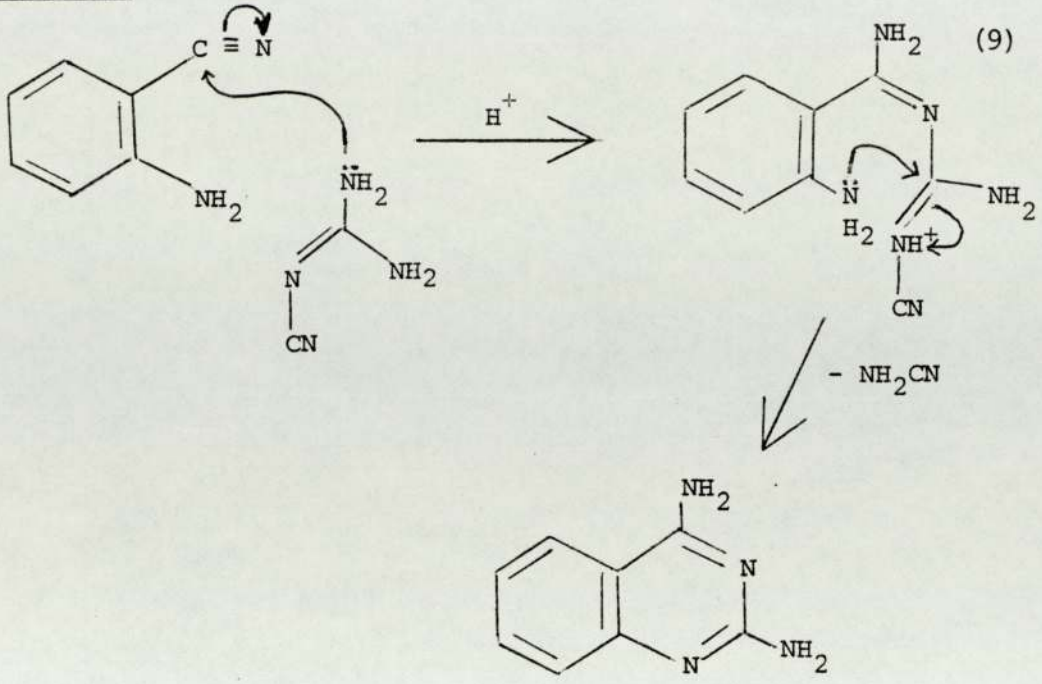
The nitro compound was reduced using the tin(II) chloride-hydrochloric acid method which, producing the amine-stannicomplex, necessitates basification to pH 12 to liberate the free amine and soluble stannate. It was found that the quantities of alkali and water required for the base liberation depended upon the amount of stannicomplex liberated, this increasing with time; overnight standing at 4° optimised the stannicomplex yield. The addition of too little alkali caused incomplete liberation of the triamine and only partial conversion of the insoluble tin(III) hydroxide to

Scheme II.2-1 : Quinazolines

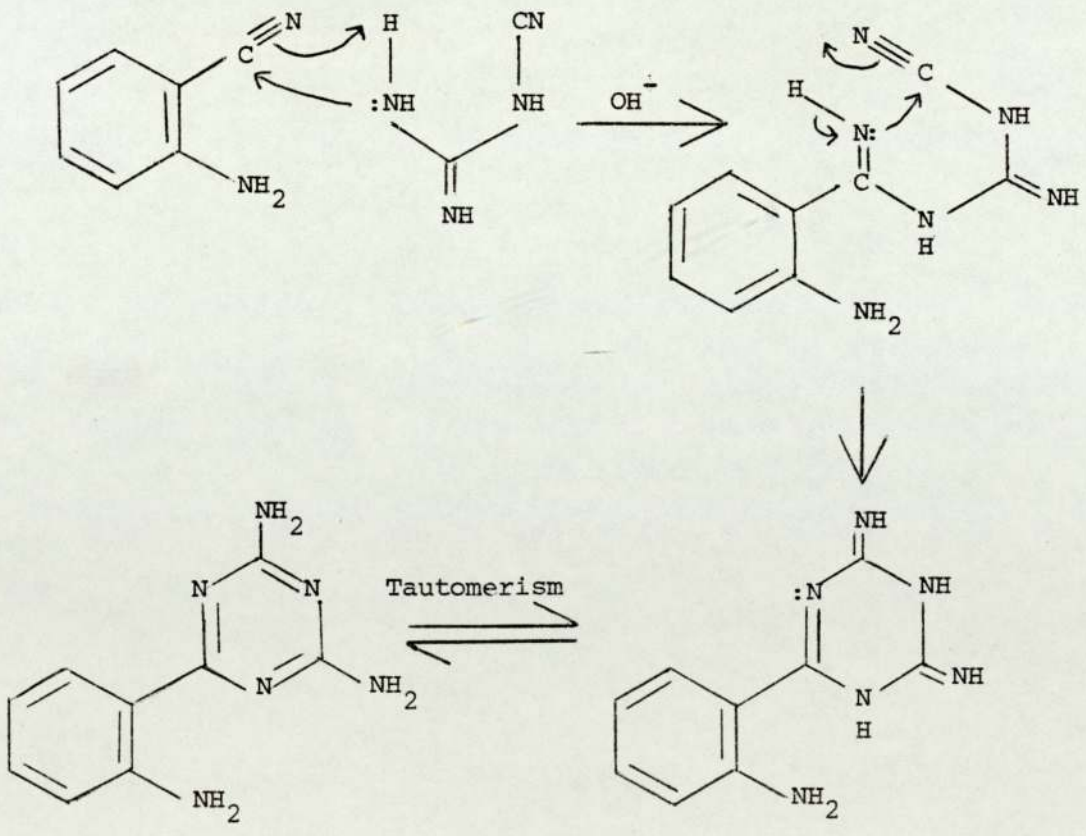


(a) 2N HCl
(b) Nitration with H_2SO_4/HNO_3
(c) Reduction with $SnCl_2/HCl$ and finally basification.
(d) Diazotisation and reaction with NaN_3.
(e) Diazotisation with $HBF_4/NaNO_2$.
(f) Coupling with 2-naphthol in N-KOH.
(g) $(CH_3CO)_2O$: Reflux.

Scheme II.2-2



Scheme II.2-3



(60)

soluble stannate. The use of too little water caused either premature amine crystallisation or precipitation of both amine and tin fractions together: excess water prevents fractional crystallisation of the amine (3). In addition, a certain proportion of the tin is rendered irreversibly colloidal by the basification process.

Yellow or green felted needles of the amine were only produced from the primary crystallisation above. However crystallisation of the crude product from water consistently produced the triamine in amber prism form. The Rf. values (t.l.c.), m.p., n.m.r. and u.v. spectra of both forms were identical but the i.r. spectra were dissimilar in the "fingerprint region" both as KBr discs and as nujol mulls. This was attributed to the existence of two solvates.

Alternative reduction routes were tried with limited success: catalytic hydrogenation and the use of Raney nickel/hydrazine hydrate were hampered by the insolubility of the nitro compound in appropriate solvents. The formation of two other products (t.l.c.) was encountered with the use of hydrazine hydrate; the displacement of the 2- and 4-amino groups by hydrazine is discussed below

The 6-azido compound (4) was formed from 2,4,6-triamino-quinazoline in the normal way. It was not found possible to produce this azido derivative directly from the crude amine stannicomplex.

Attempts to produce 2,4-diamino-6-acetylaminoquinazoline as a possible metabolite reference compound by simple acetylation procedures by using base catalyst with acetyl chloride at room temperature produced only starting materials, and the use of undiluted acetic anhydride gave a mixture of the mono-, di- and tri-acetylated amine which gave three spots on t.l.c. and three successive losses of $^{m}/_{e}$ 42

in the mass spectrum. However, the triacetylated material (8) was useful in confirming the chemical shifts of the 5, 7 and 8 protons of the quinazoline ring system. The n.m.r. spectra are discussed below.

The synthesis of the 6-diazonium tetrafluoroborate (6) and diazonium chloride hydrochloride (5) were straightforward although the latter salt was found to be unsuitable for biological testing due to its instability in water.

This diazonium chloride hydrochloride readily coupled with 2-naphthol in alkali to produce a deep red dye (7) with a broad absorbance band at 450 nm, characteristic of azo compounds.

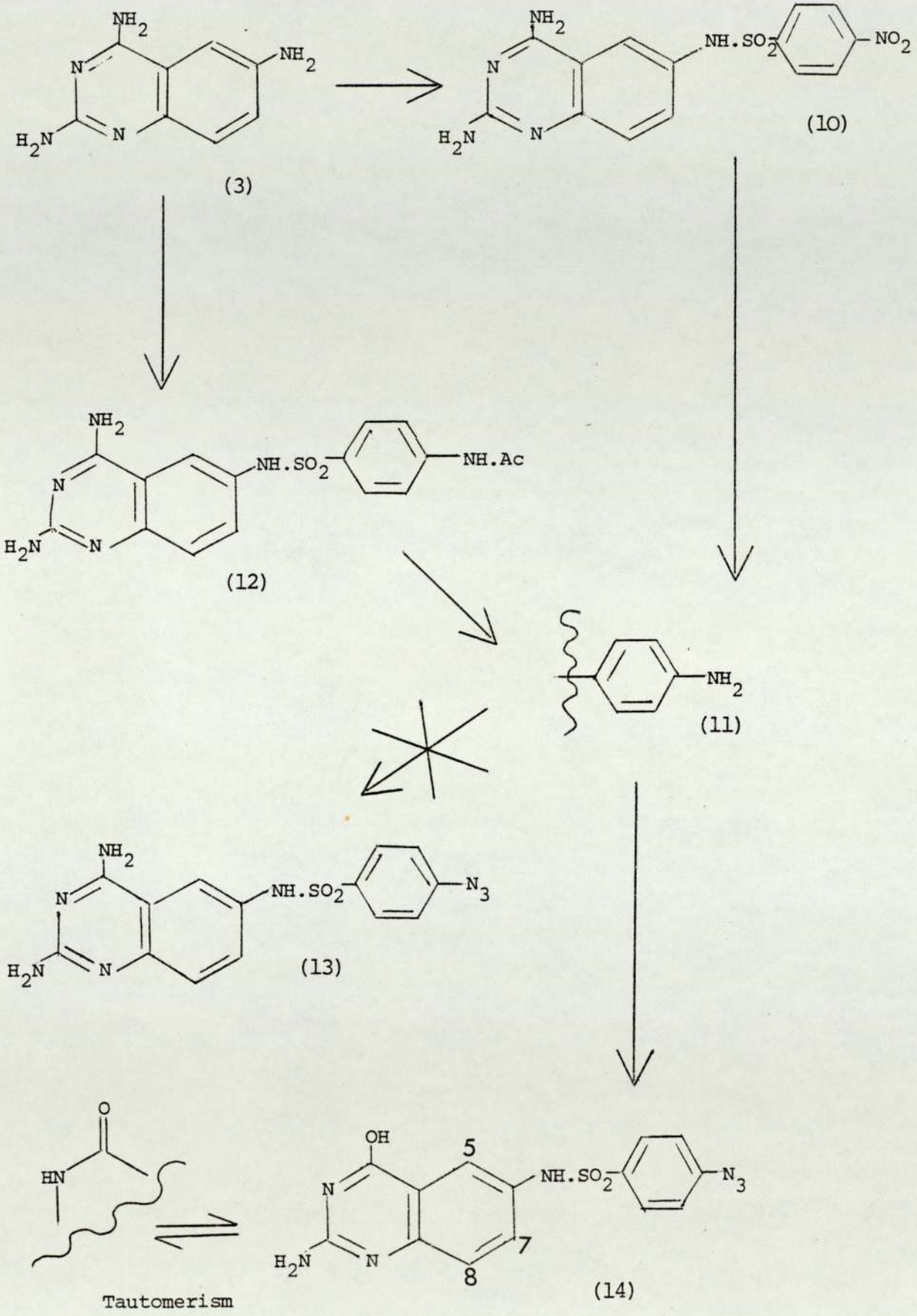
Synthesis of the sulphonamides in the quinazoline series

Scheme II.2-4 illustrates the synthetic strategy that yielded the candidate RAR 2-amino-4-hydroxy-6-[4 -azidobenzenesulphonamido]-quinazoline (14).

Numerous attempts, using pyridine as solvent and base catalyst, or triethylamine in acetonitrile, or combinations of pyridine and triethylamine with other organic solvents, all failed to effect the efficient condensation between triaminoquinazoline and p-nitrobenzene-sulphonyl chloride, (scheme II.2-5).

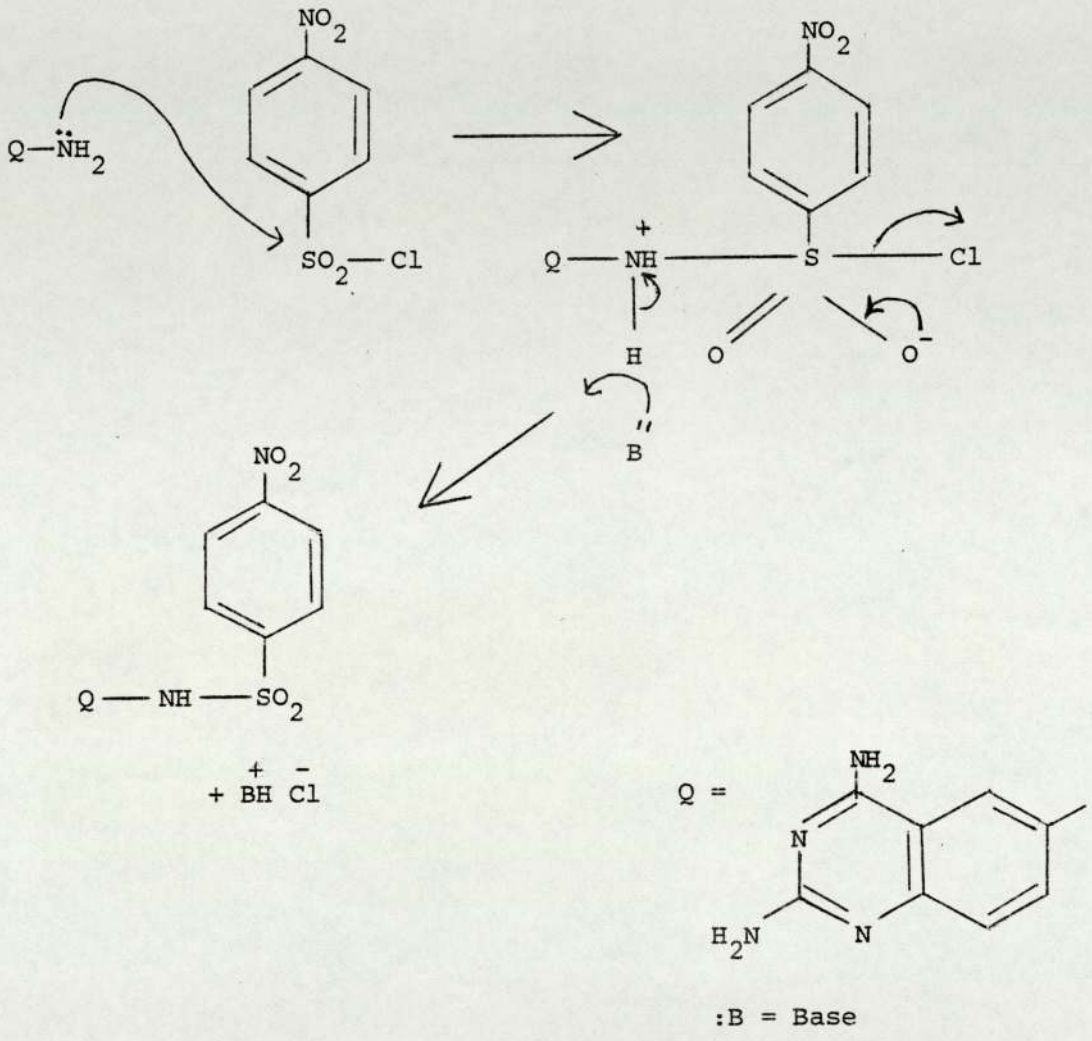
For example, overnight reflux of equimolar sulphonyl chloride and the amine in acetonitrile containing 1.1 mol.equiv. triethylamine afforded starting materials and two unidentified products (t.l.c.). Eventually it was found that the presence of water blocked the reaction and that by refluxing the sulphonyl chloride and amine in dry pyridine a high yield of the required 4-nitrobenzenesulphonamide was achieved. Northey[56] has pointed out that amino heterocycles

Scheme II.2-4 : Synthesis of sulphonamides



being weak bases, rarely give appreciable yields of sulphonamides in aqueous media. Instead he recommended the use of dry arylsulphonyl chloride and dry pyridine.

Scheme II.2-5



As was found with 2,4,6-triaminoquinazoline (3), different solvates of the nitrophenyl sulphonamide (10) having the same n.m.r. and m.s. but differing i.r. spectra, were isolated.

The nitrophenyl sulphonamide (10) was reduced to the amine (11) using hydrazine hydrate with Raney nickel and by catalytic hydrogenation under pressure, although low yields by both routes were obtained. Catalytic hydrogenation at atmospheric pressure

afforded starting materials only. A red dye was produced when the reduction product was diazotised and coupled with 2-naphthol, and t.l.c. showed a single spot which fluoresced bright blue under 254nm light. The i.r. spectra of the products obtained from the two reduction methods were similar and lacked the bands at 1350cm^{-1} and 1530cm^{-1} (NO_2).

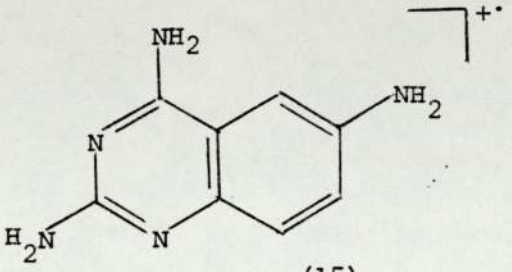
The same product (t.l.c.) was obtained by hydrolysis of the acetylamino compound (12). However, this amine was not isolated since various attempts at its crystallisation from common solvent systems failed.

The synthesis of the required candidate RAR, the azidophenylsulphonamide (13) was finally attempted via the acetylaminophenyl analogue (12) which was eventually obtained in good yield by gently heating equimolar triaminoquinazoline and 4-acetylaminobenzenesulphonyl chloride in a minimum quantity of dry pyridine containing 1.5 mol. equiv. triethylamine at 85-90° for 6h. Partial condensation results from the omission of triethylamine.

The hydrolysis of the acetylamine (12) was efficiently achieved using 2N-hydrochloric acid. Since it proved impracticable to isolate the amine base (11) (and since acetic acid was the only other reaction product) the amine group was directly converted to the azide. The product was subsequently recrystallised as a hydrochloride salt, or as the base from aqueous dimethylformamide.

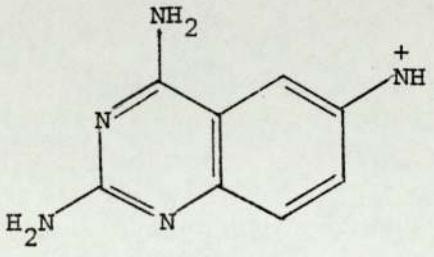
The i.r. of the pure (t.l.c.) product showed a sharp band at 2160cm^{-1}, and the n.m.r. integrated for four exchangeable protons with chemical shift 5.5 - 8.5 δ . The aromatic multiplet was clearly resolved with D_2O, and showed 5, 7 and 8 protons of the quinazoline moiety as a (*meta* split) doublet at 7.39 δ (5-H), and a broad singlet at

7.23 δ (7,8-H_2), being superimposed on the expected AA'BB' quartet which was centred at 7.51 δ . However, the m.s. strongly suggested that the 2,4-diaminoquinazoline moiety had undergone a change since no ions at $^{m}/_{e}$ 175 or 174 corresponding to structures 15 and 16 were observed. Instead there occurred a large peak at $^{m}/_{e}$ 176 (60%) whose accurate mass corresponded to the 2-amino-4-hydroxy structure (17), or (18).



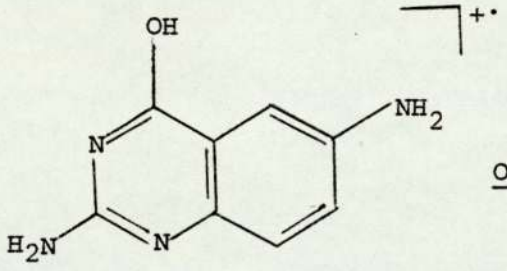
(15)

$^{m}/_{e}$ 175



(16)

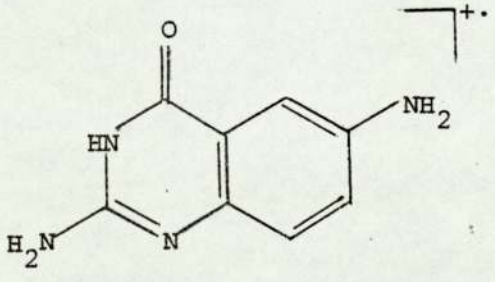
$^{m}/_{e}$ 174



(17)

OR

$^{m}/_{e}$ 176



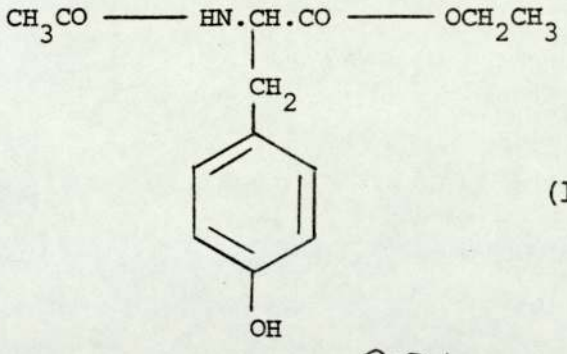
(18)

Therefore, since the structure of the starting material (12) was confirmed by n.m.r., i.r., microanalysis, and m.s. with accurate mass, it was deduced that the 4-amino group had been displaced during the process of diazotisation in nitrous acid of the aryl amine of the sulphonamide moiety.

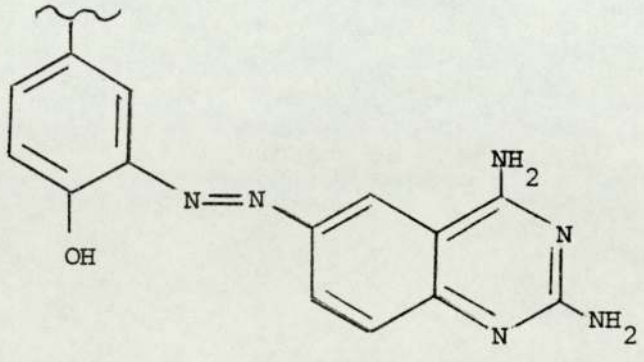
Quinazoline series : Further reactions

(a) General

Since tyrosine is known[17] to be present in the hydrophobic bonding region of DHFR, the diazonium salt (6) was coupled with an isolated tyrosine residue N-acetyl-L-tyrosine ethyl ester (19) in an attempt to support the hypothesis that this tyrosine-labelling ability, when incorporated into an active-site-targeted molecule, might be useful in elucidating the structural rôle of tyrosine at this particular DHFR site; it is thought that the presence of a polar residue in a hydrophobic centre may be of mechanistic significance. However, although the diazonium salt coupled with (19) at pH > 9 affording a brown precipitate, it did not do so at physiological pH. Attempts at isolation of the pure dye (20) were unsuccessful due to its poor solubility characteristics.



(19)

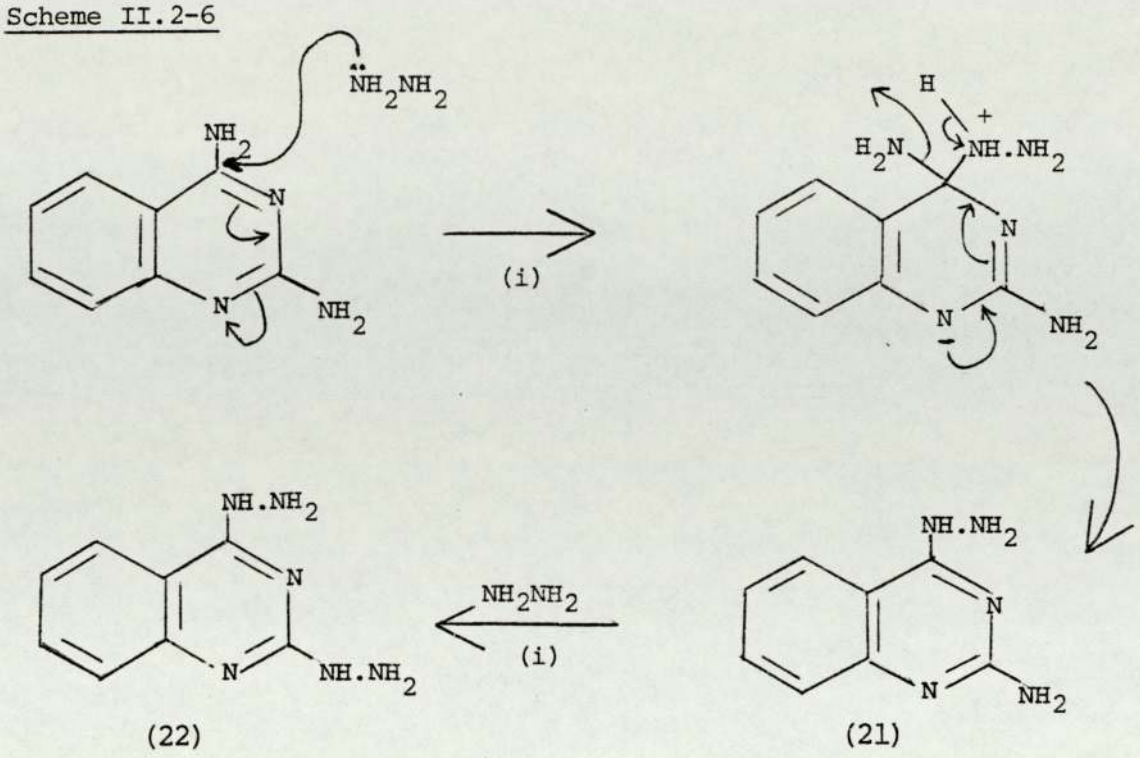


(20)

When aryl azides are refluxed in hydrazine hydrate for short periods they undergo reductive deazidation[57] . However, refluxing 2,4-diamino-6-azidoquinazoline in hydrazine hydrate for 6h not only removed the azide group but yields 2,4-dihydrazinoquinazoline. This secondary substitution reaction was confirmed by treating 2,4-diamino-quinazoline in a control experiment. When the reaction was followed by t.l.c. it was found that the diamine disappeared after 1 hour, giving rise initially to a second spot, later identified as the 2-amino-4-hydrazino compound (21), and then to a third spot, the 2,4-dihydrazino compound (22), which grew at the expense of the second (scheme II.2-6). Since it has been demonstrated that the 4-position in 2,4-dichloroquinazoline is the more susceptible to nucleophilic substitution by hydroxide[58] and alkoxide[59] ions and by ammonia[60] , it is inferred that monohydrazination took place on the 4-position. The identities of the mono- and di-hydrazino derivatives were confirmed by microanalyses and accurate mass measurements. In addition, the reported [61] m.p. of 2-hydrazino-4-aminoquinazoline (23) is 232°; this is clearly distinct from that of the 4-hydrazino isomer (21 ; m.p. 206-208°). The 2,4-dihydrazino compound (22) was found to melt at 226-228° (lit.[61] m.p. 226-227°).

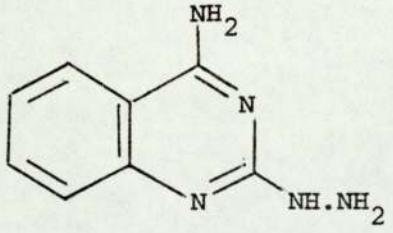
2,4-dihydrazinoquinazoline (22) was screened against DHFR to investigate the possibility that the more strongly basic hydrazino groups might afford a more tightly binding residue than the corresponding 2,4-diamino moiety.

Scheme II.2-6



(22) (21)

(i) Reflux in hydrazine hydrate.



(23)

(b) The photolysis and thermolysis of 2,4-diamino-6-azidoquinazoline (4)

Since it is known[62] that aryl azides thermolyse and photolyse to form transient nitrene intermediates, these reactions of (4) were studied primarily to confirm the chemical feasibility of the use of this compound as a RAR, and although a knowledge of the lysis mechanisms is not relevant in the RAR context *in vivo*, the isolation of lysis products was of general chemical interest. As the azide

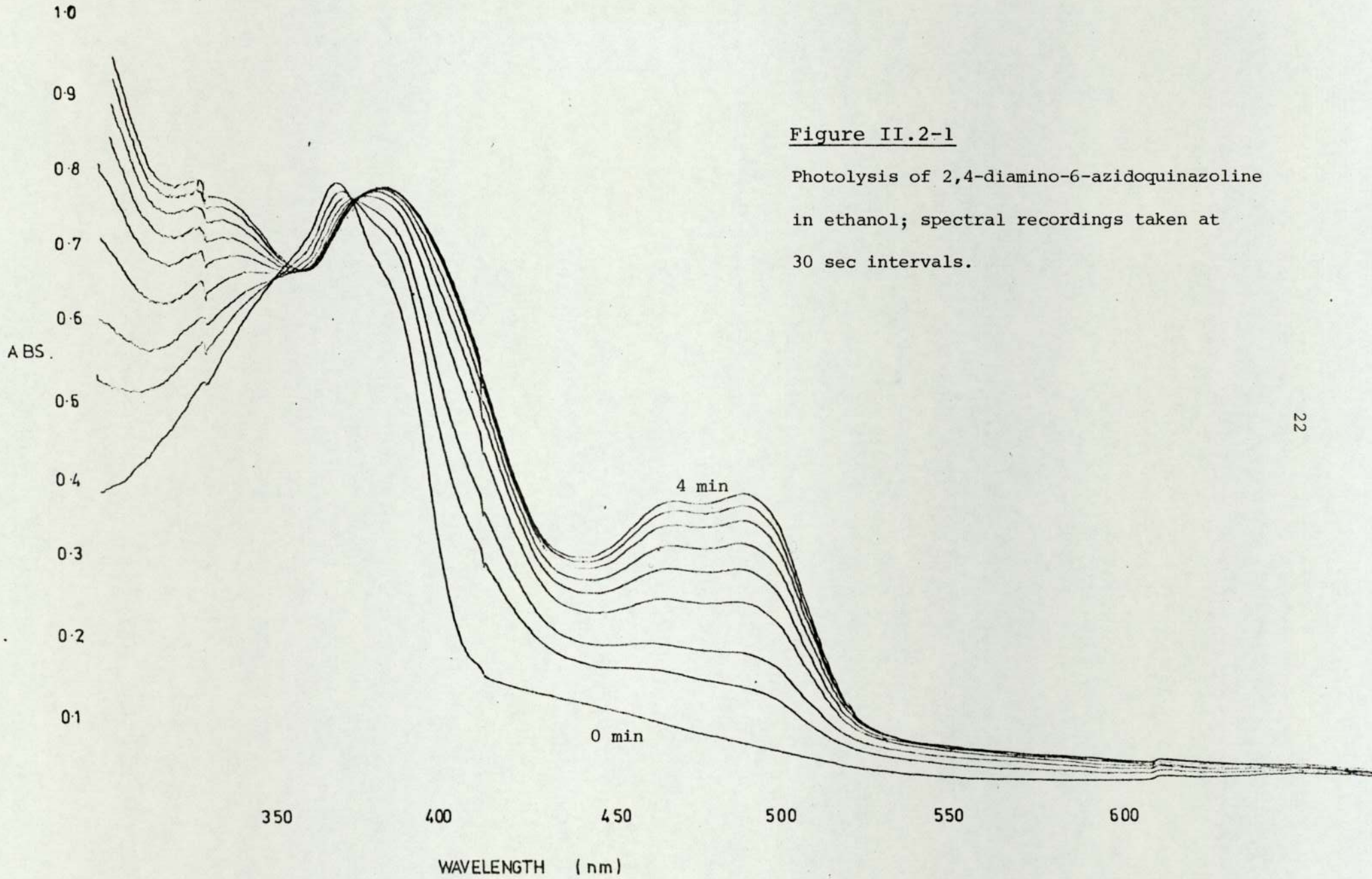


Figure II.2-1

Photolysis of 2,4-diamino-6-azidoquinazoline in ethanol; spectral recordings taken at 30 sec intervals.

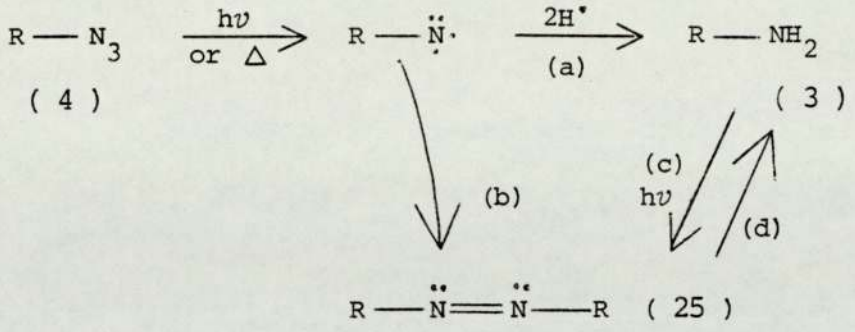
base was photolysed with both visible and ultraviolet irradiation in methanol the growth of the azo chromophore at 480 nm was spectroscopically monitored (figure II.2-1). Preparative photolysis yielded the 6,6'-azo compound (25) as the main photoproduct whereas photolysis of the azide hydrochloride in water or methanol yielded the amine, 2,4,6-triaminoquinazoline (t.l.c.).

Thermolysis of the azide base in dekalin yielded the amine (3), proton abstraction from the solvent by a triplet nitrene being favoured, while thermolysis of the same base in 2-nitrobenzene (an aprotic solvent) yielded the azo compound only. The structure of the azo compound was confirmed spectroscopically, and by its reduction to 2,4,6-triaminoquinazoline (3) with sodium dithionite. In the mass spectrum the amine is formed ($^{m}/_{e}$ 175) as a major breakdown product directly from the parent molecular ion ($^{m}/_{e}$ 346). The azide was recovered unchanged when refluxed with ethanol in the dark.

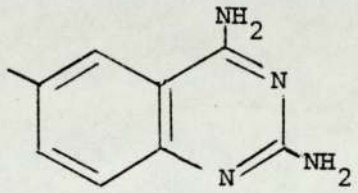
Preparative photolysis ($\lambda > 310$ nm) of the amine (3) was found to yield the same azo compound as that produced from the azide, possibly via the oxidative dimerisation of the amine to the hydrazo and hence azo compounds, or via the oxidation of the amine to the hydroxylamine and nitroso compounds, and hence the azo compound via the azoxy adduct. This amine photolysis was very slow when compared with that of the azide.

Thus, as in similar cases cited in the literature (e.g. Ref 13) these photolytic and thermolytic reactions indicate the generation of the nitrene which has been demonstrated to react in (at least) two ways, summarised in scheme II.2-7.

Scheme II.2-7

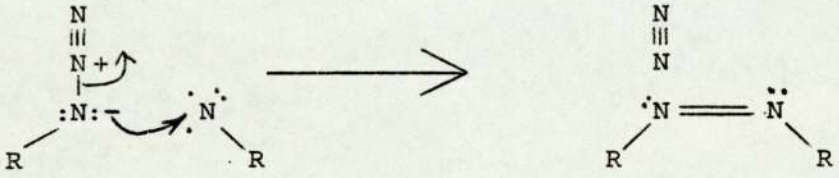


R =



(a) Hydrogen abstraction from solvent via triplet nitrene.

(b) Hydrogen radical abstraction not possible in nitrobenzene: see text.



(c) Probably via two oxidation intermediates: see text.

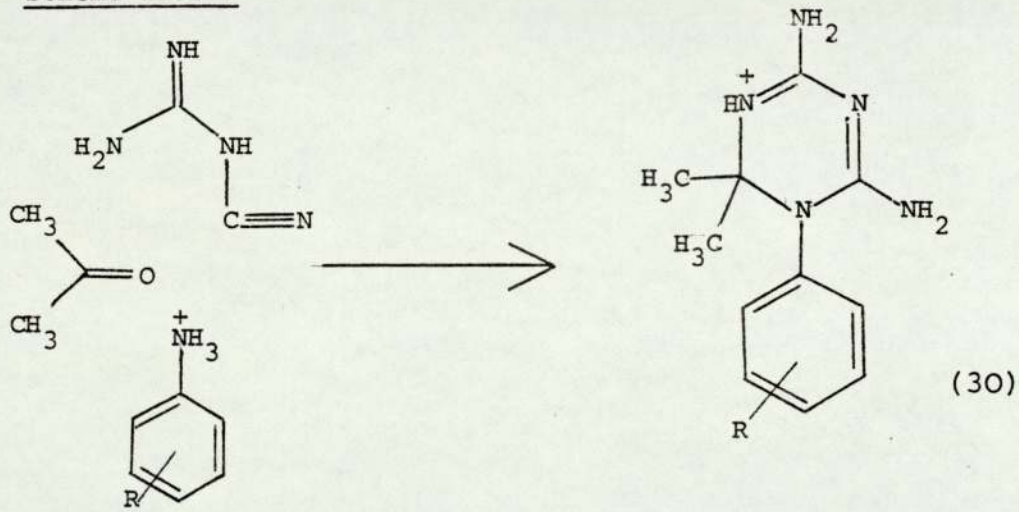
(d) Dithionite reduction.

PART II CHAPTER 3

Synthesis of the dihydrotriazines

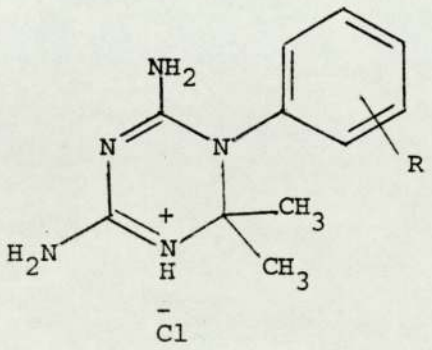
The three component synthesis of Modest [63] used as the key synthetic step involves the condensation of molar equivalents of an arylamine acid salt, dicyandiamide and acetone, with the loss of one molecule of water (scheme II.3-1). Usually the reagents are refluxed for a number of hours and the product crystallises out directly. The dihydrotriazine salts are stable and moderately soluble in water. For a successful synthesis the amino group of the arylamine must be unsubstituted.

Scheme II.3-1



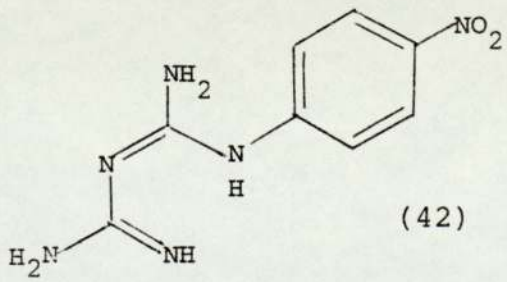
(30)

TABLE II.3-1 Structures of the dihydrotriazines



R	Compound Number
H	31
3-NO_2	32
4-NO_2	33
2-Cl	34
4-Cl	35
3-NH_2 [a]	36
4-NH_2	37
2-N_3	38
3-N_3	39
4-N_3	40
4-NHAc	41

[a] Isolated as hydrochloride-stannicomplex.



(42)

The synthesis of the unsubstituted- (31), 3-nitro- (32), 2-chloro-, and 4-chloro-phenyl (34 and 35) derivatives was straightforward where the appropriate substituted anilines were used. However, repeated attempts under various conditions of temperature, reflux time, solvent and acid used, and relative reagent concentrations in the synthesis of the 3- and 4-aminophenyl (36, 37), 4-nitro- (33) and 4-acetylaminophenyl (41) derivatives failed to afford acceptable yields of the required compounds.

In the attempted synthesis of the 4-nitrophenyl derivative (33) the reaction was complicated by persistent contamination of the product with the intermediate 4-nitrophenylbiguanide (42). Minimal contamination was effected by continual agitation of the reagents at 0° for not less than 8h ; 8h reaction time gave the required dihydrotriazine and biguanide in respective yields of 52% and 14%, while 36h reaction time gave 61% yield of the dihydrotriazine with only traces (t.l.c.) of the biguanide. A pure sample of this biguanide was made by refluxing equimolar quantities of the aniline, dicyandiamide and concentrated hydrochloric acid in acetone for 5h. 4-Nitrophenylbiguanide was identified by its m.p.[63] ; by its ability to give a coloured copper complex which is formed characteristically by arylbiguanides with cuprammonium ion but not formed by aryldihydrotriazines; by the disappearance of the 2200 cm^{-1}, 2230 cm^{-1} (CN) peaks corresponding to dicyandiamide starting material; and by the emergence of two prominent peaks at 1350 cm^{-1} and 1550 cm^{-1} (NO_2). The i.r. spectrum of this biguanide was fundamentally different from that of the dihydrotriazine and showed neither the aliphatic CH stretching at $< 3000\ cm^{-1}$ (Me) nor the recognisable pattern common to

the dihydrotriazines synthesised in the 900-1100 cm^{-1} and 1400-1600 cm^{-1} regions.

Purification of the 4-nitrophenyldihydrotriazine (33) by fractional crystallisation and by solvent extraction were tried because Modest's method, based on differential water solubility (aqueous extraction and lyophilisation), proved impracticable. Lack of success was more probably due to the close similarity in physical properties of the two species rather than to the facile loss of the elements of acetone from this dihydrotriazine analogue, and simple recrystallisation attempts from common solvents gave several unidentified products which included the biguanide (t.l.c.), rather than the biguanide alone as Modest [63] implied.

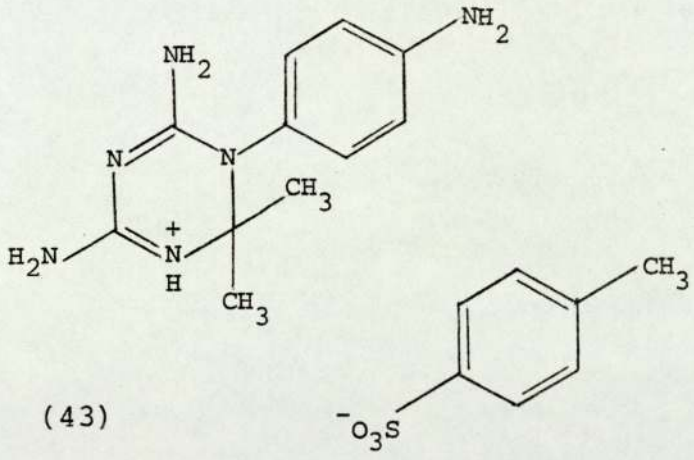
In contrast, the synthesis of the 3-nitrophenyldihydrotriazine (32) went smoothly and this was attributed to the greater nucleophilicity of the amino group.

The 3-nitrophenyldihydrotriazine was reduced smoothly with tin(II) chloride and hydrochloric acid to the amine (36), isolated as a stannicomplex rather than the free amine since this would have necessitated the use of strong alkali and this would have effected a Dimroth rearrangement characteristic of the dihydrotriazines [63-66] . This amine (stannicomplex) was diazotised and reacted with sodium azide in hydrochloric acid by Stevens [57] so that the resulting 3-azidophenyldihydrotriazine hydrochloride (39) precipitated, leaving stannic ions in solution.

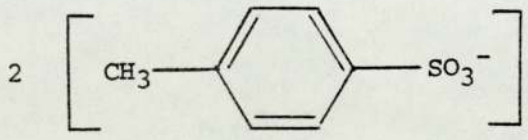
The direct synthesis of the 4-aminophenyldihydrotriazine (37) proved singularly difficult. 4-Phenylenediamine reacted neither as mono or dihydrochloride nor as free base, and the addition of HCl either as gas or in solution during or after extended reflux

invariably effected the precipitation of the unchanged diamine hydrochloride, and it is perhaps significant that this dihydrotriazine has not previously been reported. The peak at 2200 cm^{-1} (CN) of cyanoguanidine remained in solid samples isolated from attempts at the three-component synthesis with 2-, 3- or 4-phenylenediamine and with 4-acetylaminoaniline.

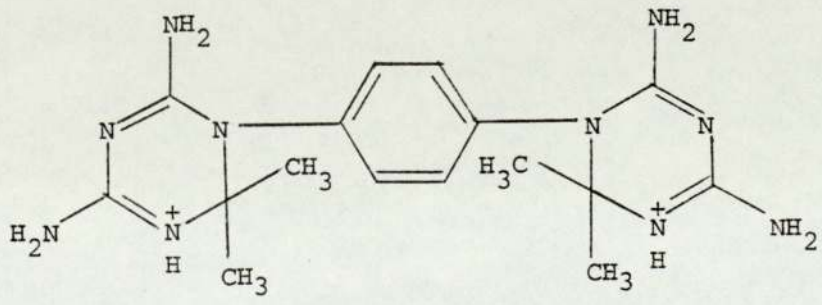
The yield of this aminophenyldihydrotriazine was unaffected by the use of DMSO or ethanol as additional solvents but was raised with water or 2-ethoxyethanol to 14% and 20% respectively, although the latter product was of poor quality. A disappointing yield of 12.4% of the amine toluene-4-sulphonate (43) resulted from the use of toluene-4-sulphonic acid which was tried as an alternative to hydrochloric and acetic acids.



(43)



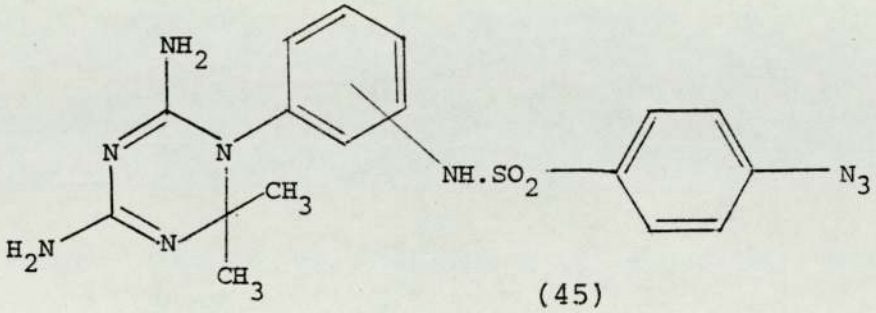
(44)



The identity of the small quantities of the 4-aminophenyl-dihydrotriazine hydrochloride and toluene-4-sulphonate (37 , 43) were confirmed primarily by n.m.r. Characteristically the 3- and 4-substituted dihydrotriazines exhibit a singlet at about 1.4 δ corresponding to the two equivalent methyl groups. In the case of the toluene-4-sulphonate salt the deuterated n.m.r. spectrum showed two singlets, at 1.4 δ and 2.3 δ , integrating for 6 and 3 protons respectively, thus indicating that the two organic species were equimolar, and there were two superimposed AA'BB' patterns, integrating for 8 protons, at 7.4 δ .

Although t.l.c. revealed only one spot under three different solvent systems the m.s. revealed peaks above the molecular ion at $^{m}/_{e}$ 232. Were a dimer (44) to be present the n.m.r. would have given an integration ratio of 12 aliphatic (dihydrotriazine methyls): 6 aliphatic (toluene-4-sulphonate):8 aromatic (dihydrotriazine and toluene-4-sulphonate). An explanation of this inconsistency is that the dimer is present at levels too low to be detected with t.l.c. and n.m.r., but that this dimer was significantly volatile at the moment the m.s. was recorded. The m.s. may have been complicated by the presence of HCl. This compound underwent the usual Dimroth rearrangement at spectroscopic concentrations, the $\lambda_{max}^{H_2O}$ shifting from 243 nm to 264 nm. However, curiously it was not found possible to convert this arylamine into the azide (starting materials being recovered) even though the compound gave a red dye upon coupling with 2-naphthol in base (confirming the presence of arylamino group). Because basification of the stannicomplex or salts of the 3- or 4-aminophenyldihydrotriazines resulted in partial Dimroth rearrangement, the free base was not

available for either mass spectrometry or for condensation with a substituted-phenylsulphonyl chloride to reach the novel sulphonamide series (45).



(45)

Various attempts to reach the 4-aminophenyldihydrotriazine (37) by reduction of the corresponding nitrocompound (33) were abortive: the use of tin(II) chloride and hydrochloric acid was unsuccessful, 4-nitrophenylbiguanide and other products (t.l.c.) being formed, and no reduction to the desired compound took place with catalytic hydrogenation, or with zinc and hydrochloric acid, or with hydrazine hydrate and Raney nickel in ethanol.

Synthesis of the azidophenyldihydrotriazines (38 - 40)

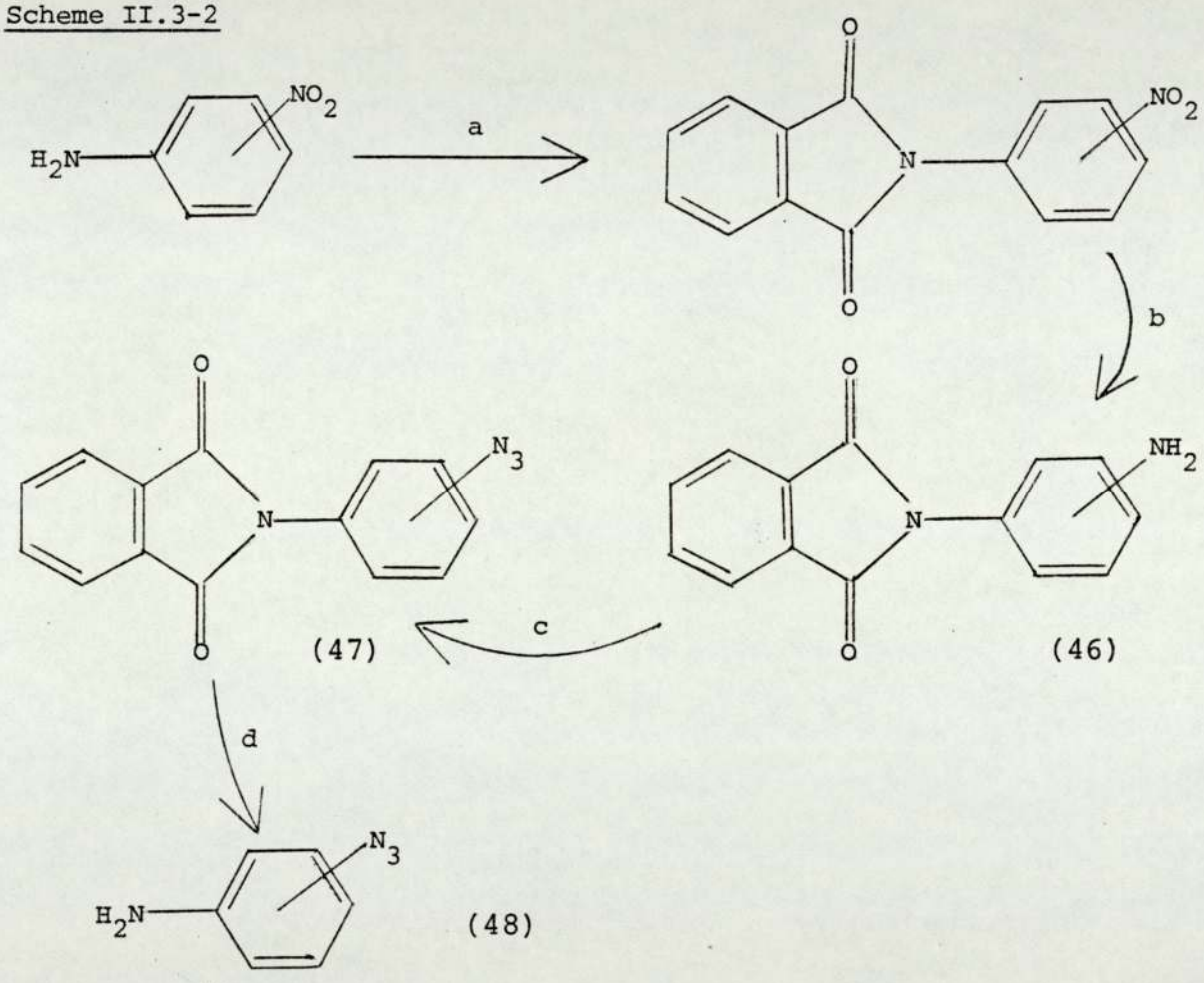
The 2-, 3- and 4-azidoanilines have previously been synthesised *via* the intermediate nitro-, amino-, and azidophenylphthalimides by Smith, Hall and Kan [67] (scheme II.3-2), although in the course of this work an essentially similar method was independently developed.

Diazotisation of the N-(4-aminophenyl)-phthalimide, being impossible in hydrochloric acid owing to the insolubility of the amine hydrochloride, was effected in acetic acid with prior heating to ensure total solution. In addition to external cooling with constant vigorous agitation, ice was added to the solution in order to allow the temperature to fall below 5° without the solution freezing. A 94%

yield of the required *para* azide (47) was obtained by this procedure. This method is simpler, quicker and more efficient than that reported by Smith *et al* [67].

Diazotisation of the 2- and 3-amino compounds (46) was best achieved in hydrochloric acid, and yields were obtained comparable to those of Smith *et al* (96%): diazotisation of the 2-amino compound in acetic acid gave only 47% yield.

Scheme II.3-2



a : Phthalic anhydride; fusion

b : Iron and acetic acid reduction

c : Diazotisation $< 5^{o}$; reaction with NaN_3

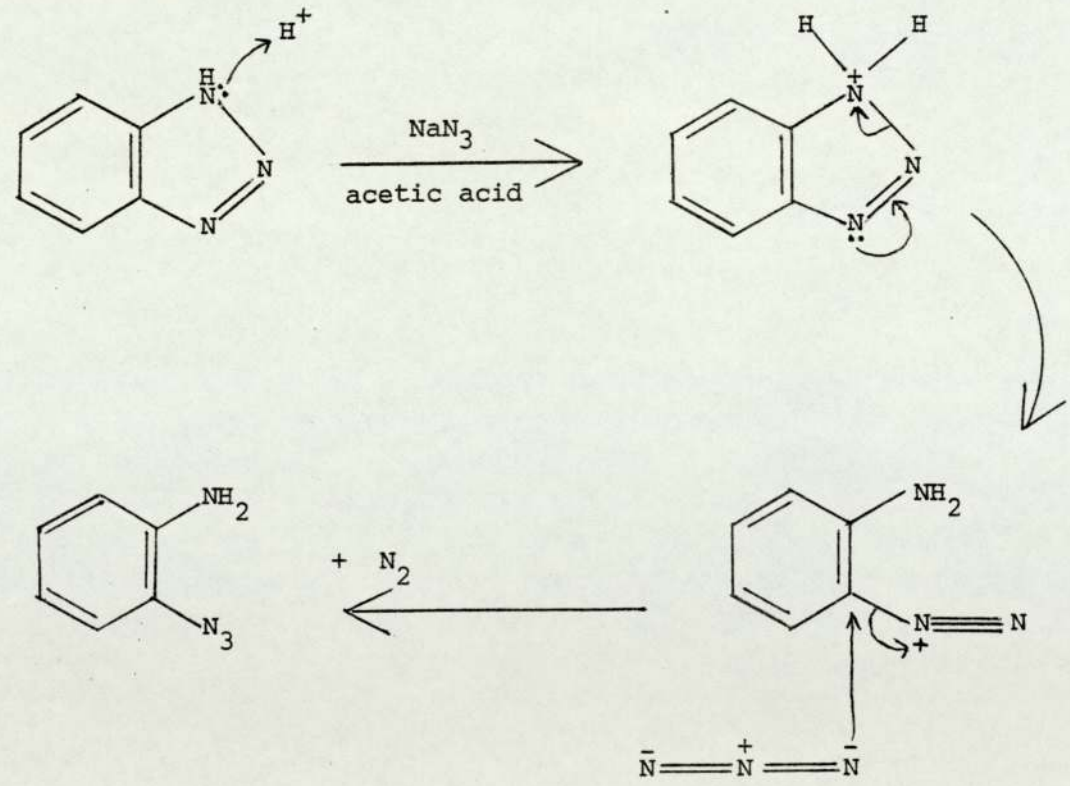
d : Hydrazinolysis; hydrazine hydrate

Hydrazinolysis at room temperature of the azidophenylphthalimides thus obtained afforded the required azidoanilines (48) for eventual successful use in the three component synthesis of the corresponding dihydrotriazines (38-40).

The use of mild temperature for the hydrazinolysis is essential since the azidoanilines melt and decompose above 63° (3-azidoaniline is an oil at room temperature).

A very low yield of 2-azidoaniline was obtained by reacting benzotriazole with sodium azide (3 mol. equiv.) in acetic acid at 50-55° (scheme II.3-3). However, the product was contaminated with a red decomposition product.

Scheme II.3-3

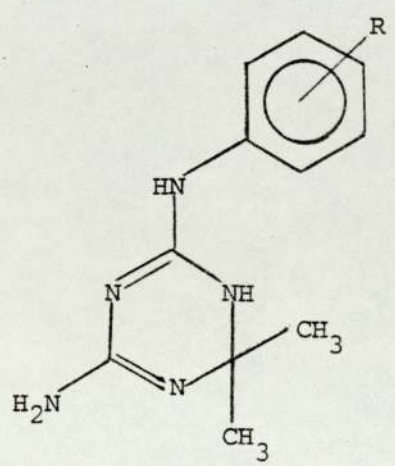


Properties of the dihydrotriazines

The spectroscopic characteristics of samples of 3-azidophenyl-dihydrotriazine (39) prepared by two different routes were identical. The i.r. spectra of all three azido derivatives showed the sharp band at 2150 cm^{-1} characteristic of the azide group.

The dihydrotriazines synthesised all show strikingly similar patterns in the "fingerprint" region of i.r. (especially in the regions 900-1100 cm^{-1} and 1400-1600 cm^{-1}) and all exhibit a CH stretching band at < 3000 cm^{-1} due to the two methyl groups. The u.v. spectra are also characteristic in that the $\lambda_{max}^{H_2O}$ varies little about 240 nm, and this maximum is characteristically irreversibly shifted, on (usually) boiling with base for 10 min, to about 260 nm (Lit. 255[63]-262[65] nm), being indicative of the Dimroth rearrangement that invariably takes place.

This irreversible rearrangement characteristic of the aryldihydrotriazines in alkali that yields arylaminodihydrotriazines has been described by Carrington[68] and Modest[63], and the products were found by these authors to be devoid of antibacterial activity. In this regard the rearrangement products of the 4-chlorophenyl- and 3-nitrophenyldihydrotriazines (49, 50) were synthesised for DHFR screening to check that any lack of DHFR inhibition was reflected in a lack of *in vivo* antitumour activity.



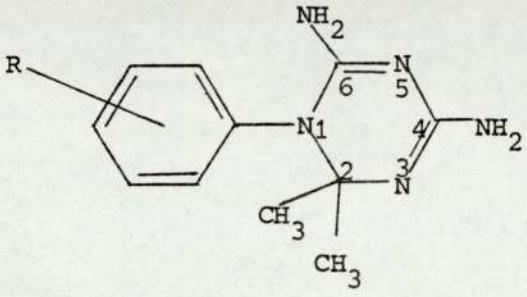
R = 4-Cl (49)
3-NO_2 (50)

^{1}H n.m.r. spectra of the dihydrotriazines

In the n.m.r. the geminal 2,2-dimethyl groups of the 2-azidophenyl- and the 2-chlorophenyl-dihydrotriazines (38, 34) show as two sharp singlets near 1.9 δ , whereas these methyl groups of the 3- and 4-substituted analogues show as singlets (Table II.3-2). In the case

---

TABLE II.3-2 : Chemical shifts of the 2-methyl protons in the dihydrotriazines[d]



Compound Number	R	Solvent	2-Methyl Chemical Shift(δ)	Lit[e] Shift Value
31	H	DMSO-d_6	1.37	1.78[c]
34	2-Cl	TFA	1.70, 1.99	1.70, 1.98
38	2-N_3	TFA	1.78, 1.90	
	3-Cl	TFA		1.85
39	3-N_3	TFA	1.91	
36	3-NH_2	DMSO-d_6	1.40	
32	3-NO_2	DMSO-d_6	1.42	
43	4-NH_2[a]	DMSO-d_6	1.40	
37	4-NH_2[b]	TFA	1.84	
40	4-N_3	TFA	1.77	
33	4-NO_2	TFA	1.80	
35	4-Cl	TFA	1.80	1.80

[a] as toluene-4-sulphonate

[b] as dihydrochloride

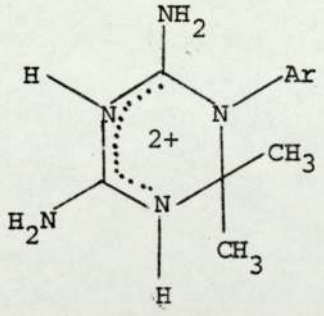
[c] solvent was TFA

[d] Spectra were determined at 60 MHz.

[e] Ref.69

of the 4-substituted derivatives the chemical shifts are essentially independent of the substituent (δ 1.79-1.80 in TFA)[69] whereas in the 3-substituted compounds, the position of the 2-methyl singlet is more substituent-dependent although the effect is small. However, the *ortho*-substituted molecules are constrained to adopt more rigid conformation, and the rotational barrier that results from steric interaction between the *ortho*-substituent on the phenyl group and both the geminal methyl groups and the 6-amino group gives rise to nonequivalence of the geminal methyls, thus giving two singlets. This doublet is produced only if the rotation about the C-N bond is slow on the n.m.r. time scale and if the chemical shift difference between the methyl groups is sufficient[69] .

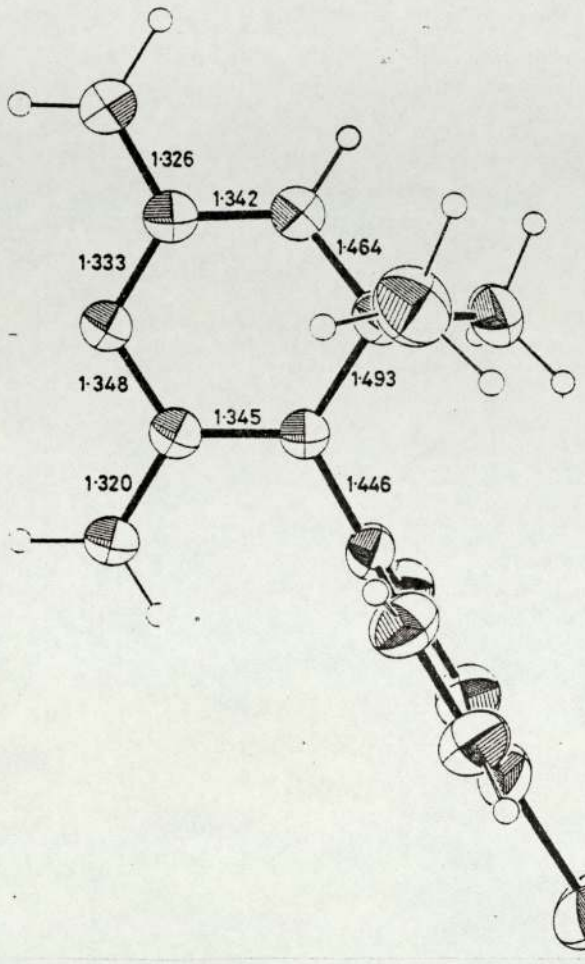
From the table II.3-2 it can be seen that the methyl groups are relatively shielded when the n.m.r. spectra are run in DMSO-d_6. The dihydrotriazines are probably diprotonated in TFA solution, forming resonance-stabilised amidinium ions (structure 51).



(51)

The crystal structure of the 4-chlorophenyldihydrotriazine hydrochloride was solved by Schwalbe and Hunt both by X-ray[70] and by neutron diffraction[71] techniques. The crystal structure (fig II.3-1) shows that the least squares plane of the (approximately planar)

Figure II.3-1 : ORTEP drawing of cycloguanil hydrochloride (35)
(Some bond distances (Å) involving the heterocycle are shown).
Reproduced, with permission, from Ref.70 .



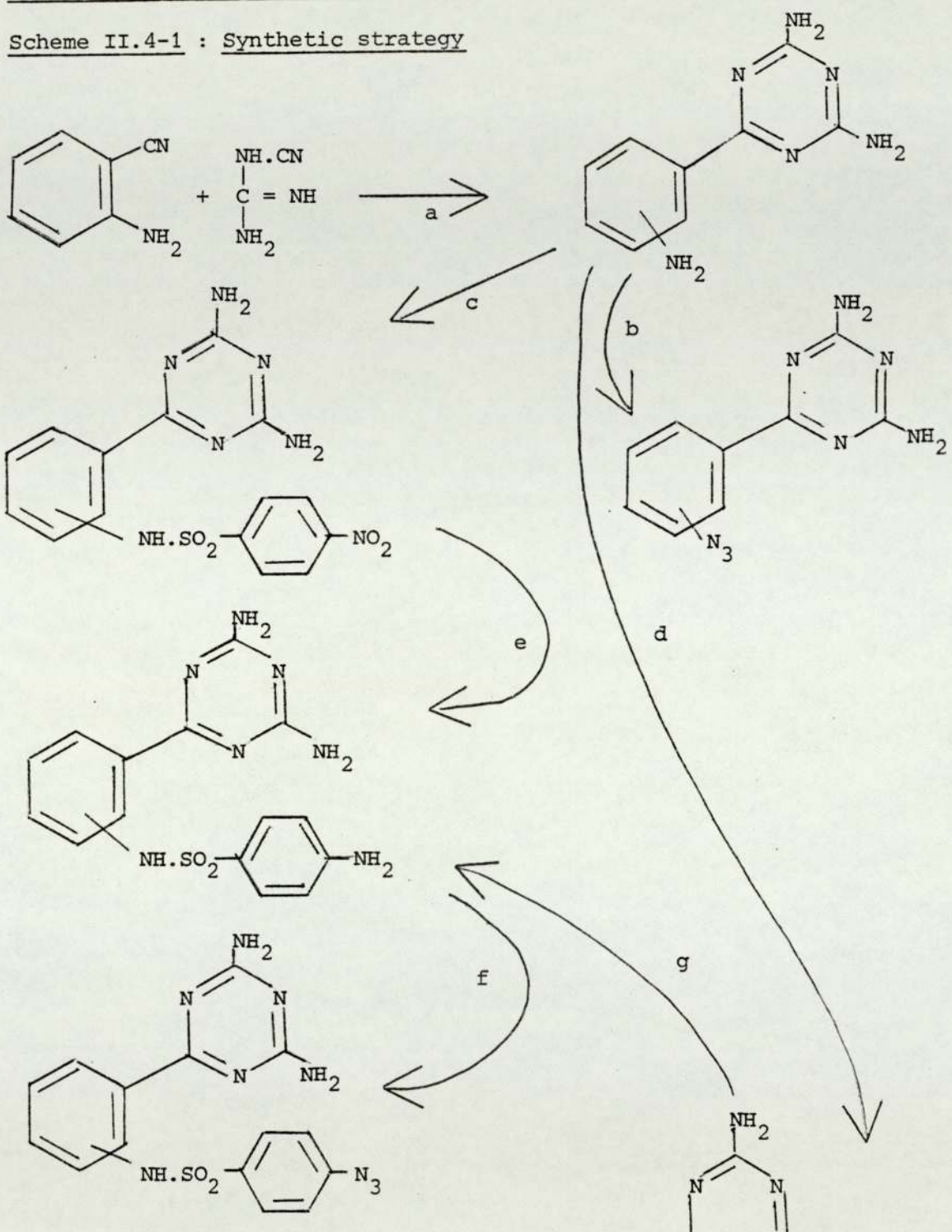
dihydrotriazine ring intersects the phenyl plane at an angle of 80°. This implies that even without any *ortho*-substituent there exists a degree of steric interaction between the two rings and the conformation of lowest energy is the one depicted in the drawing.

In this context it is interesting to note that Roth *et al* reported that *ortho*-substituted phenyldihydrotriazines have relatively high anthelminthic activity against intestinal parasites and negligible microbiological activity [65], and yet in antitumour [51], antibacterial [72] and *in vitro* DHFRI screens [15, 73-75] the *meta*- and *para*-substituted compounds have been found to be more active.

PART II CHAPTER 4

Synthesis of the aromatic-*s*-triazines

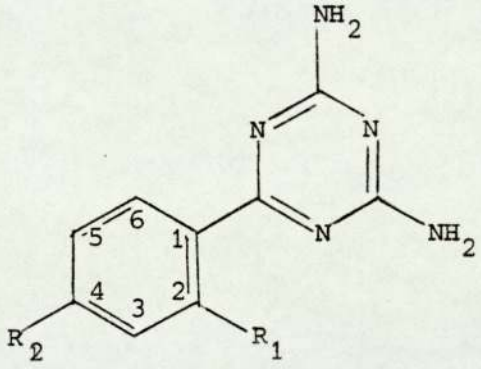
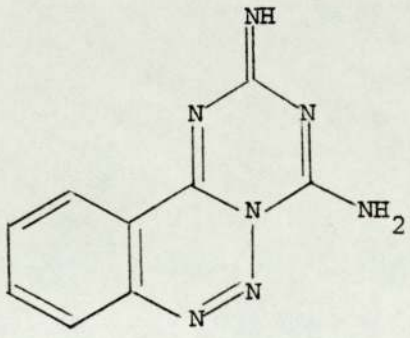
Scheme II.4-1 : Synthetic strategy



a KOH/2-Ethoxyethanol/Reflux
b $HNO_2/NaN_3/<5^o$
c $O_2N.C_6H_4.SO_2Cl$/Pyridine/Reflux
d $Ac.NH.C_6H_4.SO_2Cl$/Pyridine/Reflux
e Hydrazine hydrate/Raney nickel/Ethanol
f $HNO_2/NaN_3/<5^o$
g 2N-HCl

The well documented alcoholic-base catalysed reaction of cyanoguanidine with an arylnitrile yields the corresponding 2,4-diamino-6-[substituted phenyl]-*s*-triazine [76, 77] (scheme II.4-1(a); scheme II.2-2). The 2'- and 4'-aminophenyl derivatives (60 and 61) served as intermediates to the corresponding 2'- and 4'-azidophenyl derivatives (62 and 63) being candidate RAR, and to the novel 2'- and 4'-nitro-, amino- and azido-benzenesulphonamide compounds, as indicated in the scheme II.4-1.

TABLE II.4-1

Structure	R_1	R_2	Number
	NH_2	H	60
	H	NH_2	61
	N_3	H	62
	H	N_3	63
	$NH.SO_2.C_6H_4.NO_2$-*p*	H	64
	H	$NH.SO_2.C_6H_4.NO_2$-*p*	65
	$NH.SO_2.C_6H_4.NH_2$-*p*	H	66
	H	$NH.SO_2.C_6H_4.NH_2$-*p*	67
	$NH.SO_2.C_6H_4.N_3$-*p*	H	68
	H	$NH.SO_2.C_6H_4.N_3$-*p*	69
	$NH.SO_2.C_6H_4.NHAc$-*p*	H	70
	NO_2	H	71
	H	NO_2	72
	H	H	73
			74

The analogue 4-amino-2(2H)-imino-*s*-triazino[1,2-c][1,2,3]-benzotriazine, (74) was first prepared by Mackenzie[54] as a potential DHFRI whose masked diazonium character confers on it a potential for irreversible covalent binding. This compound was obtained as a precipitate following the diazotisation of the 2'-aminophenyl compound (60) in ice-cold 2N-hydrochloric acid with a molar equivalent of sodium nitrite (aqueous solution), and subsequent basification with concentrated ammonia solution.

The 2'- and 4'-nitro compounds (71 and 72) were primarily synthesised as intermediates to the corresponding hydroxylamines, but since such partial reduction was found to be difficult this pursuit was abandoned in favour of testing the RAR hypothesis using the azido group alone.

The condensation of 4-nitrobenzenesulphonyl chloride and 2,4-diamino-6-[4-aminophenyl]-*s*-triazine (61) in refluxing pyridine yielded, after trituration with ice-water, 2,4-diamino-6-[4-(4-nitrobenzenesulphonamido)-phenyl]-*s*-triazine (65) as indicated in scheme II.4-1(c). Similarly prepared from the corresponding 4-substituted-benzenesulphonyl chlorides were the 2-nitrobenzene-sulphonamidophenyl (64) and 2-N^4-acetylaminobenzenesulphonamidophenyl (70) analogues (scheme II.4-1,(c) and (d)). The latter analogue (70) is a potential metabolite of the azide (68).

From the two nitro compounds (64 and 65) were prepared the corresponding amines (66 and 67) by reduction with hydrazine hydrate and Raney nickel in ethanol. The reaction was monitored both by t.l.c. and by the disappearance of the absorption band at ~ 1350 cm^{-1} (NO_2).

These amines (66 and 67) were diazotised and reacted with sodium azide to yield the corresponding azides (68 and 69) being the required candidate RAR.

Acid hydrolysis of the 2-(N^4-acetylaminobenzene)-sulphonyl analogue (70) followed by diazotisation *in situ*, and subsequent reaction with sodium azide afforded a second route to the 2-(4-azidobenzene)-sulphonamide (68).

The structures of these novel sulphonamides were primarily confirmed with mass spectroscopy: this is discussed in Chapter II.6. The n.m.r. of the 4-substituted-benzenesulphonamides each showed two clearly defined superimposed AA'BB' patterns: the spectrum of the amine (67) is shown in figure II.4-1 as an example. In contrast, the n.m.r. of the 2-substituted-benzenesulphonamides showed an AA'BB' pattern superimposed on a complex *ortho*-disubstituted multiplet. The n.m.r. spectrum of the 2-(N^4-acetylaminobenzene)-sulphonamide (70) showed a singlet at 2.05 δ (CH_3), and the integration confirmed the aliphatic ^1H : aromatic ^1H ratio of 3 : 7.

In the i.r. spectra, the azido compounds 68 and 69 showed a sharp absorption band at $\sim$2157 cm^{-1} (N_3), and the 2-(N^4-acetylamino-benzene)-sulphonamide (70) exhibited a strong broad band at 1660 cm^{-1}.

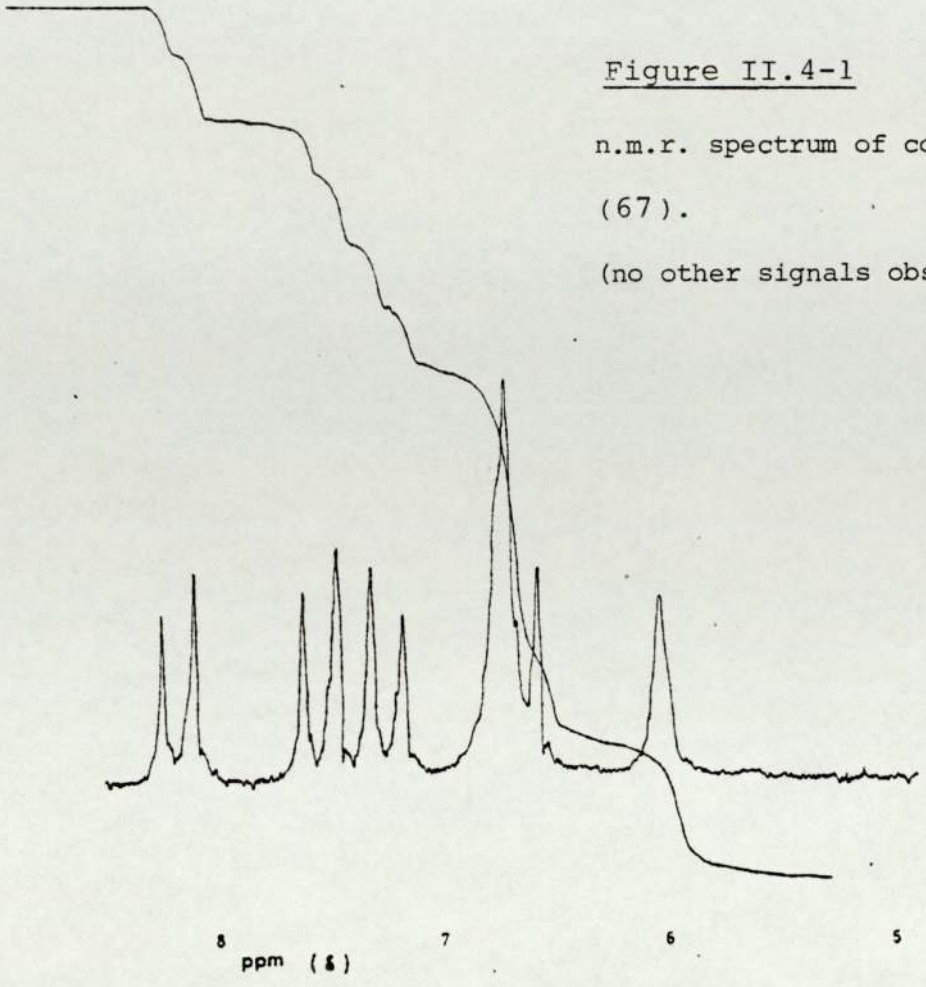
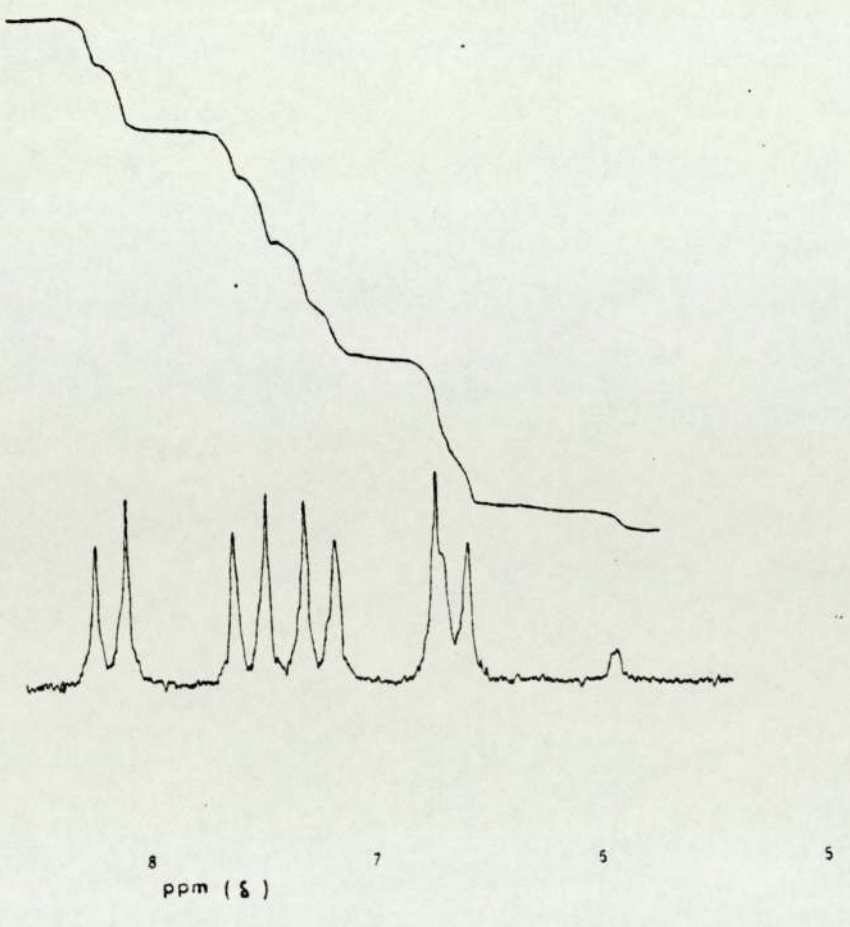


Figure II.4-1

n.m.r. spectrum of compound (67).

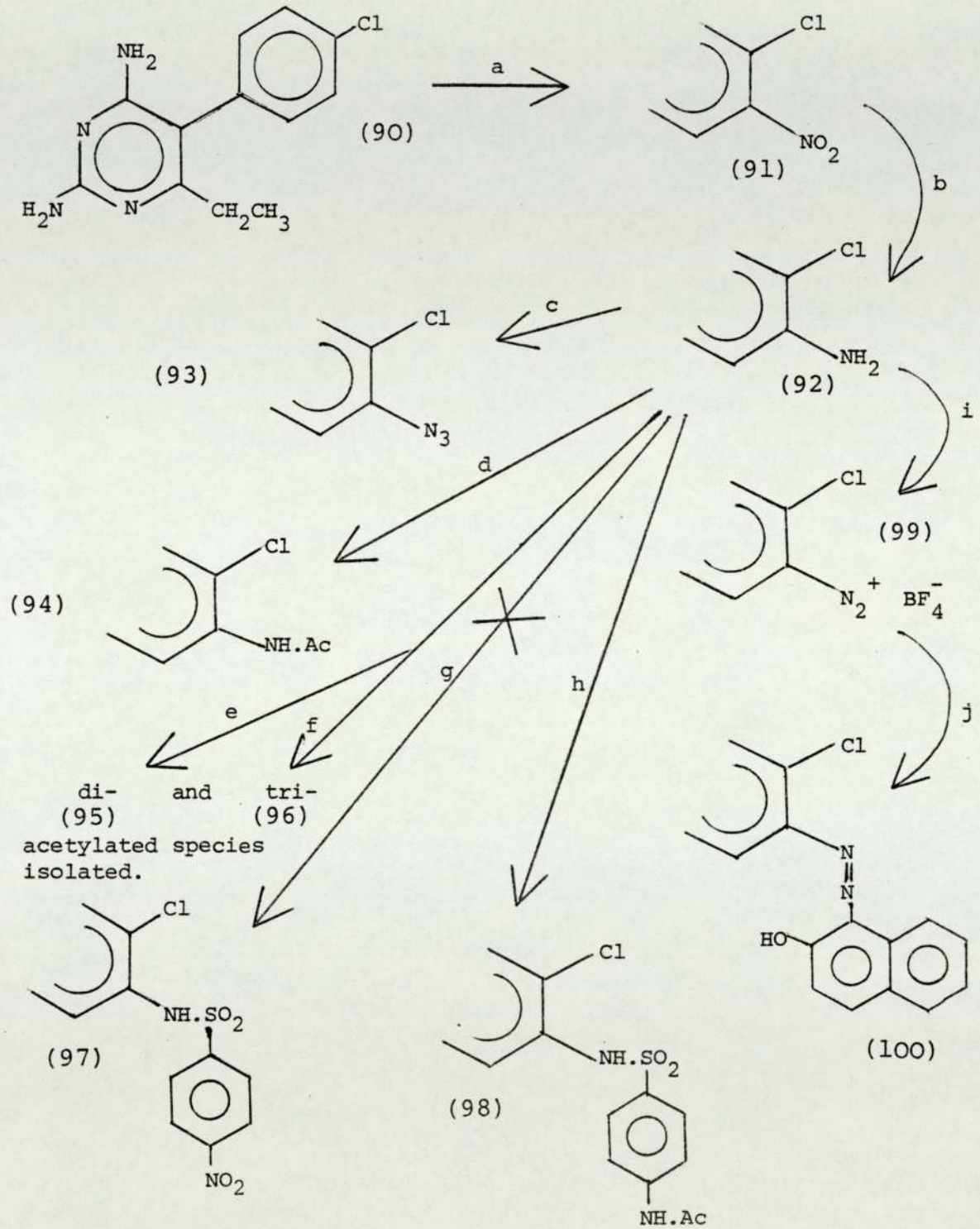
(no other signals observed)



PART II CHAPTER 5

Syntheses in the pyrimethamine series

Scheme II.5-1 : Synthetic strategy



a HNO_3/H_2SO_4 at 50°
b $SnCl_2/HCl$ or Hydrazine hydrate/Ni
c $HNO_2/NaN_3/<5^{\circ}$
d CH_3COCl/Pyridine/Reflux
e $(CH_3CO)_2O/CH_3COOH$/Reflux
f $(CH_3CO)_2O$/Reflux
g $NO_2.C_6H_4.SO_2Cl$-*p*
h $Ac.NH.C_6H_4.SO_2Cl$-*p*
i $NaNO_2/HBF_4/<5^{\circ}$
j 2-Naphthol/ N-KOH

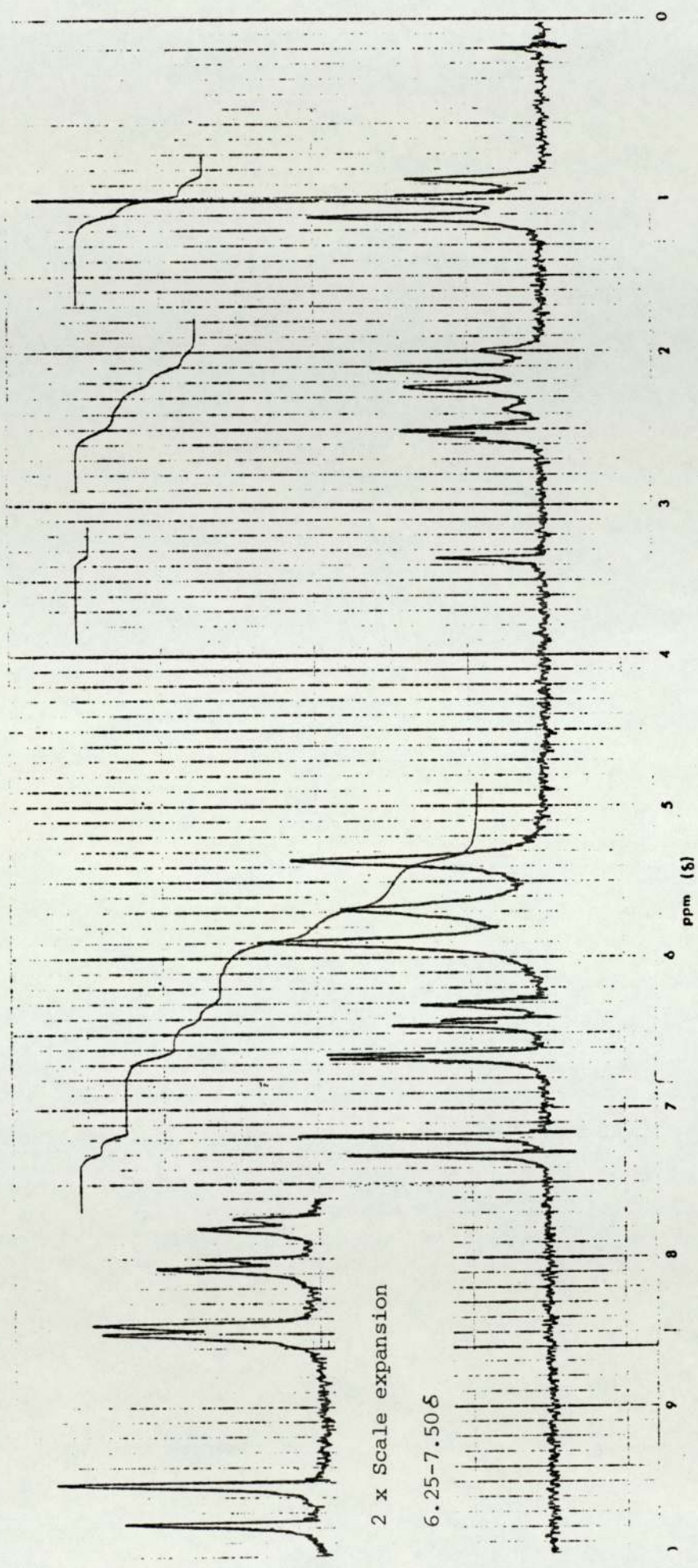


Figure II.5-1 N.m.r. spectrum of compound (92)

Pyrimethamine (90) nitrated in the 3'-position when treated with a mixture of concentrated nitric and sulphuric acids at $< 15^{\circ}$ for 1h followed by 50° for a further 2h. The activity of the 3'-position was attributed to both the *ortho* activating effect (+M) of the chloro and the *ortho* deactivating, *meta* directing effect (-M) of the diaminopyrimidino moieties.

The corresponding amine (92), produced by the reduction of the nitro compound with tin(II) chloride and hydrochloric acid or with hydrazine hydrate and Raney nickel, served as a starting material in all subsequent synthetic reactions (scheme II.5-1). This compound was crystallised both as the anhydrous base (prisms) from 100% ethanol and as the monohydrate (needles) from 50% ethanol. However, the differences that occurred in the i.r. spectrum as a result of solvation were not as pronounced as those which resulted from the solvation of 2,4,6-triamino-quinazoline (3).

The n.m.r. spectrum of this compound (92 ; fig II.5-1) shows a triplet at 1.00δ (CH_3), a quartet at 2.18 δ (CH_2), three broad exchangeable singlets (each integrating for 2 protons) at 5.35 δ , 5.69 δ and 5.90 δ (NH_2), and an ABX splitting pattern consistent with the 1,3,4-trisubstituted benzenoid moiety centred at $\sim$6.80 δ .

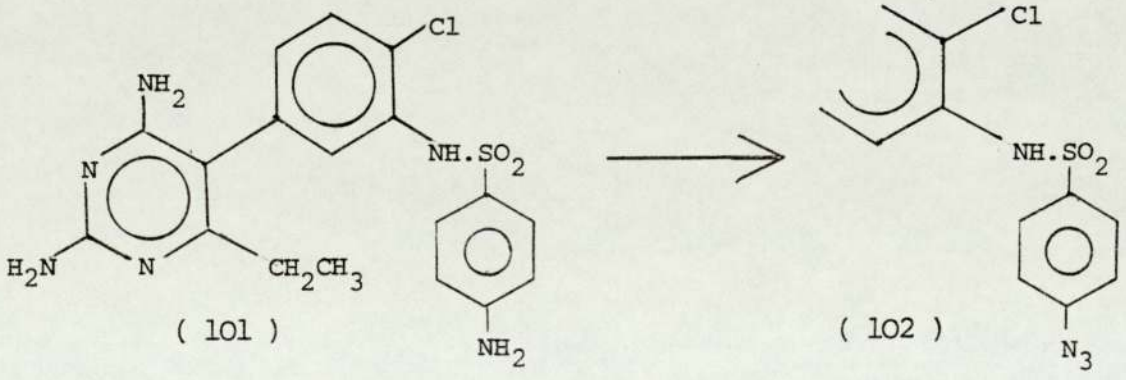
The candidate RAR, the 3'-azido analogue of pyrimethamine (93) was synthesised from the amine (92) by the usual route being first isolated as the base. The acetate and hydrochloride salts of this azide (93) were prepared from the base with acetic and hydrochloric acids respectively. These azido compounds each showed the characteristic sharp absorption band at $\sim 2170\ cm^{-1}$ in the i.r. spectrum.

The stable diazonium tetrafluoroborate (99), being a potential irreversible DHFR inhibitor *per se* by virtue of the covalent labelling capacity of the diazonium function, was coupled with 2-naphthol to yield the azo dye (100) in good yield. The diazonium salt exhibited a sharp band at 2300 cm^{-1} (N_2^+) and a very strong broad band centred at 1080 cm^{-1} (BF_4^-), and was so stable that it was recovered unchanged when crystallised from ethanol.

The three amino groups of the 3'-amino derivative (92) were acetylated to yield the mono-, di- and tri-acetylamino analogues (94, 95 and 96). Each acetylation step is characterised in the n.m.r. spectrum by the loss of one of the initial three exchangeable singlets at 5.35-5.90 δ (NH_2), and the appearance of a mobile singlet integrating for one proton at 9.18-10.45 δ (acetamido). The acetylamino group is more deshielding than amino, and the downfield shift of the phenyl protons consequent on monoacetylation is not further increased by di- or tri-acetylation. Therefore monoacetylation takes place on the 3'-amino group of the phenyl ring, and di- and tri-acetylation must involve the pyrimidine amino groups.

Attempted synthesis of sulphonamide derivatives in the pyrimethamine series

Scheme II.5-1(g and h) indicates the two synthetic routes taken in an attempt to obtain the 4-aminobenzenesulphonamide (101) being the required intermediate to the 4-azidobenzenesulphonamide (102), a candidate DHFR targeted RAR.



(101) (102)

From the condensation attempts of 4-nitrobenzenesulphonyl chloride and the 3'-amino analogue of pyrimethamine (92) in pyridine, or acetonitrile with triethylamine, or by fusion, only starting materials were obtained. When 4-acetylaminobenzenesulphonyl chloride and the amine (92) were refluxed for 8h in dry pyridine, t.l.c. examination of the reaction mixture indicated that the amber gum which was isolated was a mixture of the amine starting material and a blue fluorescent product. Because the use of additional triethylamine did not improve the yield, the development of this synthesis was abandoned. The inability of the 3'-amino group to react with benzenesulphonyl chloride can presumably be attributed to the steric hindrance of the 4'-chloro group.

PART II

CHAPTER 6

Mass spectroscopy of sulphonamide derivatives

The mass spectra of ten novel sulphonamides of the type 127 including three azides have been determined and listed in table II.6-1 together with those of N^4-acetylsulphadimidine (112), and the azido derivatives of sulphadimidine, sulphamerazine, sulphadiazine and sulphanilamide (111, 114, 117 and 120 respectively).

Table II.6-1 Mass spectra of sulphonamide derivatives

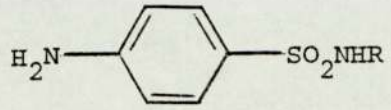
Compound Number												
10	m/e	360	330	186	175	174	157	147	132	122	105	92	64
	I	23	3	3	45	100	20	23	31	13	23	10	7
12	m/e	372	330	308	265	198	176	175	174	173	157	156	146
	I	90	7	5	5	6	19	100	100	12	35	7	36
	m/e	140	134	133	132	131	119	115	92	91	86	79	78
	I	11	22	47	100	24	5	86	26	11	54	19	28
	m/e	65	64	43									
	I	44	14	100									
14	m/e	176	159	134	125	106	93	79	66	64	52	48	46
	I	37	5	11	12	14	81	9	35	100	10	54	60
	m/e	36	32										
	I	20	50										
64	m/e	387	357	323	293	202	201	160	159	117	92	90	69
	I	69	8	19	10	25	27	11	100	9	11	9	11
	m/e	43											
	I	37											
65	m/e	387	357	202	201	184	159	117	92	90	76	43	
	I	85	16	93	100	26	100	74	33	59	41	100	
66	m/e	357	293	202	159	156	118	108	92	73	69	65	60
	I	47	63	100	53	11	12	14	33	18	19	18	28
	m/e	43											
	I	36											
67	m/e	357	293	202	159	156	108	92	65	43			
	I	77	20	100	23	57	40	66	34	31			

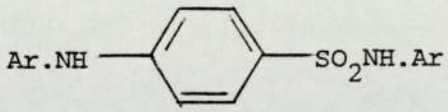
/ cont.

Table II.6-1 cont./

Compound Number													
68	m/e	383	357	293	290	202	159	118	108	92	65	64	43
	I	36	46	57	13	100	54	19	12	30	19	12	52
69	m/e	357	294	293	202	201	159	156	108	92	65	64	
	I	77	12	17	100	40	21	48	28	50	32	27	
70	m/e	399	384	335	294	293	202	201	159	118	92	69	65
	I	100	13	46	13	13	46	25	67	14	14	10	12
	m/e	43											
	I	40											
111	m/e	240	239	214	213	212	211	198	172	158	123	108	95
	I	7	24	100	100	54	42	14	14	15	42	34	27
	m/e	93	82	65	64	63	42						
	I	36	19	34	34	34	38						
112	m/e	256	255	214	213	212	198	165	148	140	134	123	108
	I	100	100	100	100	14	13	9	15	16	33	61	100
	m/e	96	92	82	65	64	63	42					
	I	56	46	64	42	35	22	39					
114	m/e	226	225	200	199	171	109	108	95	92	82	68	65
	I	7	17	64	100	79	51	28	13	57	24	16	66
	m/e	64	63										
	I	67	23										
117	m/e	212	211	186	185	184	183	157	141	126	95	80	68
	I	7	19	10	10	10	8	6	8	9	100	12	24
	m/e	64	63	42									
	I	88	11	27									
120	m/e	198	172	170	156	125	108	106	65	64	63		
	I	14	38	25	44	19	38	67	54	100	92		

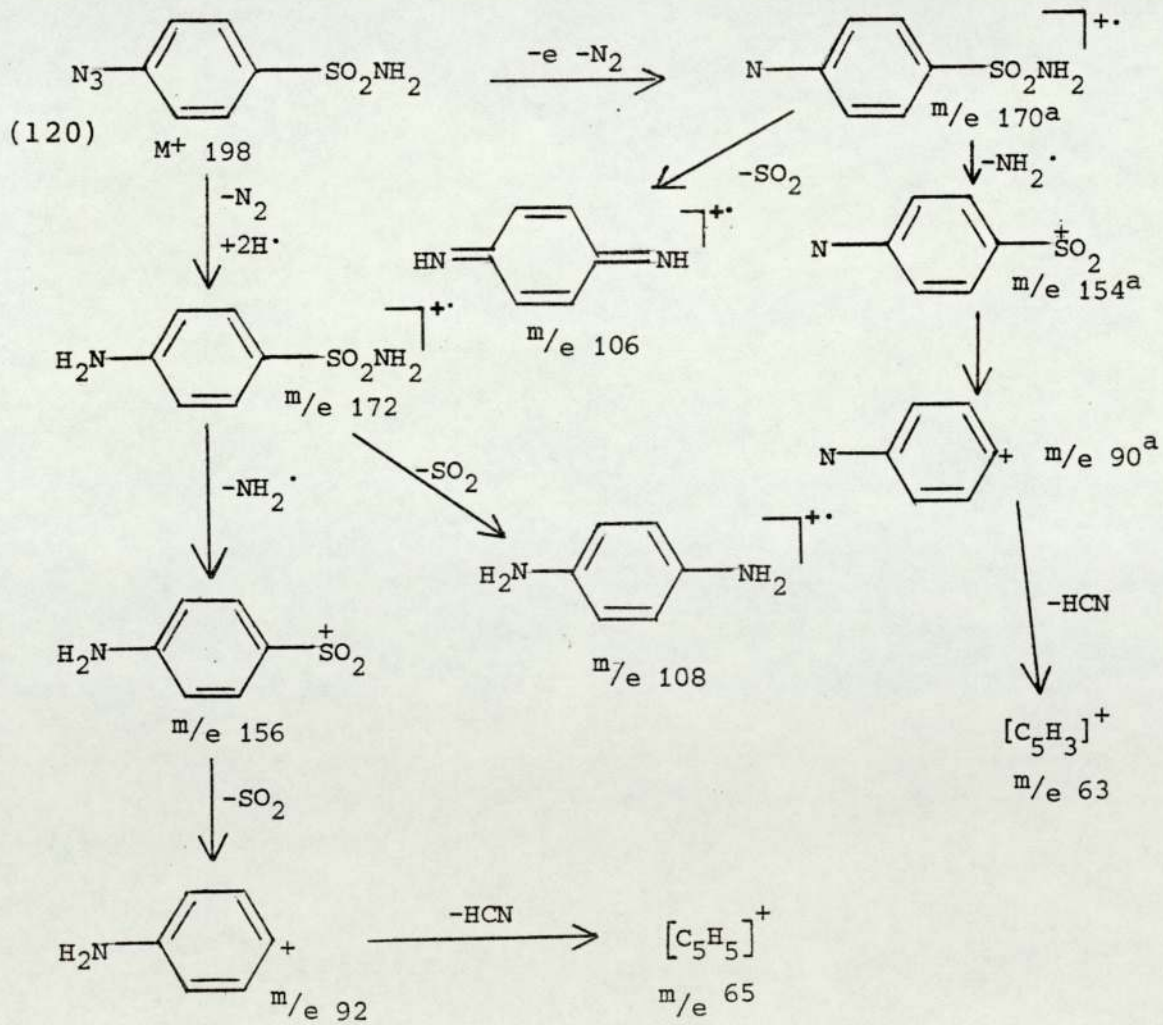
It has been documented that aryl azides do not always give rise to molecular ions and that loss of molecular nitrogen is most prevalent[78] This has been observed to be the case with all the azides herein reported.

(126)  (R = H or aryl)

(127) 

It can be seen from scheme II.6-1 that the major fragmentation pathways of azidosulphanilamide (120) are *via* skeletal rearrangements

Scheme II.6-1 Fragmentation of azidosulphanilamide



[a] This ion may exist in the corresponding ring expanded azepine form.

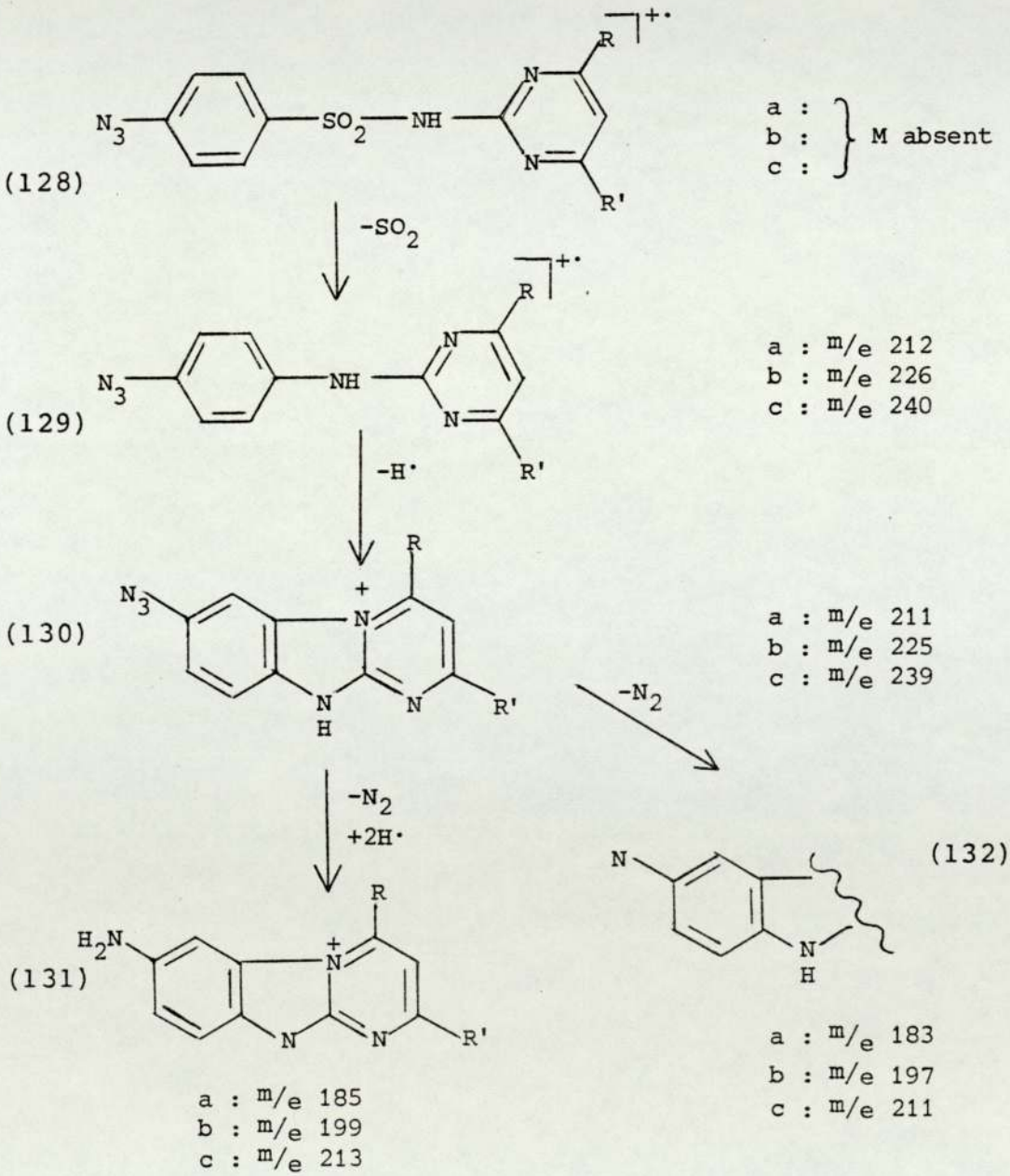
involving the loss of SO_2 which follows the initial loss of a nitrogen molecule from the azido group. However, the fragmentations of the N'-pyrimidinylbenzenesulphonamides (schemes II.6-2 and II.6-3) are

Scheme II.6-2

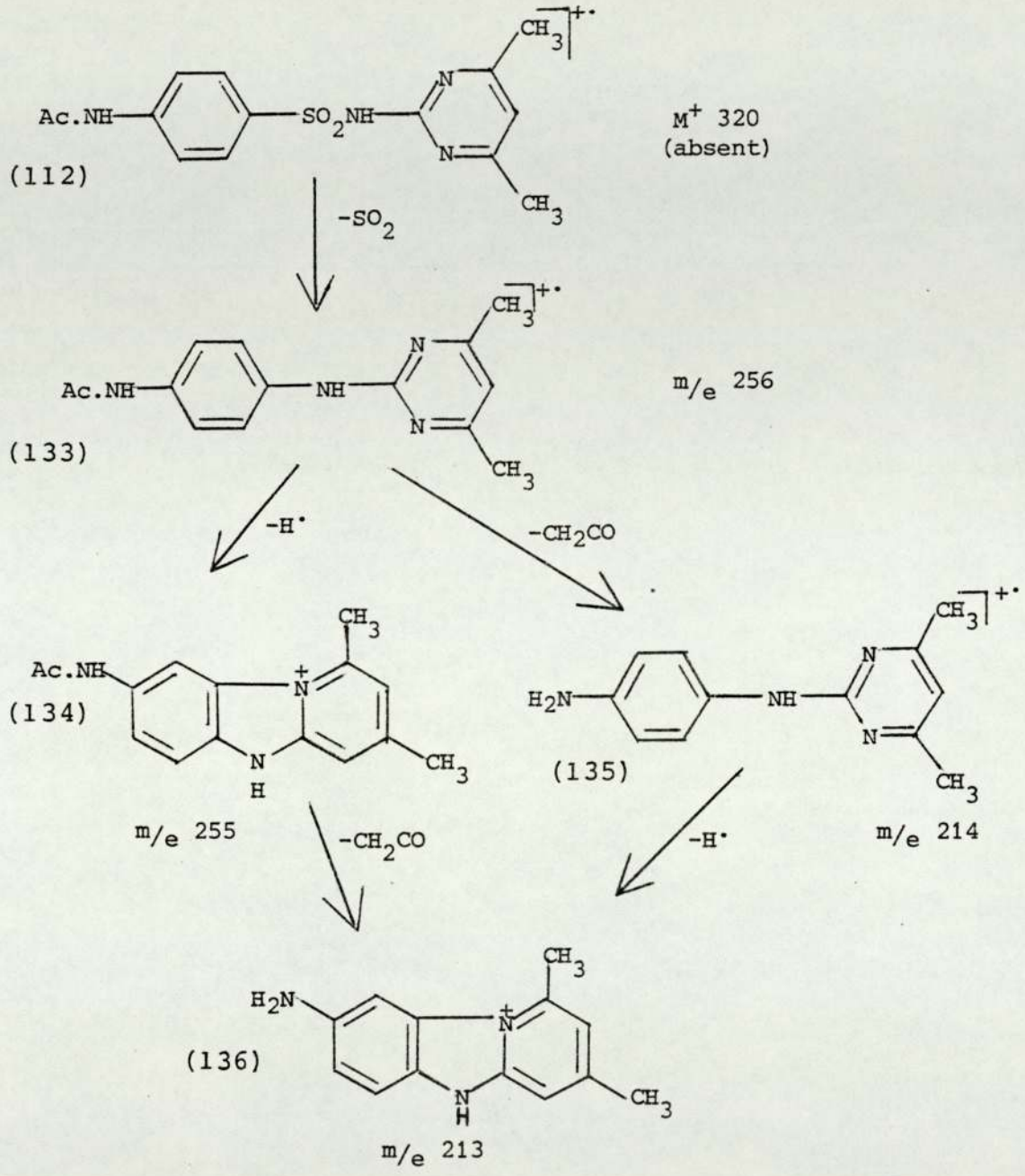
a : R = R' = H : Azidosulphadiazine, M 296

b : R = Me R' = H : Azidosulphamerazine, M 290

c : R = R' = Me : Azidosulphadimidine, M 304



Scheme II.6-3 Fragmentation of N^4-acetylsulphadimidine

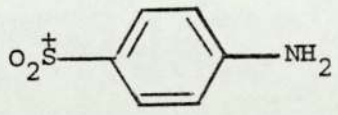


characterised by the initial loss of SO_2 with no molecular ions being observed, and the major peaks that are produced correspond to structures 130 and 131 in scheme II.6-2, and structures 133-136 in scheme II.6-3. Accurate mass measurements of the ions $^{m}/_{e}$ 211 and

$^{m}/_{e}$ 185 produced by azidosulphadiazine confirmed the composition of the structures 130 and 131 :

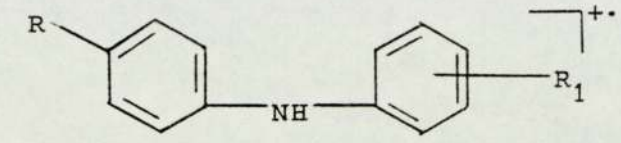
Structure 130 (a): $C_{10}H_7N_6^+$	:	Calculated	211.073800
		Observed	211.073215
		Error	2.7 p.p.m.
Structure 131 (a): $C_{10}H_9N_4^+$	:	Calculated	185.082660
		Observed	185.082717
		Error	< 1.0 p.p.m.

This accords with the results of Spiteller and Kaschnitz [79] who reported prominent M - SO_2 peaks in the mass spectra of a series of sulphonamides of the general formula 126. In contrast, Dynesen *et al* [80] have shown that secondary sulphonamides which contain two benzenoid substituents (structure 127) mainly undergo N——S cleavage although the M - SO_2 process is not a general one. Because the nitrogen containing fragment usually retains the charge this process is useful for analytical purposes[80] and has been observed with all the sulphonamides of the general structure 127 synthesised in this work. However, it is possible that the large peaks at $^{m}/_{e}$ 156 present in the spectra of compounds 66, 67, 69 and 120 are due to the relatively stable 4-aminobenzenesulphonium cation (128) also

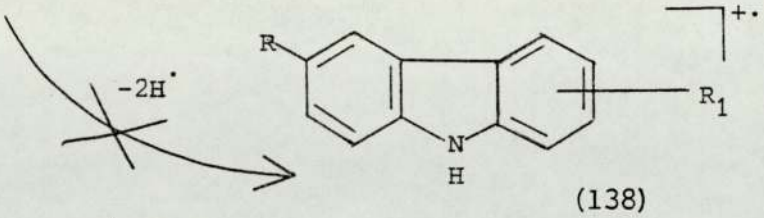
(128)  $^{m}/_{e}$ 156

formed. The composition of this ion in the m.s. of compounds 66 and 67 was confirmed by accurate mass measurements:

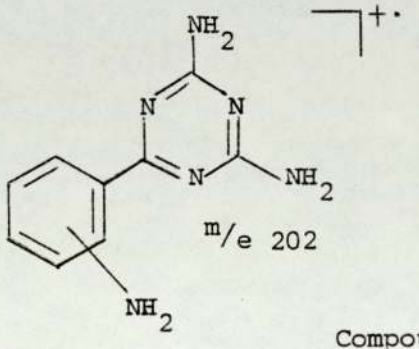
	Calculated	: 156.0118
Compound 66 :-	Observed	: 156.0107
	Error	: 8 p.p.m.
Compound 67 :-	Observed	: 156.0119
	Error	: 0.6 p.p.m.



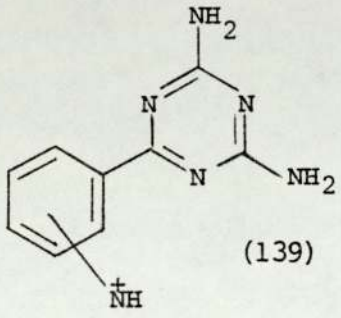
(137)



(138)



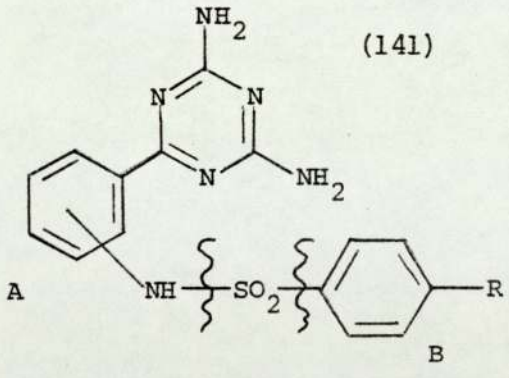
(140)



(139)

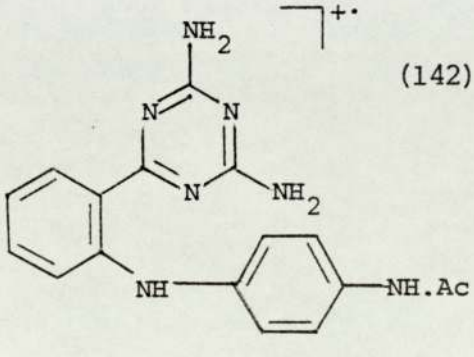
m/e 201

Compound Number	
ortho :	60
para :	61



(141)

	Compounds
R = NO_2	64, 65
NH_2	66, 67
N_3	68, 69
NH.Ac	70



(142)

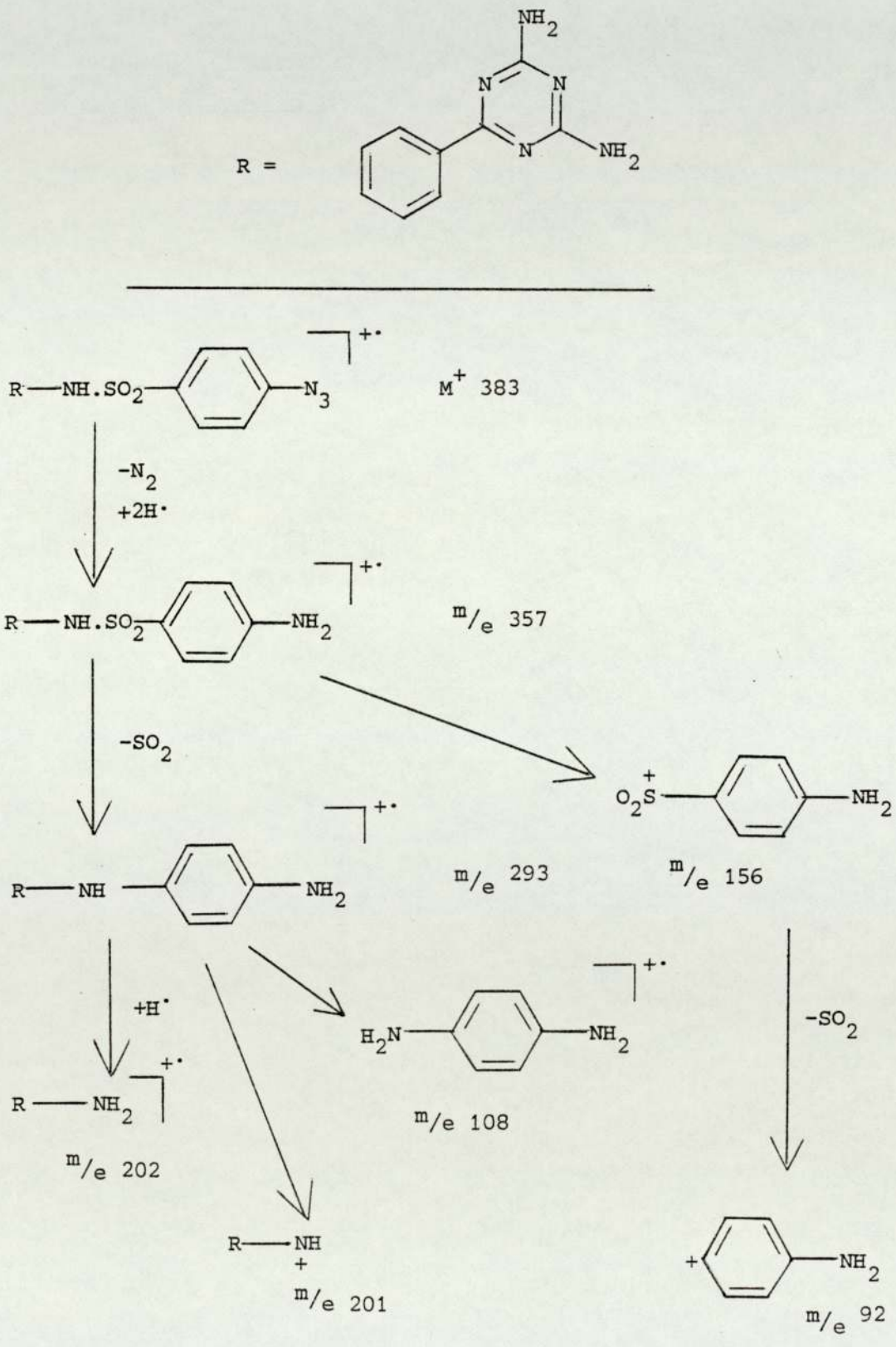
In the aryl-*s*-triazine series N——S cleavage results in the ions $^{m}/_{e}$ 201 and $^{m}/_{e}$ 202 (structures 139 and 140). The mass spectra of twenty-one aryl-*s*-triazines (e.g. structure 140) have been interpreted by Preston *et al* [81], and the ions produced by fragmentation of the $^{m}/_{e}$ 202 and $^{m}/_{e}$ 201 ions (structures 140 and 139), reported by these authors, have been observed in the spectra of the sulphonamides derived from this structure (compounds 64-70: see structure 141). Compound 64 produces a fragmentation corresponding to the successive losses of SO_2 and NO giving an intermediate of structure 137, before further decomposition to the ions at $^{m}/_{e}$ 202 and $^{m}/_{e}$ 201. However, the corresponding *para* isomer (65), rather than fragmenting *via* loss of SO_2, instead fragments to give the ions $^{m}/_{e}$ 122 ($^{+}C_6H_4NO_2$) and $^{m}/_{e}$ 201 and $^{m}/_{e}$ 202 directly.

Compounds 66, 67 and 70 degrade in very similar modes, 66 and 67 giving prominent ions at $^{m}/_{e}$ 293, and 70 giving an ion at $^{m}/_{e}$ 335 (structure 142), which correspond to M - SO_2, i.e. A + B in structure 141. The abundance of these M - SO_2 ions indicate relative stability of the skeletal rearrangement products.

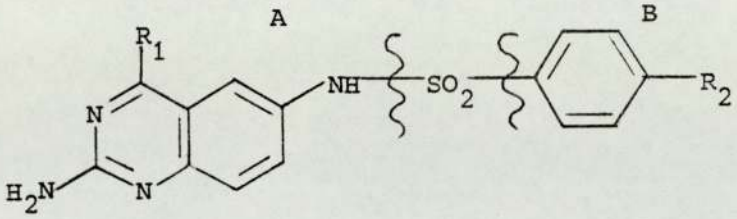
Scheme II.6-4 shows the fragmentation of the azides 68 and 69. The molecular ion of compound 68 was abundant but that of 69 was not observed. With both compounds an initial loss of nitrogen from the azido group preceded the abstraction by the nitrene of 2H· followed by N——S fragmentation to give either a 4-aminobenzene-sulphonium cation ($^{m}/_{e}$ 156) or, following the loss of SO_2, a recombination product of $^{m}/_{e}$ 293. The decomposition pathways from

the ion $^{m}/_{e}$ 293 derived from either the amines 66, 67 or from the azides 68, 69 were very similar.

Scheme II.6-4 Fragmentation of 68 and 69

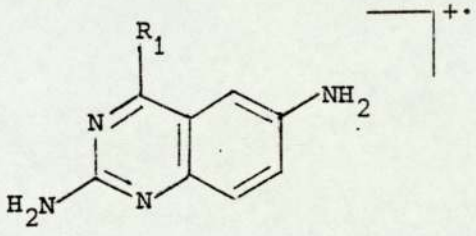


The N —— S fragmentation was the major characteristic of the three sulphonamides studied in the quinazoline series.



(143)

R_1	R_2	Compound Number
NH_2	NH.Ac	12
NH_2	NO_2	10
OH	N_3	14



(144)

R_1	$^m/_e$
NH_2	175
OH	176

Only compounds 10 and 12 showed molecular ions, while all three showed ions corresponding to structure 144. In this context it is noteworthy that while compounds 10 and 12 showed ions of $^m/_e$ 174 as well as $^m/_e$ 175 (structure 16), compound 14 showed no ion at $^m/_e$ 175 in addition to the ion at $^m/_e$ 176.

The SO_2 elimination coupled with skeletal rearrangement occurred to a small extent with compound 12, in a manner analogous to that occurring in the compounds of structure 141.

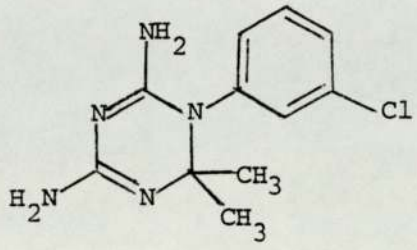
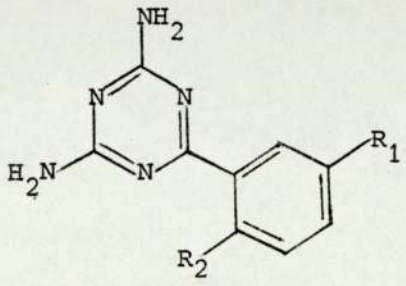
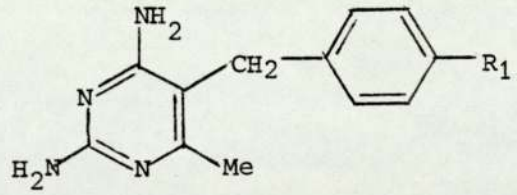
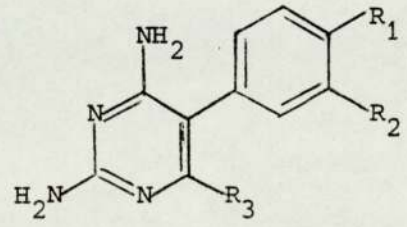
Part II

CHAPTER 7

Compounds obtained as gifts

The compounds listed in table II.7 include seven potential DHFRI (75-81) supplied by Professor M.F.G. Stevens, of which the azide (79) and the diazonium salt (78) were of particular interest, and three known inhibitors, pyrimethamine (90), etoprine (105) and metoprine (106), supplied by the Wellcome Research Laboratories.

Table II.7 Compounds obtained as gifts

Structure	R_1	R_2	R_3	Name	Number
					52
	Cl	H			75
	Me	NH_2			76
	Br	NH_2			77
	Br	$N_2^+Cl^-$		BDT	78
	Br	N_3			79
	H				80
	N_3				81
	Cl	H	Et	pyrimeth-amine	90
	Cl	Cl	Et	etoprine	105
	Cl	Cl	Me	metoprine	106

PART III

Abbreviations and Terms

A and B refer to Substrates FH_2 and NADPH

P and Q refer to Products FH_4 and $NADP^+$

E and S refer to Enzyme, i.e. DHFR and Substrate

I	Inhibitor
∞	Saturating levels
I_{50}	Inhibitor concentration required to inhibit enzyme activity by 50%
V	Maximum velocity of reaction
v	Observed velocity of reaction
K_A'	Dissociation constant of A from ternary complex EAB
K_A	Dissociation constant of A from binary complex EA
K_m	Michaelis Constant
K_I	Inhibitor Constant, i.e. dissociation constant of inhibitor from ternary complex EIB.
BDT	4-bromo-2-(4,6-diamino-1,3,5-triazin-2-yl)benzenediazonium chloride (78)
BSA	Bovine serum albumin
DMF	N,N'-Dimethylformamide
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetracetic acid
SP6	Pye-Unicam UV spectrophotometer, SP6
AC1	Pye-Unicam Automatic Chemistry Unit
PTFE	Polytrifluoroethylene (capillary tubing)
T	Test
C	Control
NCI	National Cancer Institute
6-MP	6-Mercaptopurine

Compound Reference System

Compounds of biological interest have a trivial reference acronym in addition to a number as follows:-

4	ZQ	32	*m*NG	62	*o*ZT	91	*m*NP
6	DQ	38	*o*ZG	63	*p*ZT	92	*m*AP
14	ZSQ	39	*m*ZG	69	*p*ZST	93	*m*ZP
		40	*p*ZG	78	BDT	99	*m*DP

where B = Bromo; N = Nitro; A = Amino; Z = Azido; Q = quinazoline series; G = dihydrotriazine series (guanils); T = aryl-*s*-triazine series; P = pyrimethamine analogue; S = 4-substituted-benzenesulphonamide derivative; *o* = ortho; *m* = meta; *p* = para.

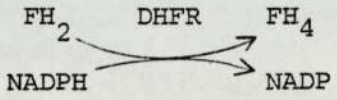
D = Diazonium salt

PART III

CHAPTER 1

The Kinetic Mechanism of DHFR

The chemistry of the DHFR reaction depicted requires both substrate (FH_2) and cofactor (NADPH).



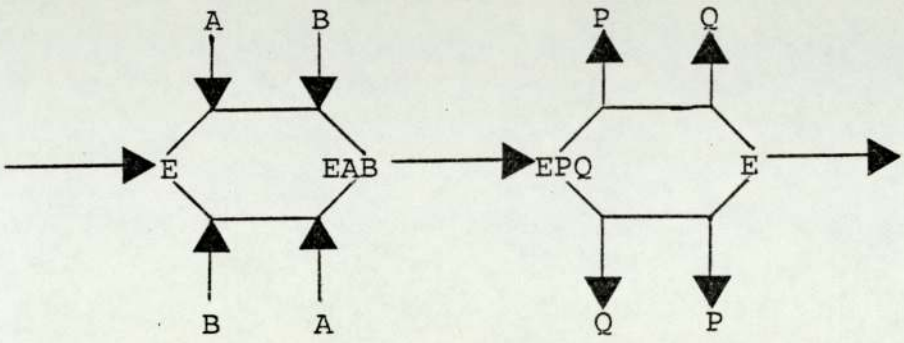
In this work the initial reaction velocity was monitored by utilising the decrease in absorbance at 340nm as NADPH and FH_2 are converted to $NADP^+$ and FH_4, respectively [82].

In kinetic terms this is a two substrate reaction with two possible mechanisms, ping-pong or sequential. The ping-pong mechanism would require NADPH to reduce the enzyme first which itself then reduces the FH_2. There is no evidence that a reduced enzyme form exists, and the X-ray and sequence analysis of the enzyme shows nothing obvious in the structure to support this hypothesis. Indeed, the crystal structures of the binary complexes DHFR.MTX[83] and DHFR.NADPH[84] and of the ternary complex NADPH·DHFR·MTX[85], using enzyme from *E.coli* (form I)[83], and *L.casei*[84,85] have been solved. Since MTX, which is a close structural analogue of FH_2, is an inhibitor having a greater affinity for the enzyme than and being competitive with, FH_2, and since the crystallographic studies have provided direct evidence that there are two discrete but linked binding sites for both NADPH and inhibitor, it is not unreasonable to infer that FH_2 and inhibitor bind similarly to a common site. Also, positive cooperativity between NADPH and FH_2 has been observed using n.m.r.[17] and kinetic[86] techniques.

Therefore, all the evidence from a number of species is indicative of a sequential mechanism and published kinetic data on various DHFRs supports the *random* sequential model (e.g. refs[82-88]). The two substrates may bind in any order and products may leave in any order (random bi bi).

In the equations and figures below DHFR, FH_2, NADPH, FH_4, $NADP^+$ and inhibitor are represented by E, A, B, P, Q and I, respectively.

SCHEME III.1 The forward reaction scheme of DHFR



The kinetic model

In the inhibitor screening experiments and K_I determinations [NADPH] was fixed at saturating so that the results obtained followed pseudo first-order kinetics and fitted equation I:

$$v = \frac{VA}{A + K_m(1+I/K_I)} \qquad (I)$$

where V = maximal velocity of the uninhibited reaction;

v = velocity of the reaction observed when the FH_2 and inhibitor concentrations are fixed at A and I respectively;

K_I is the dissociation constant of the inhibitor from the ternary complex EIB.

The rate equations for both the uninhibited and inhibited DHFR reactions are derived, and the assumptions used are set out in Appendix I.

DHFR does not behave in a simple kinetic manner and the precise meaning of the parameter K_m is open to debate (see Appendix I).

The I_{50} of a compound is its concentration required to inhibit the enzyme activity by 50% and depends upon the substrate concentration (A) used in the experiment. It is possible to estimate K_I from I_{50} from equation (ix) derived in Appendix II.

PART III

CHAPTER 2

Objectives of the kinetic study

The first objective was to characterise the two DHFRs from L1210 cells and *E.coli* RT500 (form I), by obtaining reliable and reproducible values of K_m for both FH_2 and NADPH, by using a new automated assay system, since the literature values of K_m have varied as enzyme purity and assay techniques have both improved over the years. For example, for *E.coli* the values for FH_2 and NADPH were first reported by Burchall and Hitchings in 1965[89] as 25μM and 10μM respectively, and later in 1971 Burchall[90] reported values of 10μM and 8μM respectively. Baccanari *et al* in 1975 reported yet lower values, 3.2μM and 6.8μM[91] . Finally, in 1979 Poe *et al* reported the K_m for FH_2 for *E.coli* MB1428 as 0.5μM[92] . However, in attempting to kinetically characterise DHFR, problems arose due to the nonlinear rate plots shown by the enzyme (particularly from *E.coli*) which are probably due to conformational changes occurring in the molecule especially at low substrate concentrations[93] , and possibly to as yet unidentified trace contaminants found to adulterate all commercial NADPH preparations[94] . The phenomenon of nonlinearity was coupled with the later finding that the K_m values of L1210 DHFR for FH_2 and NADPH were 0.29 ± 0.02μM and 2.14 ± 0.27μM respectively[95] (Cf. literature [82] values of 0.30 ± 0.01μM and 1.36 ± 0.05μM respectively). Thus the K_m for FH_2 was only one order of magnitude greater than the concentration of enzyme necessary to give a measurable rate with the equipment used. At high (above 95% saturating) concentration of one substrate, say NADPH, better linearity could be achieved but double reciprocal plots were near parallel to the ^{1}/[substrate] axis and K_m could not be determined. It was concluded

that despite the high efficiency of the automation hardware the UV spectrophotometer [Pye-Unicam SP6] linked into the system was inadequate, being insufficiently stable and sensitive to monitor the very low rates afforded by the low levels of substrate necessary for the K_m determination. The use of high precision spectrophotometers with long light path spectrophotometer cells has recently allowed several research groups (e.g. Refs. 31,82) to make more accurate measurements with lower rates. Since one of these instruments was not available at the time, the task of independently characterising the enzyme was abandoned in favour of using the most recent literature values of K_m in order to screen the compounds, and Dixon's method was chosen for the K_I determinations as described below.

The second objective was to screen the compounds against both enzymes, and to select ten inhibitors most interesting in terms of structure, activity and the aims of this work (azides), for K_I rather than I_{50} determination, since I_{50} depends upon the substrate concentration used in the experiment, and since I_{50} gives no indication of the mode of inhibitor binding, or of the dissociation rate of the enzyme-inhibitor complex. For this, substrate levels were to be rationally selected from the K_m values determined first.

The third objective was to test the hypothesis *in vitro* with the enzyme, by observing a radiation-mediated lowering of K_I. However it became apparent that solutions of both enzyme and substrates were photolabile, suffering extensive damage from UV irradiation.

Dixon's method for K_I determination [96]

From an examination of the mathematical basis of this method discussed below it was observed that K_m and V as well as K_I could be obtained accurately using the unmodified automated assay system

without the need for the very low concentrations and high instrument sensitivity previously required. Thus, in this laboratory, Lee[95] was able to determine for both enzymes K_m for both NADPH and FH_2 by using, in the former case $NADP^+$ as a competitive inhibitor at variable concentration, saturating FH_2 and several fixed non-saturating values of NADPH, and in the latter case similarly, TMP as inhibitor, saturating NADPH and several fixed values of FH_2.

Subsequently, Lee's value for K_m for FH_2 was confirmed in the course of this work both by Dixon's method, and by using a computer programme which calculated K_m, K_I and V by fitting the raw data [S], [I] and observed v to the hyperbolic Michaelis-Menten model equation I.

A compromise was made in the choice of buffer so that it could be used for both L1210 and *E.coli* enzymes, and therefore all soluble compounds were tested against both enzymes under similar conditions.

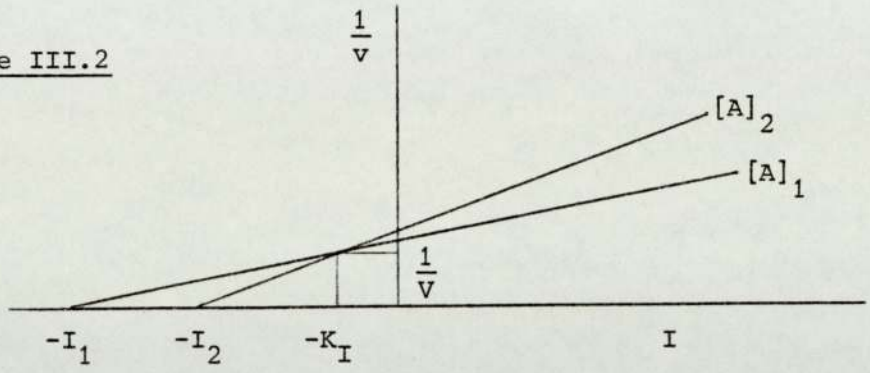
Inversion and rearrangement of the hyperbolic equation (I) which is derived in Appendix I, yields

$$\frac{1}{v} = \frac{1}{V} + \frac{K_m}{VA} + \left[\frac{K_m\, I}{K_I\, VA}\right] \qquad \text{(II)}$$

where K_A', K_I' have been substituted by K_m and K_I respectively, since B $\longrightarrow \infty$ and the reaction is pseudo first-order.

Thus a plot of $\frac{1}{v}$ against [I] for a fixed value of [A] gives a straight line with an abscissa intercept ($-I_x$) which is an apparent K_I (see figure III.2).

Figure III.2



(a) $[A]_1$ and $[A]_2$ are two fixed levels of A

(b) $-I_1$ and $-I_2$ being two intercepts with the I axis are the values of "apparent-K_I" from each line.

(c) The ordinate intercept is $\frac{1}{V}$ for competitive inhibitors.

For each individual line the I intercept occurs when $\frac{1}{v} = 0$, i.e. from equation (II),

$$\frac{1}{V} + \frac{K_m}{VA} + \frac{K_m\, I_x}{K_I\, VA} = 0$$

where I_x is the apparent K_I

$$\therefore \quad \left[\frac{1}{V} + \frac{K_m}{VA}\right] \frac{K_I\, VA}{K_m} = -I_x$$

$$\therefore \quad \left[1 + \frac{K_m}{A}\right] \frac{K_I\, A}{K_m} = -I_x$$

$$\therefore \quad \frac{K_I\, A}{K_m} + \frac{K_m}{A} \cdot \frac{K_I\, A}{K_m} = -I_x$$

$$\therefore \quad -I_x = K_I \left[1 + \frac{A}{K_m}\right] \qquad \text{(III)}$$

Thus each individual line affords a value of $-I_x$ from which K_I can be calculated.

For more than one substrate concentration the lines which result intersect at a point in the fourth quadrant if the inhibition is *competitive*, or on the I axis if the inhibition is *noncompetitive*. The abscissa [I] coordinate of this point is the K_I (figure III.2), and can be read from the plot directly.

PART III

CHAPTER 3

Instrumentation; the automated enzyme assay system

This comprises three units: an automatic chemistry unit (Pye-Unicam AC1 shown in figure III.3), a single beam spectrophotometer with a narrow 8 nm bandpass (Pye-Unicam SP6) and a programmable calculator (Hewlett Packard HP 9815A). The AC1 performs the functions of sample dilution, reagent addition, mixing, incubation and transfer of the final reaction mixture to the SP6 for measurement. The calculator controls and monitors the system by accepting sample identification data from the AC1 and absorbance data at 50 msec intervals from the SP6.

In this work the calculator was programmed to integrate the data input from the SP6 to give the required rate measurements ($\Delta A.min^{-1}$) after a short (equilibration) delay, and to check these against programmed limits. If, for example, the initial absorbance were to lie outside preset limits a warning message would be printed and no calculation would be performed. The number of (consecutive) integrations to be performed and the time periods for the delay and integration allowed considerable scope for optimisation, and are discussed below. The integration results per sample were electronically printed in the sequence: RATE 1, ... RATE n, MEAN of rates 1 to n. Equal integrations indicate rate linearity.

In the AC1 the reagents are delivered to the processing stations *via* syringes and capillary bore PTFE tubing. All syringe volumes are fixed and determined by means of a pre-cut metal template "key" which acts as a stop for the syringe plungers. Samples are processed in racks, each rack bearing ten reaction tubes and ten small plastic reagent cups mounted alongside. Lugs, on the side of the cups, are

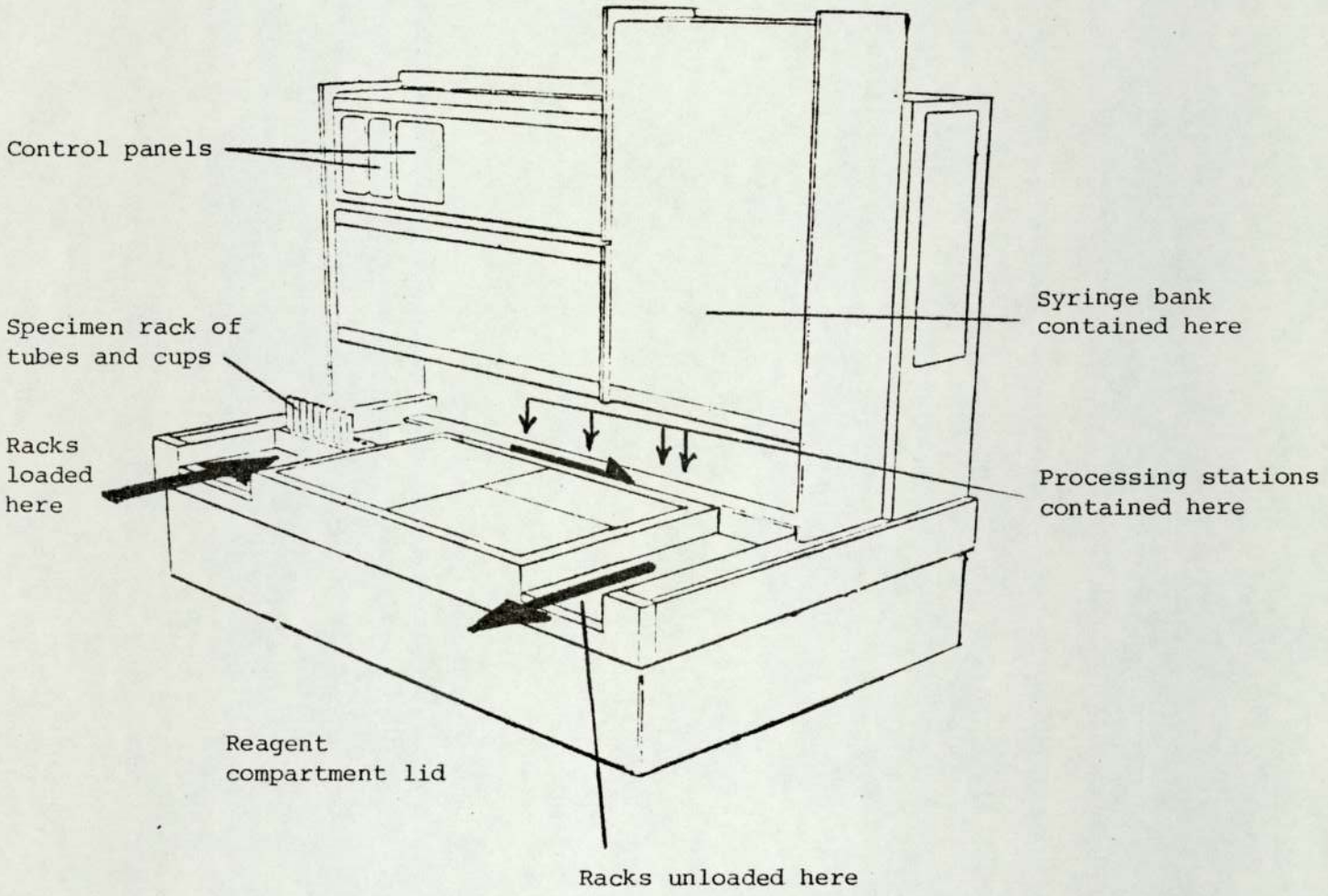


Figure III.3 Diagram of AC1

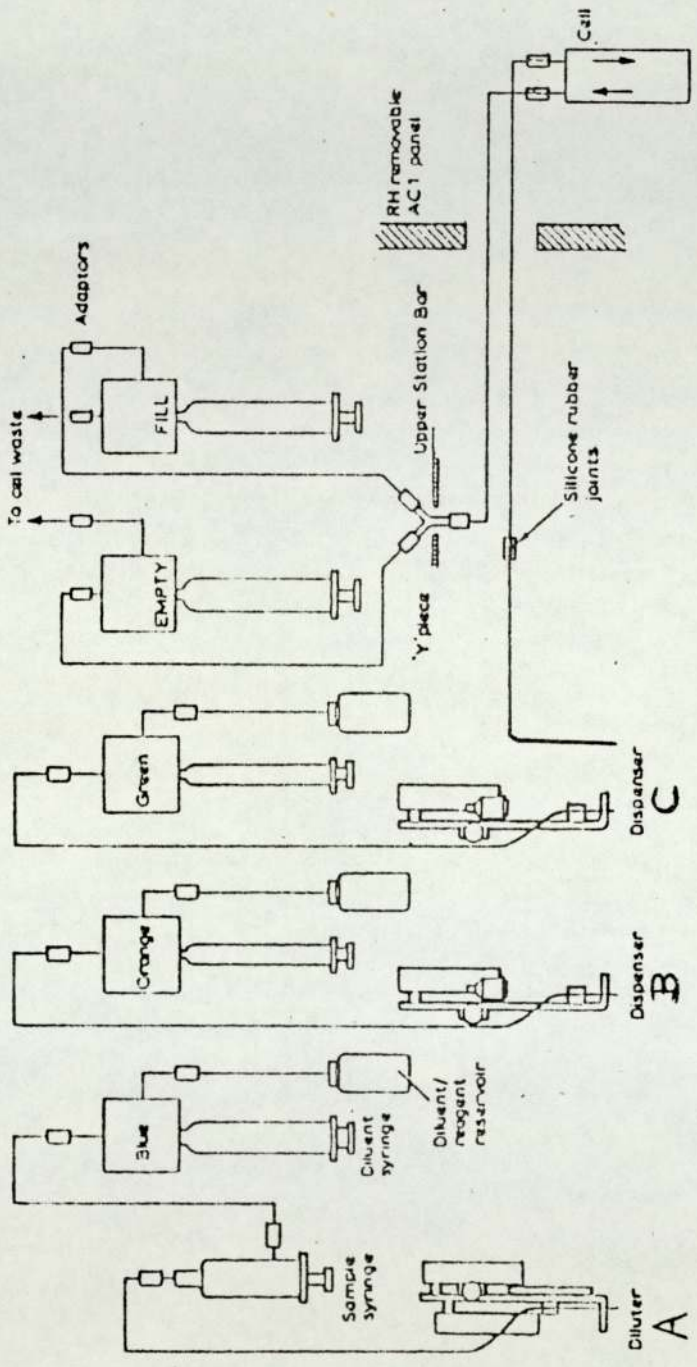


Figure III.3-2 Liquid connections

automatically sensed as the racks move through the system, and thus actuate the appropriate reagent addition, mixing, and transfer operations at the various processing stations. The liquid connections are shown in figure III.3-2.

At the first processing station, the diluter (A), each reaction tube is pushed down into the waterbath (held at a fixed temperature) and a programmed volume of sample is then withdrawn from the cup and washed into the tube with the desired volume of diluent. The tube then moves to the dispenser (B) where a second reagent is added and where efficient (automated) stirring takes place.

The distance between diluter (A) and dispensers (B) and (C) may be altered in order to vary the incubation time. In DHFR assays the dispenser (C) was located next to the transfer station to permit initiation by enzyme immediately prior to transfer.

Thus, for example, for most of the assays of the uninhibited reaction FH_2 was placed in the tubes, and NADPH was placed in the cups (alongside) being picked up and washed into the tubes with the fixed volume of buffer at station (A). Following the programmed incubation the reaction was initiated with enzyme at dispenser (C), and after the delay the contents of the tube were aspirated and transferred to the flowcell in the SP6.

Sample incubation and measurement are performed at the same temperature as the cell holder in the spectrophotometer is connected to the waterbath. In the AC1 all reagent stockbottles are housed in lightproof compartments containing ice.

The automated assay system has a capability of 80 DHFR assays per hour and through its speed and uniformity in sample treatment it hastens the kinetic analysis of inhibitors, involving the variation

of substrate and inhibitor concentrations, and reduces errors due to operator fatigue and enzyme or substrate lability. However, as already discussed, the advantages of automation were only realised in the inhibitor screening and K_I determinations. Here the effects of the limited sensitivity and stability, and noise, were effectively minimised by the relatively high reaction rates.

The order of reagent addition in the inhibitor studies is discussed in section III.3-5.

Optimisation of delay and integration times

The specific delay and integration times used resulted from extensive experience with the system. In the early work on kinetics and crude screening these time periods had been varied both with respect to each other and with respect to the substrate concentration.

The measurement of the low initial rates resulting from very low substrate levels was further complicated by significant substrate depletion. In extreme situations the most linear period was only observed with minimal (nominally zero) delay and with two 7 sec integrations.

The use of two rather than one integration period allowed routine in-system checking of rate linearity. Because the absorbance data from the SP6 is integrated at 20 times per second, the longer the integration period the smaller the effect of noise and the greater the sensitivity in detecting small differences in rate.

In later experiments with 5μM FH_2, two 25 sec integrations were chosen at first because the linear initial velocity was only observed for 60 sec in the manual experiment. However, the presence of

inhibitor was found to cause deviations from this linearity even early in the reaction. Therefore in accord with manual results a total integration time of 30 sec was used. Two identical printouts from the two 15 sec integrations confirmed linearity. Thus in the development of the protocols the total integration time was progressively shortened, but at the same time it was ensured that a sufficient sensitivity was retained.

Since the mixing-time cannot be avoided without some loss in reproducibility a minimum delay of 5 sec was resolved.

Additional precautions

Care was taken to check for air bubbles in the system which cause incorrect volumes to be delivered and anomalous rates to be observed.

The laboratory window blinds were drawn to exclude sunlight, and tinfoil was used to protect the processing track (with its racks of tubes and cups), syringe banks and PTFE tubing containing photosensitive reagents.

PART III

CHAPTER 4

Development of the assay method

Routinely, prior to all experiments using the automated assay system, the activity of the enzyme and the linearity of the rate were checked in a preliminary manual experiment with an SP1800 spectrophotometer coupled with an AR25 chart recorder. Throughout this work, "manual" relates to this context.

In all experiments blanks were run to correct for the slow non-enzymic oxidation of FH_2 by NADPH by omission of enzyme, and for possible NADPH oxidase activity by omission of FH_2.

The temperature for all assays here reported was 25° which was found to give optimal stability of reagents and rates of FH_2 and NADPH conversion of between 0.01 and 0.04 $\Delta A.min^{-1}$, except for some assays with the highest inhibitor concentration. Baccanari [97] has noted that the rates of substrate decomposition at 37° are approximately double those at 30°, and S. Smith [98] has observed that the purified enzyme preparation used in this work was unstable to prolonged exposure to 30°.

Initially the nominal concentrations of the stock solutions were checked and adjusted when necessary, using the extinction coefficients of 2.8×10^4 at 282 nm [99] and 6.2×10^3 at 340 nm [100] for FH_2 and NADPH respectively. Throughout the experiments these stock solutions were kept in light protected iceboxes and were regularly checked for possible decomposition by monitoring their absorbances.

Reagent solutions (a) buffers and excipients

In earlier studies with DHFR not reported here imidazole chloride buffer pH 7.0 was used, and 2-mercaptoethanol was included in stock solutions of FH_2 to prevent its oxidation. However, the work was frustrated by spurious kinetic results and, since the separate absorbances of both FH_2 and NADPH stock solutions remained unchanged from the theoretical values, substrate decomposition was excluded. Then, in November 1978 Baccanari demonstrated that such results were due to the very rapid oxidation of NADPH to $NADP^+$ in neutral imidazole chloride buffer at 30° in the presence of 2-mercaptoethanol or dithiothreitol (DTT)[101]; $NADP^+$ inhibits DHFR competitively with respect to NADPH. This reaction had time to take place during the incubation period prior to the initiation of the reaction by enzyme. Baccanari showed that this reaction requires oxygen, is stimulated by traces of metal ions (e.g. Fe^{++}), is inhibited by ethylenediaminetetracetic acid (EDTA), and occurs only in imidazole and histidine buffers [101]. The mechanism that he proposed involved the oxidation of NADPH by a superoxide radical or by hydrogen peroxide and hydroxyl radicals which are generated from the imidazole- and metal-catalysed oxidation of thiols.

Tris buffer (100mM) at pH 7.5 was found suitable for both *E.coli* and L1210 enzyme assays, and Hillcoat *et al*[102] showed that FH2 decomposition in this buffer at pH 7.3 was minimal at 0°, such a solution being suitable for use the same day without the introduction of significant errors.

Even though the presence of reducing agents is felt by some workers (e.g. 31) to be capable of altering the kinetic properties of the enzyme the thiol containing system was found to give optimal enzyme stability.

DTT was substituted for mercaptoethanol since it was less offensive and more convenient to use, and is judged as efficient a reducing agent.

EDTA became routinely incorporated into all solutions to remove trace metal contamination.

The use of DTT in enzyme solutions was intended (a) to prevent the conversion of active monomer to an aggregate form of lower specific activity, as demonstrated with *E.coli* DHFR by Baccanari [91], and (b) to protect the cysteine residues of the same enzyme from trace metal oxidation as suggested by Williams and Bennett[103] .

Bovine serum albumin (BSA) at 3 $\mu g.ml^{-1}$ was found necessary to stabilise the enzyme (L1210 and *E.coli*) at protein concentrations below 3 $\mu g.ml^{-1}$.

Reagent solutions (b) Ionic conditions

There are two major effects that have a direct and crucial bearing on the design of any kinetic analysis of DHFR *in vitro*: firstly, the order of addition of substrates to the reaction mixture influences the curvilinearity, maximal curvilinearity occurring in assays initiated with FH_2 and minimal curvilinearity in assays initiated with enzyme. Secondly, and even more importantly, the presence of salts[104] and urea[105] exert a profound effect on the enzyme activity-pH function, and drastic changes in the DHFR rate

profiles can occur [93]. Kaufman[105] has shown that the activated enzyme has a considerably increased K_m for NADPH as well as an increased maximal velocity V. Thus the meaning of K_m as an absolute descriptor of this enzyme is called into question (see Appendix I).

Reyes and Huennekens [104] have shown that chloride and bromide ions at 0.8-1.0 M produce about fivefold activation of L1210 DHFR, and that although divalent cations contribute an activating effect monovalent cations give very little such effect. They showed that the activation was freely reversible, did not change the apparent molecular weight of the protein, did not increase the number of binding sites for either NADPH or MTX, but converted the double pH optimum (at 4.5 and 7.0) of the nonactivated enzyme to a single optimum at pH 7.0 and also increased the K_m value for NADPH about fivefold. These findings were interpreted in terms of a degree of unfolding of the protein conformation.

Urea is generally believed to alter the tertiary structure of proteins by breaking hydrogen bonds, but the mechanism by which salts produce conformational changes is not clear.

In contrast Baccanari *et al* [91] have shown that monovalent cations inhibit DHFR* and that, at a given ionic strength, the degree of inhibition is a function of the ionic radius of the cation. Anions have no effect.

For mammalian DHFR, literature values of the optimal ionic concentration of monovalent cations required to produce maximal activation varies between 150mM[106] and 800-1000mM[104].

In the kinetic studies of the uninhibited reaction in the present work, no added salts were used with the *E.coli* DHFR, while 750mM KCl

*from *E.coli*

was used with the L1210 DHFR. However, because of the uncertainty surrounding the mechanism of ionic effects on the enzymes, it became desirable to establish identical experimental conditions for studying each enzyme in order to attempt direct comparisons between them, both with and without inhibitors. Therefore the concentration of potassium chloride eventually used was 100mM, this being a compromise which afforded experimentally similar uninhibited rates for the two enzymes.

Thus, considering (a) and (b) above, both L1210 and *E.coli* DHFRs were assayed under similar conditions in a common buffer system containing Tris (100mM), potassium chloride (100mM), EDTA (1mM), DTT (2.2mM) and BSA (3 $\mu g.ml^{-1}$).

Because it was found that the *E.coli* DHFR under these conditions gave a shorter linear initial velocity than the L1210 enzyme, the integration periods were adjusted to accommodate this difference.

Reagent solutions (c) Effects of organic solvents on DHFR assays

Very few potential DHFRI that are synthesised are water soluble. Therefore in many studies organic solvents have been used, albeit at low final concentrations. Solvents such as dimethylformamide (DMF)[107], dimethylsulphoxide (DMSO)[108] and ethanol[109] have been shown to denature protein and Freudenthal and Hebborn[108] have demonstrated that at levels between 2%-12% these solvents act as noncompetitive inhibitors of DHFR. It is known that the tertiary conformation of an enzyme is dependent on its solvent environment in addition to pH, ionic strength and temperature, and these authors have suggested that there is a strong competition between organic solvent with water for

for either the H-bonding groups on a protein normally solvated by water, or for the structural water molecules H-bonded to the protein.

Noncompetitive inhibition by solvent would interfere with the effect of compounds tested and comparisons with the control assay in the presence or absence of solvent would not be readily analysed.

Therefore the levels of DMF used to dissolve several of the compounds (prior to dilution with water) were kept as low as possible so that in the enzyme assay mixture even the highest final concentrations of DMF (0.015%; 2mM) produced no observable inhibition.

Compound dissolution

On the day of the experiment each compound (0.6 mg) was initially dissolved in water or 1M HCl (30 µl) or 0.12M $NaHCO_3$ (400 µl) or 13M DMF (30 µl) or a combination of these according to table III.6, and was then made up with water to the volume required to give a 0.1mM stock solution which was kept in the dark. Eight compounds listed in table II.6 were insoluble and were not assayed. Although these eight compounds were soluble in organic solvents e.g. acetone, ethanol, DMF, the addition of water to give the 0.1mM stock dilution caused their precipitation or crystallisation. The use of DMSO offered no advantages over DMF.

The highest concentrations of solvents in the final assay mixture were, for the activity test 0.15mM HCl, 0.24mM $NaHCO_3$ and 2.0mM DMF and for the kinetic analysis of the ten compounds 0.027mM HCl, 0.0048mM $NaHCO_3$ and 0.36mM DMF. The higher concentrations had no effect on the enzyme as measured. 10mM DMF, five times the highest concentration used, caused 3.7% inhibition.

The various concentrations required of each compound, obtained from the 0.1mM stocks, were kept in the dark for the duration of the experiment and were only brought out immediately prior to their use.

The automated-system racks were loaded one at a time throughout the experiment in order to render pre-process waiting time minimal and uniform.

PART III

CHAPTERS 5 and 6 deal with the initial testing of sixty of the compounds whose chemistry is described in Part II.

CHAPTERS 7, 8 and 9 deal with the selection of ten of these compounds for further kinetic analysis (K_I determination).

PART III

CHAPTER 5

Parameters and method used in the activity testing

For this ranking test the FH_2 concentration was fixed at 5 μM, NADPH at 150 μM and DHFR from L1210 cells or *E.coli* at 20 nM. 5 μM was the lowest concentration of FH_2 that gave a repeatable linear rate, both manually and with the automated screen. This represents 18 x K_m, i.e. 95% saturating. NADPH was effectively saturating at 150 μM (60 x K_m).

Sixty compounds were tested initially at 10 μM against the L1210 enzyme. Depending on the results, concentrations were then reduced in steps to 10 nM until the 50% inhibition was passed as shown in table III.6 . From these results one concentration was selected per compound for testing against the *E.coli* enzyme under similar conditions allowing a direct comparison of activity to be made (table III.6). After this, compounds showing little or no inhibition at 10 μM were rejected as being insufficiently active to be of interest.

The use of the automated system

Four control assays containing water in place of compound solution, separated groups of six test assays. In each group of six tests three compounds were assayed sequentially in ascending order of expected inhibition based on previous experimental results.

The controls allowed frequent and regular checking of the (uninhibited) enzyme activity. The water also served to flush the flowcell and PTFE tubing free from any remaining active compound

and to monitor any such carryover. A complete rack of ten controls at the beginning and at the end of the experiment provided an independent estimate of the total loss in enzyme activity thus allowing corrections to be made when necessary.

A solution of compound (150 µl) was placed into each tube and excess NADPH solution was placed in the sample cups. At station (A) each tube was pushed down into the waterbath and NADPH solution (20 µl) was withdrawn from the adjacent cup and washed into the tube with buffer (1030 µl). During the incubation period at a fixed time FH_2 solution (150 µl) was added with stirring at station (B). At station (C) the reaction was initiated by the addition of enzyme solution (150 µl) and the tube contents (1500 µl) were aspirated to the SP6 for absorbance monitoring.

Assays were repeated whenever variation in observed reaction velocity greater than 0.002 $A.min^{-1}$ (i.e. 5%) for assays with the same compound occurred.

PART III

CHAPTER 6

Results of the activity testing

From table III.6 it can be seen that, in general, all the guanils (31-40, 52) except *o*ZG (38) show marginally greater activity against L1210 DHFR (between 2 and 33 times), whereas the quinazolines (1-14) and pyrimethamines (90-105) tested showed even less differential activity. Metoprine was only 14 times more active against L1210 DHFR.

The aryl-*s*-triazines (60-62) and (71-75) showed virtually no activity against either enzyme. These results were borne out by the observation that Kendrew models of these compounds could not be contorted to fit into the binding site of a scale (Kendrew) model of the *E.coli* enzyme.

Although the sulphonamides in the aryl-*s*-triazine series showed only low activity, it can be seen that with the exception of (64) these compounds gave marginally greater inhibition of the *E.coli* DHFR.

Because the % inhibition results for both L1210 and *E.coli* enzymes were similar, this comparative exercise was not pursued further.

In the quinazoline series the 6-*p*-nitrobenzenesulphonamido derivative was about ten times more active against both the tumour and bacterial enzymes than its 6-nitro analogue. Similarly the 6-*p*-acetylaminobenzenesulphonamide was at least ten times more active against both enzymes than its 6-amino analogue. Surprisingly, in contrast, 2-amino-4-hydroxy-6-[4-azidobenzenesulphonamido] quinazoline [or its quinazolone tautomer] (ZSQ; 14) showed only about 47% of the activity of 2,4-diamino-6-azidoquinazoline (4) against L1210 DHFR, and about 6% of the activity against *E.coli* DHFR.

Table III.6 Results of inhibitor screening against L1210 and *E.coli* RT500(form I) DHFR*

Compound Number	Compound Ref.	Solvent[b]	% inhibition at indicated concentration of DHFR from:- L1210 cells[a]				*E.coli* RT500(form I)			
			10nM	100nM	1μM	10μM	10nM	100nM	1μM	10μM
1		A				42				93
2		B				67				78
3		A				59				91
4[c]	ZQ	B	3	20	85	90			96	
6	DQ	A	0	7	26	77			75	
7		G								
10		E			63	94			78	
12		A		43	86	92			93	
14[c]	ZSQ	E			40	85			6	
22		A				0				0
31		A		59	88	96		8		
32	*m*NG	A	22	67	90	99		11		
34		A			66	87			2	
35		A	26	75	95	95		5		
36		E	9	41	78	98		3		
37		B				54				2
38	*o*ZG	A			10	40				59
39[c]	*m*ZG	C	7	42	84	96		9		
40[c]	*p*ZG	A	7	36	81	95		7		
42		A				35				7
49		B				0				0
50		B				0				3
52		A			54	90			26	
60		A				0				3
61		A				0				3
62	*o*ZT	E				0				3
63	*p*ZT	B				13				4
64		C				2				3
65		C				7				26
66		E				7				32
67		B				2				23
68	*o*ZST	G								
69	*p*ZST	F				2				16
70		E				1				6
71		C				0				2
72		E				2				0
73		B				7				7
74		B			4	8				0
75		B				2				4
76		A				0				10
77		B				18				83
78	BDT	B			9	78				6
79		G								

Compound Number	Compound Ref.	Solvent[b]	% inhibition at indicated concentration of DHFR from:- L1210 cells[a]				*E.coli* RT500 (form I)			
			10nM	100nM	1μM	10μM	10nM	100nM	1μM	10μM
80		B				32				0
81		C				7				9
90[c]	pyrimeth-amine	B	21	65		99		36		
91[c]	*m*NP	B	47	97	99	100	10			
92[c]	*m*AP	B	13	50	86	98		53		
93[c]	*m*ZP	A	31	82	96	100	10			
94		B		35	81	93		21		
95		B			24	70			10	
96		B			40	84		18		
99	*m*DP	A	12		90	100		29		
100		G								
105[c]	etoprine	B	52	95	99	100	13			
106	metoprine	B	68	93		100	5			
110	SDM[d]	B				0				4
111						0				0
112		G								
113						0				0
114		G								
115						0				0
116		B				0				2
117		G								
118		G								
119		A				29				0
120						0				0
121						0				0

* Conditions of assay:- [FH_2] at 5μM, [NADPH] at 150μM, [DHFR] at 20nM Buffer 100mM TRIS, pH 7.5, containing 100mM KCl, 1mM EDTA and 2mM DTT.

[a] MTX at 50nM gave 96% inhibition with L1210 DHFR in this test.

[b] Solvent used as described in Part III.4 for initial dissolution:-
A = distilled water with or without heating;
B = 1M HCl;
C = 0.12M $NaHCO_3$;
D = 13M DMF;
E = DMF + HCl;
F = DMF + $NaHCO_3$;
G = Insufficiently soluble in aqueous media.

[c] These compounds were chosen for K_I determination.

[d] SDM = sulphadimidine: this and the following eleven compounds were not expected to be DHFRI.

In the aryl-*s*-triazine series, the replacement of the nitro, amino and azido groups in the *para* position by 4-nitrobenzenesulphonamido, 4-aminobenzenesulphonamido and 4-azidobenzenesulphonamido groups resulted in an approximately 20-fold increase in activity against the *E.coli* enzyme.

With L1210 DHFR, BDT *may* show slightly increased inhibition compared to that produced by other aryl-*s*-triazines, by virtue of its covalent coupling to a reactive residue (e.g. tyrosine) in the enzyme. However, the diazonium salts DQ (6) and *m*DP (99) do not show any increased DHFRI activity in relation to their analogues, compounds (1-14) and (90-96). The type of inhibition (competitive or noncompetitive) shown by these compounds (DQ and *m*DP) was not ascertained.

2,4-Dihydrazinoquinazoline (22) shows no activity whereas 2,4-diaminoquinazoline (1) shows significant inhibition at the same concentration (10 μM). This probably indicates a lack of bulk tolerance of the active site to the two hydrazino (as opposed to the two amino) moieties; the dihydrazino compound being the more strongly basic would otherwise be expected to bind more tightly. This observation is in accord with those of Davoll *et al* [37] who reported similar lack of bulk tolerance with compounds substituted at N2 or N4.

The strong base 9-aminoacridine (not tabulated) was found in a previous experiment to be devoid of activity.

As expected, the Dimroth rearrangement products (49) and (50) showed no inhibition, while 4-nitrophenylbiguanide showed a low level of activity against L1210 DHFR.

The most potent inhibitors of the L1210 enzyme were the compounds in the pyrimethamine series (90-93), etoprine and metoprine (105 and 106), and the dihydrotriazines (31, 32, 35 and 39). The azides best suited for any experiments to test the RAR-hypothesis are *m*ZP (31), ZQ (4), ZSQ (14), *m*ZG (39) and *p*ZG (40).

PART III

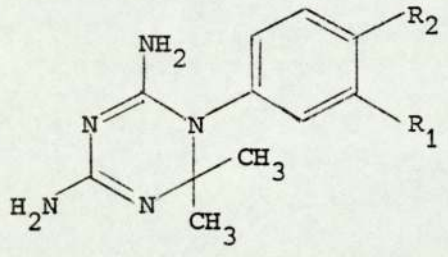
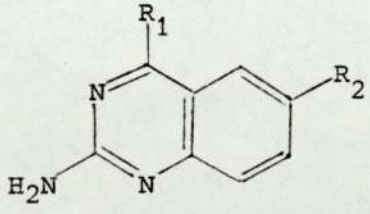
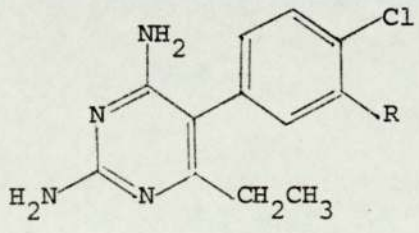
CHAPTER 7

Selection of ten compounds for K_I determination with L1210 DHFR

It was not possible to perform kinetic analyses on all compounds of interest. Those tested were chosen firstly on the basis of the activity tests, since for an ATRAR to work in the designed hypothetical mode it is essential that the compound binds reasonably tightly to DHFR prior to radiotransformation. Secondly, active non-azide analogues were required for comparison with the candidate radiolysable azides.

The numbers, trivial reference codes and structures of the ten chosen compounds are shown in table III.7.

TABLE III.7

Compound Reference	Compound Number	Structure	Parent Structure
*m*NG	32	$R_1 = NO_2$, $R_2 = H$	
*m*ZG	39	$R_1 = N_3$, $R_2 = H$	
*p*ZG	40	$R_1 = H$, $R_2 = N_3$	
ZQ	4	$R_1 = NH_2$, $R_2 = N_3$	
ZSQ	14	$R_1 = OH$, $R_2 = NH.SO_2.C_6H_4.N_3(p)$	
Pyrimethamine	90	R = H	
*m*NP	91	$R = NO_2$	
*m*AP	92	$R = NH_2$	
*m*ZP	93	$R = N_3$	
Etoprine	105	R = Cl	

In the pyrimethamine series the parent analogue served as a standard inhibitor of mammalian DHFR and had been used clinically in treating cancer[46]. The other four compounds were chosen because, being close analogues of pyrimethamine, they formed a group which might establish some structure activity correlations in terms of DHFR inhibition, antitumour activity and toxicity. In addition the chloro compound (etoprine) was probably the closest analogue of the pseudohalide (azide) *m*ZP on physico-chemical grounds (pK_a and log P; see part V).

In the guanil series the *m*-azido analogue was compared with its *p*-azido and *m*-nitro analogues.

Neither of the two quinazolines were found to be very active as DHFRI but were included because the novel principle of a single molecule DHFRI-sulphonamide combination may well be explored further, in regard to antibacterial as well as anti-tumour fields, and because it was of interest to investigate possible correlation between its potency as a DHFRI and its anticancer activity. ZQ was tested for comparison with ZSQ.

The aryl-*s*-triazine series were either too insoluble or inactive as DHFRI to be included in further kinetic experiments.

PART III

CHAPTER 8

Parameters and method used in K_I determinations

For these analyses [NADPH] and [DHFR] remained fixed at 150μM and 20nM respectively but two FH_2 concentrations of 5 and 10μM were used, although only one was essential for the analysis (see part III.2). Five compound concentrations were chosen from the activity data in table III.6 to give a range of 10-50% inhibition. Each concentration was tested in triplicate, sequentially and in ascending order of inhibition, and after five concentrations a control assay was conducted in quintuplicate. Thus five controls separated groups of fifteen tests. No changes in these controls were detected in each experimental run.

As in the activity testing a complete rack of ten controls was placed at the beginning and at the end of the experimental run to provide a check against possible loss in enzyme activity over the experimental period.

FH_2 solution at one of the two fixed concentrations (150μl) was placed in the tubes and the compounds at the required concentrations were placed in the cups (excess volume).

At station (A) each reaction tube was pushed down into the waterbath and compound solution (20μl) was withdrawn from the adjacent cup and washed into the tube with buffer (1030μl). During the incubation period at a fixed time NADPH solution (150μl) was added with stirring at station (B). At station (C) the reaction was initiated by the addition of enzyme solution (150μl) and the tube contents (1500μl) were aspirated for absorbance monitoring.

The control rates remained steady within the range 0.0440-0.0400 $A.min^{-1}$. The integration and linearity of this rate was manually checked.

For these determinations the FH_2 was placed in the tubes in order to further minimise its decomposition since it has been previously found that, despite precautions taken, a small amount of decomposition took place in the syringe and PTFE tubing that supplied processing station (B). Since the FH_2 was transferred from the dark icebox to the rack tubes immediately prior to processing, it did not have time to degenerate, and the rack itself was found to afford improved photoprotection.

Calculation of K_I

Firstly, the raw date $[FH_2]$, [I] and observed v were fitted to the model (equation I):

$$v = \frac{VA}{A + K_m\left(1 + \frac{I}{K_I}\right)}$$

where A is $[FH_2]$, using a computer programme (GENFIT; part VI) which calculated K_I and V, assuming in this case a K_m of 0.3μM for FH_2.

Secondly, following the method of Dixon (part III.2) apparent K_I values for the two concentrations of FH_2 were taken from the abscissa (I-axis) intercepts of the two lines. The slope and intercepts of each line was computed, and each line yielded a value of K_I by calculation from the formula (equation IV). The abscissa coordinate of the point of intersection ($-K_I$ in theory) of the two Dixon-plot lines merely served to confirm the order of magnitude of the two calculated values of K_I.

$$-K_I = \frac{I_x}{\left(1 + \frac{A}{K_m}\right)} \qquad \text{(IV)}$$

PART III

CHAPTER 9

Results of K_I determinations

These are summarised in tables III.9-1 and III.9-2.

From the inhibition patterns analysed by Dixon's method, and because the signs of the residual deviations, resulting from the fitting of the data to the competitive curve function, formed no discernible pattern, it is clear that all ten compounds assayed were *competitive* inhibitors of L1210 DHFR.

There was generally a reasonably close agreement between the two methods of calculation and between the duplicate experiments. Naturally K_I would vary with the chosen K_m value for FH_2 and values a little less than 0.3 μM have been reported[31], but the differences are probably not significant.

Estimates of the I_{50} values for the ten compounds were obtained graphically from plots of the concentrations of the ten compounds against percent inhibition using the activity testing data from table III.6. The I_{50} values thus obtained were used to calculate K_I from the equation $I_{50} = K_I[1 + {}^{A}/_{K_m}]$ and these are listed with the K_I values obtained by the two previous methods. The agreement is with some exceptions, reasonable. This demonstrates that, in the conditions of high FH_2 concentration and saturating NADPH used, L1210 DHFR appears to behave with these inhibitors according to the Michaelis-Menten model assumed.

Table III.9-1

Footnotes

[a] Two programmes were used:- (i) to determine the least-squares linear-regression of each line per run
(ii) to determine the coordinates of the point of intersection of the two lines per run.

[b] $-I_x$ is the apparent K_I being the negative intercept on the abscissa.

[c] K_I was calculated from $-I_x = K_I[1 + {}^{A}/_{K_m}]$, where K_m has been taken as 300nM.

[d] The calculated abscissa-coordinate of the point of intersection = K_I.

[e] Abscissa coordinate of intersection was positive, therefore not recorded.

Small variations in the two slopes produce small variations in calculated K_I but relatively large variations in the coordinates of the point of intersection. Therefore this point of intersection in practice only serves to confirm approximately the value of K_I calculated.

Table III.9-1 K_I determinations with Dixon's method[a]

Compound Ref.	Experimental run	FH_2 (μM)	No. of points	Slope of line	$-I_x$(nM)[b]	K_I(nM) calculated from each line function[c]	K_I(nM) observed as point of intersection of two lines
*m*NG	I	5	15	0.406 ± 0.022	60.5	3.42	3.81
		10	15	0.180 ± 0.005	131.7	3.83	
	II	5	12	0.419 ± 0.026	58.7	3.16	12.09
		10	11	0.203 ± 0.009	108.4	3.32	
*m*ZG	I	5	12	0.074 ± 0.004	319.2	18.07	14.36
		10	12	0.040 ± 0.003	575.8	16.77	
	II	5	12	0.110 ± 0.007	216.2	12.24	5.78
		10	15	0.051 ± 0.004	462.1	13.46	
*p*ZG	I	5	12	0.085 ± 0.005	285.4	16.16	17.66
		10	12	0.041 ± 0.003	566.6	16.50	
	II	5	15	0.086 ± 0.005	293.4	16.61	16.12
		10	15	0.038 ± 0.006	650.7	18.95	
ZQ	I	5	12	0.025 ± 0.002	991.6	56.13	20.81
		10	12	0.016 ± 0.001	1531.5	44.61	
	II	5	12	0.028 ± 0.001	965.0	54.62	171.70
		10	11	0.013 ± 0.001	1911.3	55.67	
ZSQ	I	5	11	0.015 ± 0.000	1585.4	89.74	88.59
		10	12	0.007 ± 0.001	2974.8	86.64	
	II	5	15	0.016 ± 0.002	1416.1	80.16	99.28
		10	14	0.010 ± 0.001	2205.9	64.25	
Pyrimethamine	I	5	10	0.438 ± 0.025	60.4	3.41	6.75
		10	12	0.215 ± 0.013	115.9	3.38	
	II	5	12	0.370 ± 0.031	66.7	3.77	0.55
		10	14	0.176 ± 0.008	139.4	4.06	
*m*NP	I	5	15	1.474 ± 0.041	14.3	0.82	e
		10	15	0.636 ± 0.012	38.3	1.12	
*m*AP	I	5	11	0.171 ± 0.015	145.8	8.25	9.23
		10	13	0.088 ± 0.003	275.5	8.02	
	II	5	12	0.177 ± 0.011	156.9	8.89	41.12
		10	12	0.098 ± 0.009	250.4	7.29	
*m*ZP	I	5	12	0.713 ± 0.023	32.9	1.87	2.70
		10	12	0.386 ± 0.018	58.8	1.71	
	II	5	15	0.579 ± 0.018	37.7	2.13	e
		10	15	0.271 ± 0.016	88.9	2.59	
Etoprine	I	5	6	2.440 ± 0.189	8.4	0.47	0.16
		10	11	1.503 ± 0.057	13.5	0.39	
	II	5	12	2.364 ± 0.092	8.3	0.47	e
		10	15	1.338 ± 0.054	16.3	0.47	

Table III.9-2 Values of K_I from three methods

Compound Ref.	I_{50}(nM)[a]	K_I(nM) determined from:- $I_{50} = K_I[1+A/K_m]$[ab]	Dixon method[c]	GENFIT programme[d]
*m*NG	34	1.92	3.63 3.24	3.34 ± 0.12 3.02 ± 0.13
*m*ZG	140	7.91	17.42 12.85	17.54 ± 0.84 12.56 ± 0.55
*p*ZG	300	16.95	16.33 17.78	15.04 ± 0.65 14.76 ± 1.18
ZQ	740	41.81	50.37 55.15	51.46 ± 6.30 43.17 ± 3.08
ZSQ	1800	101.69	88.19 72.21	87.15 ± 2.26 92.15 ± 6.20
Pyrimeth-amine	38	2.15	3.40 3.90	2.98 ± 0.11 3.75 ± 0.15
*m*NP	11	0.62	0.97	1.01 ± 0.07
*m*AP	100	5.65	8.14 8.09	7.90 ± 0.37 6.78 ± 0.30
*m*ZP	23	1.30	1.79 2.36	2.04 ± 0.07 2.71 ± 0.25
Etoprine	9	0.51	0.43 0.47	0.59 ± 0.06 0.60 ± 0.06

[a] I_{50} obtained graphically using data from table III.6.

[b] $A = [FH_2] = 5\mu M$; K_m taken as $0.30\mu M$.

[c] Listed are the mean calculated values from RUN I and II in table III.9-1.

[d] Curve fit to equation (I) ; $[FH_2] = A = 5.0\mu M$ and $10.0\mu M$; K_m taken as $0.30\mu M$; listed are the values from RUN I and II in table III.9-1.

Part III

CHAPTER 10

Initial attempts to test RAR with u.v. light and *in vitro* systems

Five azide hydrochlorides [ZQ (4), *o*ZG (38), *m*ZG (39), *p*ZG (40), *m*ZP (93)] were photolysed at spectroscopic concentrations in water in a quartz cuvette with a 100W medium-pressure arc in a Hanovia photochemical reactor both with ($\lambda > 310$ nm) and without ($\lambda > 270$ nm) a Pyrex filter. It was found that all five compounds were effectively photolysed after 5 minutes when no Pyrex filter was used, but the Pyrex filter effectively shielded the azide solutions so that only 50% photolysis occurred after approximately 0.5 h.

Photolysis of a solution of *m*ZP in a sterile transparent plastic container used in cell culture, using the unfiltered lamp was complete after approximately 12 minutes.

It was subsequently observed that when L1210 cells, suspended in culture medium and contained in the sterile plastic containers, were exposed to the filtered photo-irradiation for 20 min., or to the unfiltered u.v.-irradiation for 3 min., the respective cell counts after incubation for 45 h at 37^{o} were 34% and 50% of the unirradiated controls. Consequently it did not appear feasible to attempt any azide photo-activation experiments with the 100W-photolysis lamp and with L1210 cells in culture.

Although L1210 cells in suspension, contained in the plastic vials, when exposed for 1.5 h to white light emitted by an 8W fluorescent tube sustained no observable damage, only one compound (ZQ) was photolysed under the same conditions.

It was observed in the course of the work with DHFR that this enzyme, as well as its substrate FH_2, was photolabile. Therefore all kinetic experiments with DHFR were carried out with shielding from sunlight.

Since it appeared to be impracticable to test the RAR-hypothesis with L1210 cells in culture or with solutions of DHFR, on account of photosensitivity, it was decided to test the hypothesis *indirectly* using γ-irradiation and a solid murine tumour implant.

PART IV The *in vivo* studies

Introduction

One objective in this study was to assess generally as many of the compounds synthesised as possible for antitumour activity. Therefore, many of them synthesised in the course of this work, were submitted to the National Cancer Institute, U.S.A. (NCI) for routine screening. In addition, since pyrimethamine and its structural analogues metoprine and etoprine, whose ability to cross the blood-brain barrier and whose anticancer properties have been widely documented, were of special interest, six compounds in this series were tested in the Experimental Chemotherapy Unit, Aston University, Birmingham (ASTON) using a TLX5 lymphoma system in female CBA/LAC mice.

Independently, at the Wellcome Research Laboratories, Beckenham (WELLCOME), an attempt has been made to set up a novel protocol for the testing of the ATRAR hypothesis by using a solid W3129 myeloma, subcutaneously implanted in the flanks of female BALB/c mice, a Cs^{137} δ-source, and a simple means of shielding the body of the test animal while accurately collimating the δ-irradiation to the tumour site. The five azides in the group of ten DHFR inhibitors kinetically analysed in Part III were initially screened for activity without irradiation against this myeloma implant. From the screening results mZP and ZSQ were further evaluated and mZP was then chosen for the hypothesis test.

Preliminary assessment of the metabolic fate of the aryl azido group *per se*

An indication of the metabolic fate of this group was obtained from an examination of the urine of rats treated with azido derivatives of four clinically used aminobenzenesulphonamides: sulphadimidine, sulphamerazine, sulphadiazine and sulphanilamide. These are shown in table IV.1 together with two of their potential metabolites: the corresponding parent amines and N^4-acetylated amines.

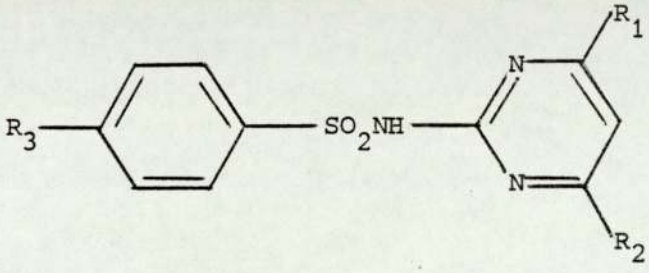


TABLE IV.1

Number	Name	Structure		
		R_1	R_2	R_3
110	sulphadimidine	CH_3	CH_3	NH_2
111	azidosulphadimidine	CH_3	CH_3	N_3
112	N^4-acetylsulphadimidine	CH_3	CH_3	NHAc
113	sulphamerazine	CH_3	H	NH_2
114	azidosulphamerazine	CH_3	H	N_3
115	N^4-acetylsulphamerazine	CH_3	H	NHAc
116	sulphadiazine	H	H	NH_2
117	azidosulphadiazine	H	H	N_3
118	N^4-acetylsulphadiazine	H	H	NHAc
119	sulphanilamide	$H_2N \cdot C_6H_4 \cdot SO_2NH_2$		– *p*
120	azidosulphanilamide	$N_3 \cdot C_6H_4 \cdot SO_2NH_2$		– *p*
121	N^4-acetylsulphanilamide	$NHAc \cdot C_6H_4 \cdot SO_2NH_2$		– *p*

These compounds were not anticipated nor found (see part III) to inhibit DHFR.

Each azidobenzenesulphonamide (e.g. 111) was administered to rats (i.p.) at a dose of 100 mg/kg as a fine suspension in 10% acetone/arachis oil. Examination of chloroform extracts of the urine, collected over 24h, by t.l.c. revealed the presence of unchanged azide, the corresponding aminobenzenesulphonamide (e.g. 110) and its acetyl derivative (e.g. 112), as indicated by table IV.2. There were also traces of up to several unidentified metabolites in each case. Clearly the azido substituent underwent metabolic reduction to the amine which was then further metabolised by acetylation, except in the case of azidosulphadimidine.

TABLE IV.2

Compound Administered	Metabolites detected in urine		
	Azide (unchanged)	Amine	Acetylamine
azidosulphadimidine	+	+++	-
azidosulphamerazine	+	+++	+
azidosulphadiazine	+	+++	++
azidosulphanilamide	+	+++	++

\+ traces

\+++ major metabolite

\- not detected

PART IV CHAPTER 1

Results of antitumour screening (without irradiation)

(1) The pyrimethamine series

The results of the screening of six analogues in this series against a TLX5 lymphoma implanted in female CBA/LAC mice at ASTON are summarised in table IV.3. The animals were inoculated subcutaneously at the inguinal site with 2×10^5 tumour cells on day 0, followed by intraperitoneal treatment with either compound suspended in 10% DMSO/arachis oil, or an equivalent volume of this vehicle, for five consecutive days starting day 2. A % IST of 20% is considered significant[110].

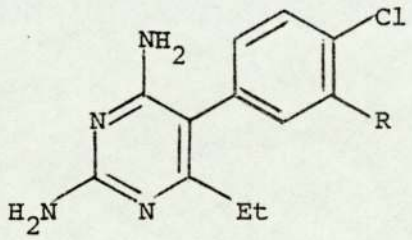


TABLE IV.3

Activity against TLX5 lymphoma in CBA/LAC mice at ASTON

Number	Trivial Ref. name	Substituent R	Dose (mg/kg)	Max % IST[b]	Toxic dose (mg/kg)
90	Pyrimeth-amine	H	25	ns[c]	25
92	mAP	NH_2	25	ns	50[d]
94		NHAc	50	25[e]	50[d]
91	mNP	NO_2	12.5	33[e]	25[d]
105	Etoprine[a]	Cl	12.5	ns	25
93	mZP	N_3	25	37[e]	100[d]
	6-Mercaptopurine		25	47	50

[a] Etoprine = DDEP; [b] Mean increase in survival time (days) of treated animals relative to the mean survival time of untreated animals; maximum % IST occurred at the indicated dose level, protocol in text; [c] ns = not significant; [d] Cf. toxicity thresholds observed by NCI with CDF_1 mice: see tables IV.5, IV.6 and intervening text; [e] 5 mice per dose level and S.E. for death days all within 5% mean.

(a) *meta*-azidopyrimethamine (mZP)

Fig IV.1 shows a dose-response curve constructed from the data in table IV.4 for this compound in the TLX5 lymphoma screen.

TABLE IV.4 mZP against TLX5 lymphoma (ASTON)

Dose (mg/kg)	Log Dose	Mean Death Day (± S.E.)	n	% IST
25	1.40	12.60 ± 0.24	5	36.9
12.5	1.10	11.60 ± 0.60	5	26.1
6.25	0.80	10.40 ± 0.40	5	13.0
3.125	0.49	9.80 ± 0.49	5	6.5
1.563	0.19	9.40 ± 0.60	5	2.2
Untreated	-	9.20 ± 0.37	5	0

The compound has also been found significantly active in the P388 lymphocytic leukaemia model in male CDF_1 mice by the NCI. The animals were inoculated into the ascitic fluid with 10^6 tumour cells on day 0, followed by nine daily intraperitoneal injections of a suspension of mZP in water/Tween 80, starting on day 1. The preliminary results for two separate experiments are summarised in table IV.5 below.

TABLE IV.5 mZP against the P388 leukaemia (NCI)

Dose (mg/kg)	Toxicity (survivors on day 5)	Median survival time: Test T	Control C	T/C %
50	6/6	14.4	10.9	132
25	6/6	12.0	10.9	110
12.5	6/6	11.7	10.9	107
6.25	6/6	11.2	10.9	102
3.13	6/6	10.8	10.9	99
200	6/6	17.0	11.2	151
100	6/6	14.0	11.2	125
50	6/6	12.3	11.2	109
25	6/6	10.0	11.2	89

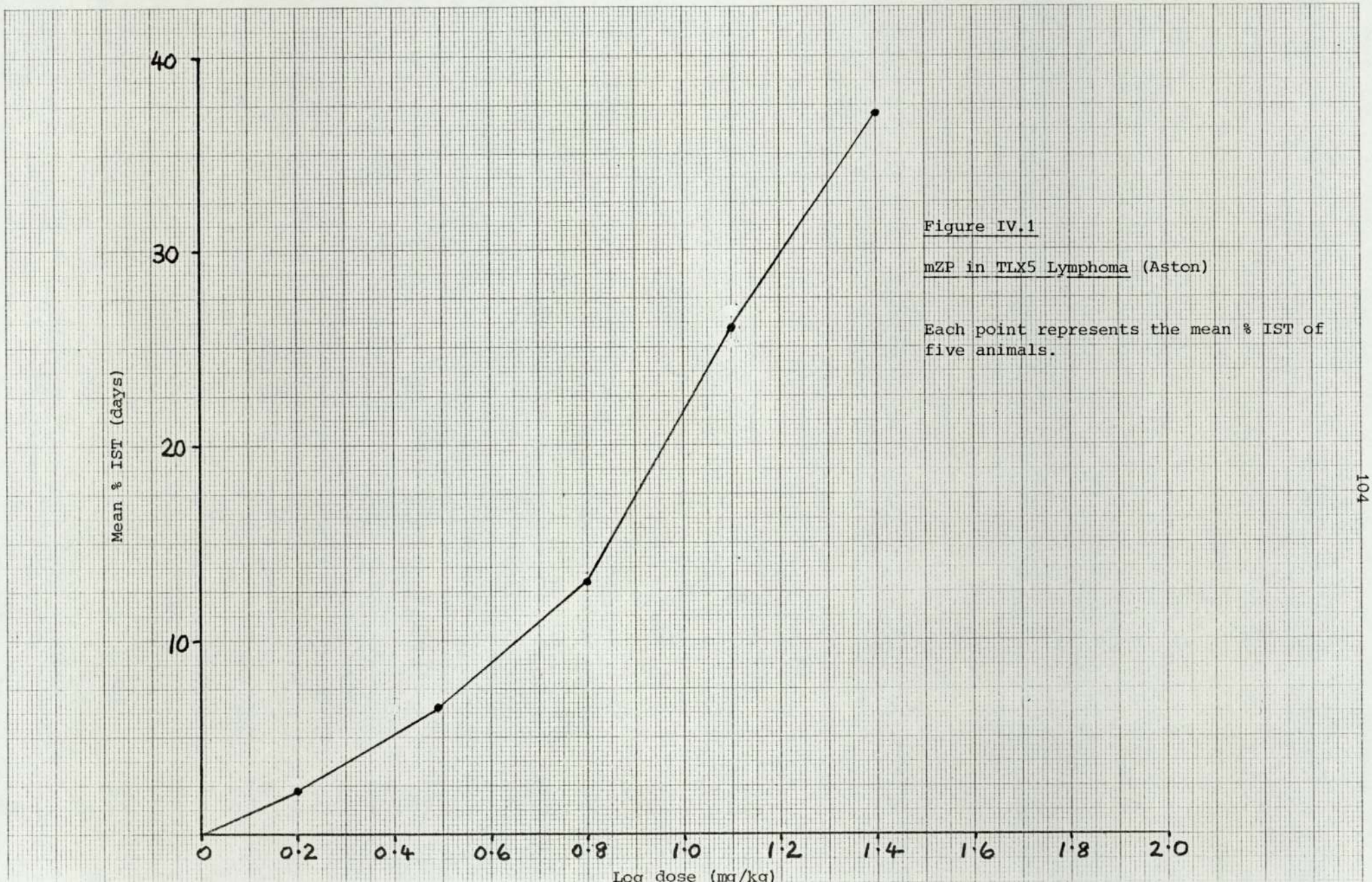


Figure IV.1

mZP in TLX5 Lymphoma (Aston)

Each point represents the mean % IST of five animals.

The NCI have requested a further 25 g of this compound for a more detailed examination and the results are awaited.

(b) Other pyrimethamine analogues

mAP (92) and the 3'-acetylaminopyrimethamine (94) derivatives were found by the NCI to be inactive in the P388 leukaemia model with male CDF_1 mice and under the same protocol as that used for mZP. However, with both of these compounds no toxicity was reported at 200 mg/kg. This contrasts with the findings at ASTON, where toxicity began to occur at 50 mg/kg in CBA/LAC mice.

The 3'-nitro-analogue (mNP : 91), however, was shown to be significantly active in the TLX5 lymphoma model (at ASTON : table IV.3), and in the L1210 lymphoid leukaemia, Walker sarcoma 256 (subcutaneous), P388 lymphocytic leukaemia, and the B16 melanoma of the NCI. Significant activity was seen in the Colon 38 carcinoma model, but no clear dose-response was apparent in either of two separate experiments. No activity was reported for the NCI Lewis Lung carcinoma. The protocols and results for these screens are summarised in tables IV.6(a) and IV.6(b). The compound was administered *via* the intraperitoneal route as a fine suspension in either water/Tween 80 or normal saline/Tween 80.

The potential usefulness of mNP as an anti-tumour agent is further discussed in part V.

TABLE IV.6(a) Screening of mNP by the NCI; experimental protocols

Tumour; animals; assay parameter[b]	Inoculum site; and level (Day 0)	Day of experiment termination[a]	Treatment schedule[c]
L1210; male CDF_1 mice; 2	Intraperitoneal; 10^5 cells	30	A
W256; male rats; 3	Intraperitoneal; 10^5 cells	60	B
P388; male CDF_1 mice; 3	Intraperitoneal; 10^6 cells	30	A
Colon 38; female BDF_1 mice; 7	Subcutaneous; 10^6 cells as a solid tumour fragment	20	C
B16; male $B_6C_3F_1$ mice; 3	Intraperitoneal; 1/10 dilution of homogenate melanoma tissue	60	A

[a] Any animals surviving on this day were killed and examined for signs of disease.

[b] These are given as NCI codes:

2 = Mean survival time

3 = Median survival time

7 = Median tumour weight estimated from tumour diameter

[c] A = nine daily injections starting day 1.

B = five daily injections starting day 1.

C = two injections (total) given on days 2 and 9.

TABLE IV.6(b) Screening of mNP by the NCI. Results

Tumour test system[c]: assay parameter[d]	Dose (mg/kg)	Toxicity: Survivors on day ( )	Assay parameter evaluations: Test T	Control C	T/C %
L1210:	50	6/6 (5)	11.2	8.6	130
	25	6/6 (5)	11.2	8.6	130
2	12.5	6/6 (5)	10.8	8.6	125
	6.25	6/6 (5)	9.2	8.6	106
	3.13	6/6 (5)	8.7	8.6	101
W256:	50	6/6 (5)	7.0	8.0	87
	25	6/6 (5)	60.0	8.0	750[a]
3	12.5	6/6 (5)	60.0	8.0	750[a]
	6.25	6/6 (5)	12.3	8.0	153
	3.13	6/6 (5)	9.4	8.0	117
	50	4/6 (5)	6.3	7.6	-
P388:	12.5	4/6 (5)	17.0	10.4	163[b]
	6.25	4/6 (5)	15.3	10.4	147
3	3.13	4/6 (5)	12.8	10.4	123
	1.56	4/6 (5)	13.0	10.4	125
	0.78	4/6 (5)	11.0	10.4	105
	50	4/6 (5)	6.0	11.0	-
Colon 38:	100	7/10 (20)	800	1529	52
	50	9/10 (20)	700	1529	45
7	25	10/10 (20)	1120	1529	73
	12.5	10/10 (20)	1584	1529	103
	6.25	10/10 (20)	995	1529	65
Colon 38:	100	0/10 (20)	-	768	-
	50	8/10 (20)	481	768	62
7	25	10/10 (20)	810	768	105
	12.5	10/10 (20)	749	768	97
	6.25	10/10 (20)	592	768	77
	3.13	10/10 (20)	544	768	70
B16:	50	10/10 (5)	21.0	15.9	132
	25	10/10 (5)	20.0	15.9	125
3	12.5	9/10 (5)	20.0	15.9	125
	6.25	10/10 (5)	17.8	15.9	111
	3.13	10/10 (5)	16.8	15.9	105
	1.56	10/10 (5)	17.0	15.9	106

[a] 750 is a maximum value in this test and indicates cures.

[b] 288 is the maximum value in this test.

[c] See text.

[d] 2 = mean survival time
3 = median survival time
7 = median tumour weight estimated from tumour diameter.

(2) Results for other compounds tested by NCI

ZQ (4) was found to be inactive against the P388 lymphocytic leukaemia (see table IV.7(a) and (b)). However, it may be significant to note that this compound was tested by NCI as the base which is water insoluble, rather than as the hydrochloride which is water soluble having a K_I for L1210 DHFR of < 58nM (part III). Precipitation may have occurred in the peritoneum from the single injection of the base given as a suspension.

In the aryl-*s*-triazine series, compounds (60-63) and (71-73) were inactive against the L1210 leukaemia model in mice. Neither *E.coli* DHFR nor L1210 DHFR were inhibited by any of the six screened sulphonamide derivatives in the aryl-*s*-triazine series (part III). However, the four *ortho* analogues (64, 66, 68, 70) each showed significant activity in the W256 model (table IV.7(a) and (b)). Results for the *para* analogues (65, 67, 69) are awaited.

The anti-tumour activities of many of the dihydrotriazines are known by the NCI but not published. However, the novel mZG (39) and its precursor (36) were significantly active in the W256 screen (table IV.7(b)) which is not normally sensitive to MTX [112]. On the basis of its pronounced activity in this screen together with its water solubility, mZG was submitted to the Imperial Cancer Research Fund (ICRF) for further detailed evaluation as a potential ATRAR for which the results are awaited. Corresponding *in vivo* results for the *ortho* and *para* isomers of this compound, oZG and pZG (38 and 40), both candidate ATRAR, are also awaited.

Most of the compounds found to be active *in vivo* were also found to be inhibitors of DHFR (L1210 and *E.coli* : part III).

However, the Dimroth-rearrangement product of the 3'-nitrophenyl-dihydrotriazine, compound (50), while showing no activity against DHFR (L1210 and *E.coli*) was significantly active against the W256 carcinoma of NCI (table IV.7(b)).

TABLE IV.7(a) NCI Protocols

Tumour: animals: assay parameter	Inoculum site and level (Day 0)	Day of experiment termination[a]	Treatment Schedule
P388; male CDF_1 mice; 3	Intraperitoneal $x\ 10^6$ cells	30	D
W256; male rats; 3	Intraperitoneal $x\ 10^5$ cells	60	B

[a] Animals surviving on this day were killed and examined for signs of disease: this day marks a maximal survival time.

3 = Median survival time.
D = One injection on day 1.
B = Five daily injections, starting day 1.

TABLE IV.7(b) NCI Results

Tumour test system[a]	Compound number and Ref.	Vehicle or solvent used for administration	Dose (mg/kg)	Toxicity: Survivors on day ( )		Median Survival Time of: Test	Control	T/C %
P388	4 (ZQ)	hydroxypropyl-cellulose	400	0/6	(5)		11.9	
			200	6/6	(5)	12.0	11.9	100
			100	6/6	(5)	11.8	11.9	99
W256	50	Water/Tween 80	100	6/6	(60)	10.4	7.6	136
			50	6/6	(60)	8.2	7.6	107
			25	6/6	(60)	7.4	7.6	97
			12.5	6/6	(60)	7.4	7.6	97
			6.25	6/6	(60)	8.1	7.6	106
W256	36	Aqueous solution	200	0/6	(5)	-	7.6	-
			100	6/6	(5)	12.7	7.6	167
			50	6/6	(5)	14.3	7.6	188
			25	6/6	(5)	12.0	7.6	157
			12.5	6/6	(5)	8.4	7.6	110
W256	39 (mZG)	Aqueous solution	100	2/6	(5)	0.0	7.6	
			50	5/6	(5)	60.0	7.6	789[b]
			25	6/6	(5)	14.3	7.6	188[c]
			12.5	6/6	(5)	9.0	7.6	118
			6.25	6/6	(5)	8.0	7.6	105

/cont.

TABLE IV.7(b) (cont.)

Tumour test system[a]	Compound number and Ref.	Vehicle or solvent used for administration	Dose (mg/kg)	Toxicity: Survivors on day ( )		Median Survival Time of: Test T	Control C	T/C %
W256	64	Water/Tween 80	400	6/6	(60)	18.0	7.6	236
			200	6/6	(60)	11.3	7.6	148
			100	6/6	(60)	8.8	7.6	115
			50	6/6	(60)	9.0	7.6	118
W256	70	Water/Tween 80	100	6/6	(60)	11.0	7.6	144[c]
			50	6/6	(60)	12.0	7.6	157
			25	6/6	(60)	8.4	7.6	110
			12.5	6/6	(60)	8.4	7.6	110
			200	5/5	(5)	13.5	7.5	180
			100	5/5	(5)	9.5	7.5	126
			50	5/5	(5)	9.5	7.5	126
			25	5/5	(5)	8.5	7.5	113
W256	68 (oZST)	Water/Tween 80	100	4/4	(60)	10.0	7.6	131
			50	4/4	(60)	11.0	7.6	144
			25	4/4	(60)	8.0	7.6	105
			12.5	4/4	(60)	9.0	7.6	118
			6.25	4/4	(60)	10.8	7.6	142
W256[d]	66		200	6/6	(60)	[e]	[e]	194

[a] See accompanying table IV.7(a): [b] 789 is a maximum value in this test (see table IV.7(a)): 5/6 cures reported: no traces of disease found: [c] 1/6 cure reported: [d] female rats: [e] T and C figures unavailable

PART IV CHAPTER 2

ATRAR hypothesis testing with the W3129 myeloma (WELLCOME)

The five azide DHFR inhibitors chosen for anti-tumour evaluation against the myeloma were ZQ (4), ZSQ (14), mZG (39), pZG (40) and mZP (93). Groups of five animals were used for each compound/dose level and antitumour effectiveness was measured by determining with callipers the diameter of the tumour. For all measurements allowance was made for the skin thickness (2 mm). The compounds were administered intraperitoneally as either a fine suspension or solution, as indicated, on the days shown after inoculation at day 0 with 5×10^5 cells. 25% DMSO/arachis oil in a total injection volume of 0.2 ml when injected into otherwise untreated animals was found to cause neither toxic side effects nor deviation from the untreated (control) tumour growth rate.

Initially the compounds were screened by administration on days 2, 5 and 8 at 50 mg/kg. The significant tumour inhibition by ZSQ was qualitatively reproducible, although in the experiment whose results are shown in fig IV.2 and table IV.8 the tumour growth rate (after initial growth suppression) was not uniform in the test group and this is reflected by the large standard errors (e.g. tumour diameter on day 14 was 2.20 mm $\pm$ 1.19 mm).

In the same protocol ZQ and mZG showed significant though much less activity than ZSQ (table IV.8: fig IV.2), and pZG killed all the animals after the second injection on day 5 and was therefore not included in the table or figure. When mZP was injected as a fine suspension in water it consistently showed no activity at 50 mg/kg, but when given as a single dose of 100 mg/kg as a fine suspension

GROUP	Compound[a]	Mean tumour diameter (mm, with s.e.) measured on days below after inoculation[g]						T/C % (mean)[f]				
		6	8	9	12	14	19	6	8	9	12	14
I	Untreated	1.02 ± 0.28	2.40 ± 0.17	5.80 ± 0.48	9.22 ± 0.14	11.22 ± 0.19	> 13[e]					
II	ZSQ[b]	0	0	0	0.77 ± 0.77	2.20 ± 1.19	10.13 ± 0.54	0	0	0	8	20
III	mZP[c]	0	2.12 ± 0.14	5.08 ± 0.55	9.22 ± 0.22	11.02 ± 0.29	> 13	0	88	88	100	98
IV	mZG[d]	0	1.08 ± 0.50	4.50 ± 1.30	8.05 ± 0.65	9.50 ± 0.50	> 13	0	45	78	87	85
V	ZQ[d]	0.24 ± 0.24	1.22 ± 0.24	4.46 ± 0.29	8.52 ± 0.75	10.30 ± 0.60	> 13	24	51	77	92	92
VI	6-MP[c]	0	0.72 ± 0.30	2.94 ± 0.26	7.52 ± 0.55	9.08 ± 0.10	> 13	0	30	51	82	81

TABLE : IV.8

[a] All compounds at 50 mg/kg in a total volume of 0.2 ml, given on days 2,5,8: [b] Injected as fine suspension in 25% DMSO/water: [c] Injected as fine suspension in water: [d] Aqueous solution: [e] Tumour diameters > 13 mm not accurately measurable: [f] Represents % of inhibited/uninhibited tumour growth resulting from treatment: [g] Inoculation on day 0 with 5×10^5 cells.

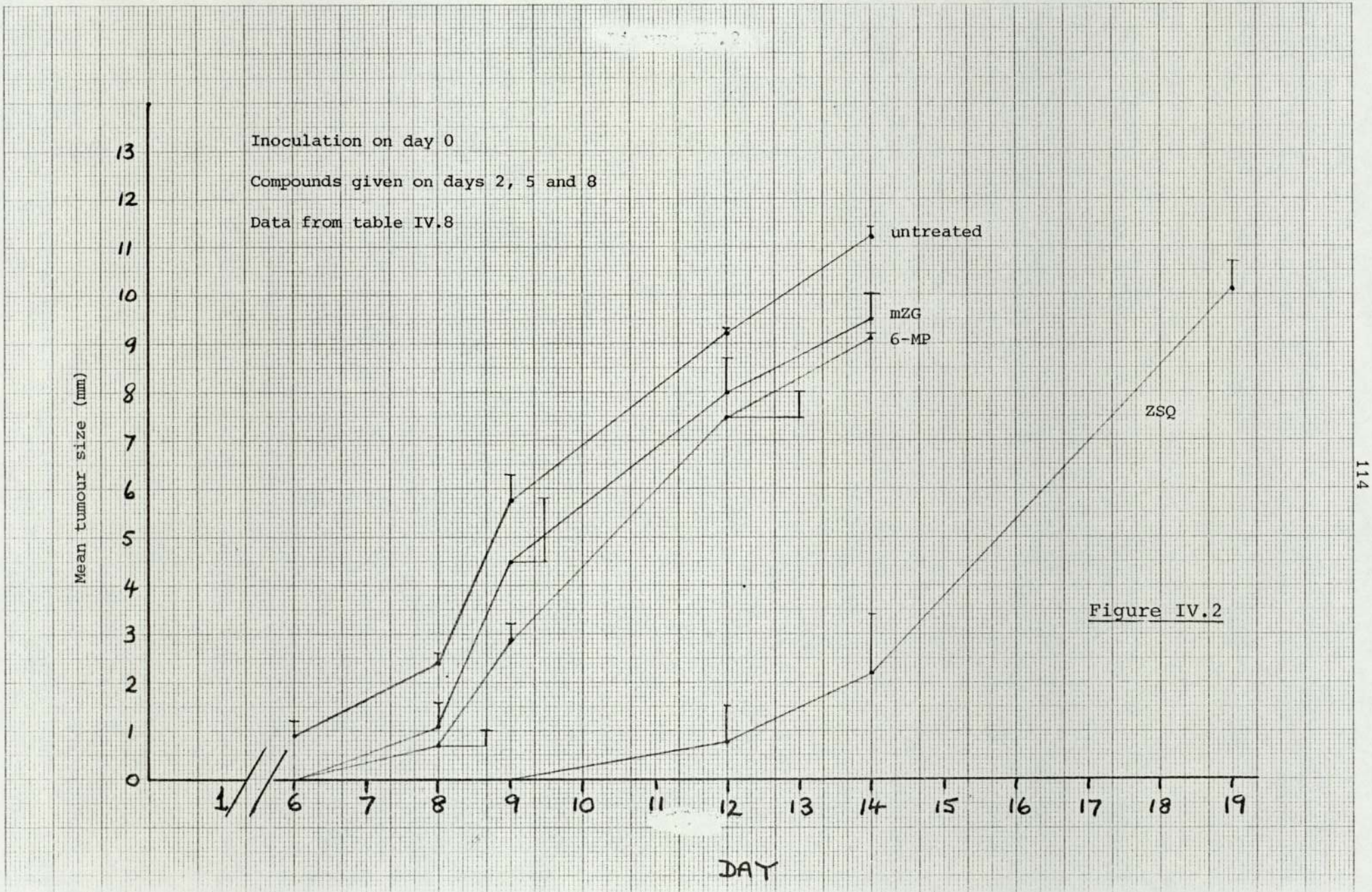

Inoculation on day 0
Compounds given on days 2, 5 and 8
Data from table IV.8
untreated
mZG
6-MP
ZSQ
Mean tumour size (mm)
DAY
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19

Figure IV.2

in 25% DMSO in water or arachis oil on day 2, it showed significant activity (table IV.10). Therefore it appears that the DMSO increases the bioavailability of the compound.

In some cases the standard errors may reflect an inconsistent bioavailability. ZSQ and mZP are markedly insoluble in water and the possibility exists that these compounds may be precipitated in the peritoneum effecting decreased or irregular bioavailability despite the formulations containing 25% DMSO with or without arachis oil.

In a similar protocol, pZG at 5 mg/kg showed neither activity nor toxicity, while ZSQ at 25 mg/kg remained more active than 6-MP at 50 mg/kg (table IV.9).

TABLE IV.9

Compound	T/C %[a] (mean tumour diameter) measured on days:					
	6	8	9	12	14	16
6-MP[b]	0	75	89	88	86	87
pZG[c]	95	104[e]	108[e]	84	82	93
ZSQ[d]	0	45	50	65	69	80

[a] T/C % as in table IV.8.
[b] 50 mg/kg : [c] 5 mg/kg : [d] 25 mg/kg.
[e] treated-tumour growth exceeds that of untreated tumour.

Of the five azides tested against the W3129 myeloma with no irradiation, ZSQ and mZP showed the most promising activity; of these two, mZP was chosen for the *in vivo* testing of the radiopotentiation effect (ATRAR hypothesis) for the following reasons. Firstly, this compound when injected in a 25% DMSO/arachis oil vehicle as a single dose of 100 mg/kg showed activity comparable with ZSQ (see table IV.10).

TABLE IV.10

Group[a]	Compound[b]	Mean tumour diameter (mm, with S.E.) on days: 8	10	11	14
I	Untreated	6.2 ± 0.7	10.3 ± 0.5	> 13.0 -	c -
II	ZSQ	2.4[d] ± 0.8	8.7[e] ± 0.9	11.2 ± 0.1	> 13.0 -
III	mZP	3.6 ± 0.3	8.5 ± 0.3	9.9 ± 0.2	> 13.0 -

[a] Five mice per group.

[b] Given day 2, 100 mg/kg, 25% DMSO/arachis oil, i.p.

[c] Tumours having diameters > 13 mm were not accurately measurable.

[d] $P < 0.005$ [e] $P < 0.01$.

Secondly, since the tissue persistence characteristics of ZSQ and mZP were unknown, it was reasoned that mZP might persist as long as its analogues metoprine and etoprine, whose plasma half-lives are known to be long, the data in the table below being taken from ref [45].

TABLE IV.11

Species comparison of plasma half-lives (hrs) of pyrimethamine analogues

Compound	Rat	Dog	Man
Pyrimethamine	9	35	92
Metoprine	18	57	180
Etoprine	12	36	unknown

A 24h delay between the injection of mZP and δ-irradiation was chosen for a pilot experiment.

For optimal experimental design the dose of compound and irradiation should separately effect minimal inhibition of tumour

growth, in order that any potentiation (rather than a simple additive effect) might be demonstrated. In the pilot experiment four groups of five mice were used, each being inoculated with 5×10^5 cells on day 0 and respectively receiving no treatment, 100 mg/kg mZP in 25% DMSO/ arachis oil on day 2, 400R γ-irradiation to the tumour site on day 3, and both 100 mg/kg mZP (day 2) and 400R γ-irradiation (day 3).

The mice were immobilised prior to irradiation with 0.01 ml/g body weight of 2% Avertin[a] in 10% absolute alcohol since physical restraint was difficult, and the irradiation was carried out with the individual animals passively spread-eagled in a well aerated perspex chamber (12 x 8 x 3 cm) and the whole body except the tumour-site shielded with 6 cm lead.

From table IV.12 and figure IV.4 it can be seen that for a simple additive effect the mean tumour diameter on (say) day 11 would be expected to be 7.8 mm. However, the observed mean diameter was 3.8 mm. Thus these preliminary results are not inconsistent with a potentiation mechanism.

These data indicate that such potentiation might be more clearly demonstrated by the separate and combined use of < 100 mg/kg of mZP and about 100R localised irradiation. In retrospect, since Nichol *et al* [45] have shown that etoprine has a plasma half life of 12h in the rat, irradiation might be better scheduled after 12h rather than after 24h.

These preliminary data would suggest that further experiments are warranted.

[a] 2-tribromoethanol in amylene hydrate

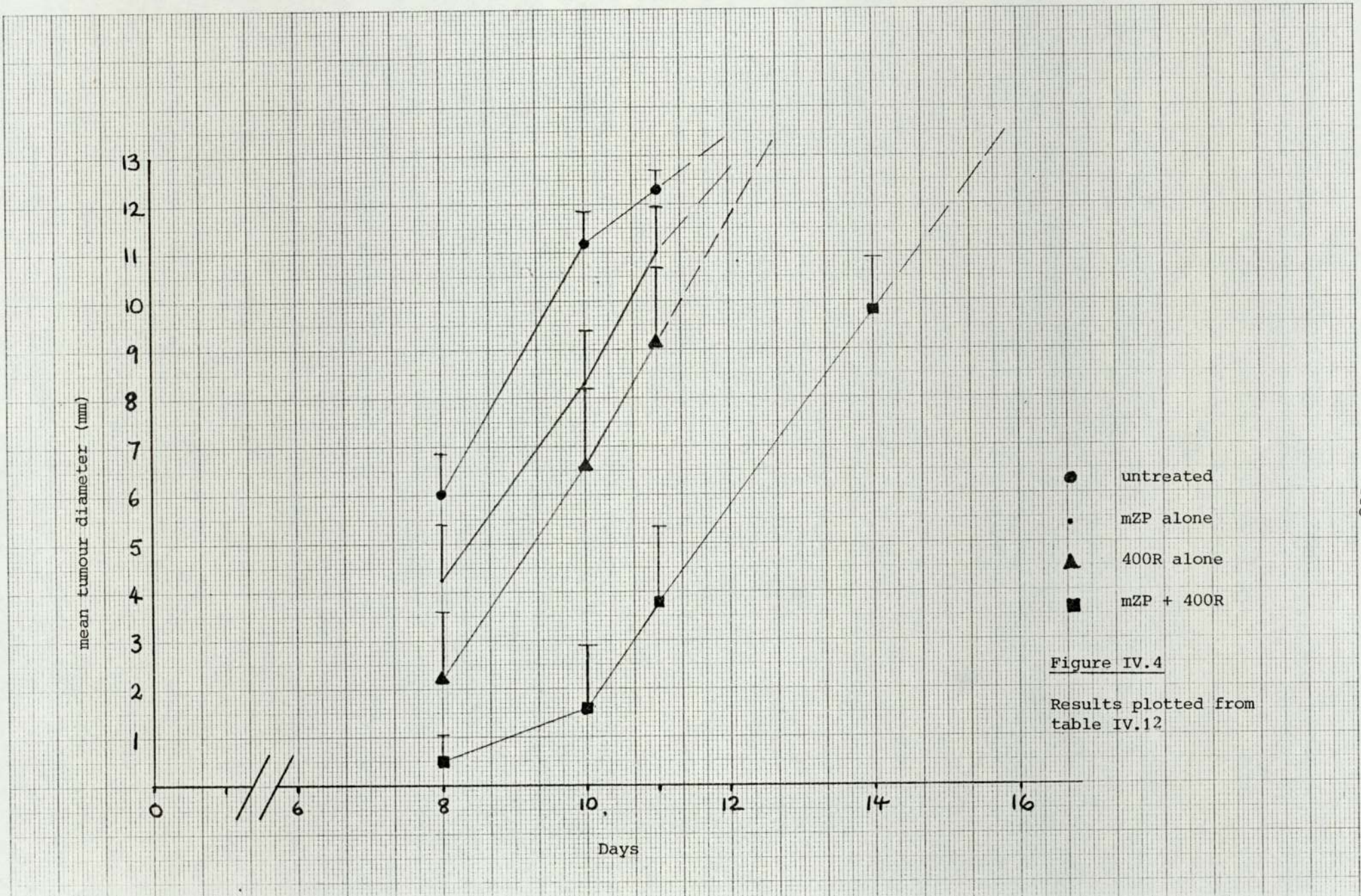


Figure IV.4

Results plotted from table IV.12

TABLE IV.12 Radiopotentiation: Pilot experiment

Group	Treatment[b]	Mean tumour diameter (mm, with S.E.) on days: 8	10	11	14	21
I	Untreated	6.0 ± 0.88	11.2 ± 0.68	12.3 ± 0.4	a	a
II	δ	2.2 ± 0.139	6.6 ± 1.62	9.1 ± 1.69	a	a
III	Z	4.2 ± 1.20	8.3 ± 1.09	11.0 ± 0.98	a	a
IV	δ + Z	0.5 ± 0.52	1.6 ± 1.30	3.8 ± 1.57	9.8 ± 1.04	a

[a] Tumours having diameters >13mm were not accurately measurable.

[b] See text for protocol:
δ = 400R δ-irradiation
Z = 100mg/kg mZP

Since mZP at >100mg/kg was found to show no toxicity in CBA/LAC mice while pyrimethamine and its 3'-substituted analogues mAP, mNP, etoprine and 3'-acetylamino-pyrimethamine showed acute toxicity between 25 and >50mg/kg (at ASTON), it was considered possible that a whole body δ-irradiation dose of 400R, causing no illness or having no toxic effects (e.g. immunosuppression), might be sufficient to radiolyse the aryl azide of mZP affording a more toxic species *via* the nitrene. However, with BALB/c mice it was found that neither the administration of mZP (in 25% DMSO/arachis oil) at 100 or 200mg/kg followed by 400R whole-body irradiation after a ½h or 24h delay nor their administration separately produced ill effects in groups of five healthy mice.* This is in direct contrast with the effects of combined and separate treatments on the W3129 tumour

*after 3 weeks.

growth shown in figure IV.4, where it can be seen that combined treatment led to a more than additive inhibition of tumour growth.

There are three possible reasons for the lack of observed toxic effects in normal control animals:- (i) Normal tissue is more resistant to the combined or separate effects of γ-irradiation and mZP because the growth rate and biosynthetic requirements are less (i.e. less sensitive target sites). (ii) as before, normal tissue is more resistant to the separate effects due to (i). In addition, since it is possible that the nitrene produced by the combination of mZP and γ-irradiation initiates a free-radical chain-reaction (giving perhaps $O_2^{\cdot -}$ radicals), normal tissues may be more resistant to the combined effect because their free-radical scavenging mechanisms (e.g. superoxide dismutase) are adequate. In tumours such free radical scavenging mechanisms have been found to be deficient[113]. (iii) Normal tissue is more resistant to the separate effects due to (i), and to the combined effect due to these concentrations not being toxic to it. Antitumour activity, however, could be achieved by selective concentration of mZP by the tumour. This may be effected by an above normal biosynthetic requirement of the tumour [as in (i)], or by above normal levels of DHFR to which the compound is targeted.

PART V

Conclusions

Were a candidate RAR (azide) bound only moderately to DHFR, the radiolytically generated nitrene intermediate would probably react preferentially with water and other intracellular nucleophilic sites. Since the RAR hypothesis could not be demonstrated directly using isolated enzyme, in this work, recourse had to be made to its indirect demonstration *in vivo*. However, only one pilot experiment was performed. Therefore the following two experiments (using the W3129 myeloma implant system at WELLCOME) are planned:

Firstly, in order to demonstrate conclusively that any radiopotentiation takes place, it is necessary to find irradiation and compound doses which separately cause minimal inhibition of tumour growth (see fig.IV.4 and preceding page), in order that the combined (greater than additive) effect of γ-irradiation and azide might be more clearly shown.

Secondly, in order to demonstrate that the nitrene species is the destructive agent it is necessary to show that no similar potentiation is produced (in the animals) either by interaction between γ-irradiation and a *non*-azide, or by the injection of an azide solution that had been irradiated *prior to injection* (i.e. by the injection of radiolysed azide).

That any such radiopotentiation operates *via* the specific mechanism of RAR-covalent binding to DHFR is inferred from the correlation between reversible DHFR inhibition and pre-irradiation-antitumour activity. Were a compound to show a selective anti-tumour effect but only a weak potency as a DHFRI, then any radiopotentiating

effect exhibited would indicate the radiolytic transformation of the azide to a more cytotoxic derivative whose site(s) of action were unknown.

Little has been published regarding the toxicity and metabolic fate of the arylazido compounds. However, the aliphatic azido group of azidomorphine (i.e. 6-deoxy-6β-azidodihydromorphine) has been shown to be relatively stable towards enzymic and chemical change in man [114]. Azidomorphine was excreted in the urine in small amounts as the unchanged azide, the corresponding primary amine, and in larger amounts as the conjugated azidomorphine [114]. In the rat, azidomorphine is both conjugated with glucuronic acid and N-demethylated, but the azido group resists biotransformation [115]. In the present work, four 4-azidobenzenesulphonamides have been shown to be excreted in the urine mainly as the corresponding arylamine together with smaller amounts of the acetylated amine and unchanged azide (Part IV.1).

The organic azido group exerts -I and +M electronic effects comparable to bromo and acetamido substituents [116]: in size and lipophilicity it closely resembles the bromo group [117]. Therefore it became of some interest to compare *m*ZP with metoprine and etoprine. These two compounds are lipid-soluble potent DHFRI and are able to cross cell membranes rapidly and independently of the MTX/N^5-methyl FH_4/N^5-formyl FH_4 transport pump [14,118,119,48,120-122]. Lipid-soluble potent DHFRI such as metoprine have potential for the treatment of tumours in fatty tissues (e.g. brain [123] and breast [120]) which are inaccessible to MTX. In addition, metoprine has been used with success clinically against tumours that are resistant to MTX [45,120,121,124]. Etoprine and *m*NP (91) were also synthesised by WELLCOME more than ten

years ago as potential anti-tumour agents but were discarded as too toxic for clinical use, although renewed interest has been shown in etoprine and this is now a candidate for human studies [45]. Metoprine and etoprine have been found to be potent inhibitors of histamine N-methyl transferase[125,126](HMT: EC 2.1.1.8) and recent spectulation[126-128] has linked their toxicity with HMT inhibition. During the course of this work both *m*NP and *m*ZP have been found to possess anti-tumour activity *in vivo* (part IV). How much of this activity depends upon DHFR inhibition, or even a differential inhibition of DHFR and HMT, remains to be elucidated.

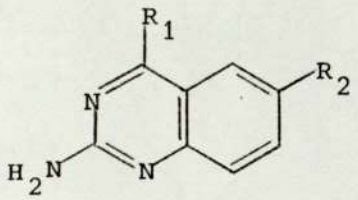
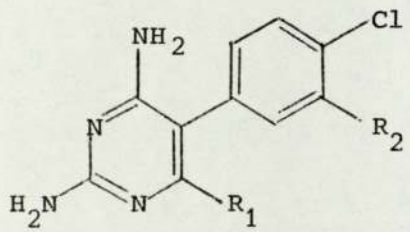
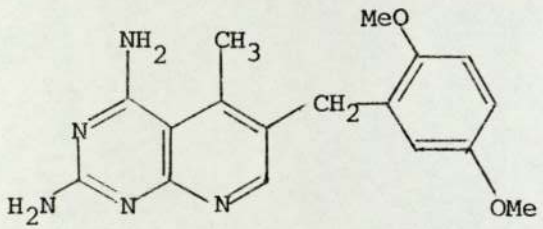
Despite its long plasma half life the toxicity of metoprine has been at least partially circumvented by the *concurrent* administration of folinic acid (CF), i.e. by CF-*protection* rather than by CF-*rescue*, by Hill, Price and co-workers [52, 120, 129-132], and these authors feel that the long half-life is actually advantageous[124,133].

Recently certain 6-substituted pyridopyrimidines such as BW301U (see table V.1) have been found to be both moderately lipophilic and much more potent DHFRI than metoprine, etoprine or pyrimethamine[45,134] and have the advantage of being relatively weak inhibitors of HMT[128,134, 135].

It has been documented that increasing activity of DHFR in the brain correlates with the course of primary tumour growth. Therefore the presence of DHFR in neoplasms of the CNS is relevant to the development of DHFRI which, unlike MTX, can pass the blood brain barrier[45,136].

The relative lipophilicity of several related 2,4-diamino-pyrimidines are shown in table V.1. It can be seen that although

TABLE V.1

Structure	R_1	R_2	Name	Log P Calculated[a]	Log P Determined	pK_a*
	NH_2	H	(1)	1.00		8.06[d]
	NH_2	N_3	ZQ (4)	1.46		7.53[d]
	NH_2	$NH.SO_2.C_6H_4.N_3(p)$	Structure (13)	1.68		-
	OH	$NH.SO_2.C_6H_4.N_3(p)$	ZSQ (14)	2.24		-
	Et	H	pyrimethamine	2.48	2.69[b]	7.34[e]
	Me	Cl	metoprine	2.73	2.82[b]	7.15[e]
	Et	Cl	etoprine	3.19	3.19[b]	7.20[e]
	Et	N_3	*m*ZP (93)	2.94		7.29[d]
	Et	NO_2	*m*NP (91)	2.20		7.02[d]
	Et	NH_2	*m*AP (92)	1.25		7.60[d]
	Et	$NH.SO_2.C_6H_4.N_3(p)$	Structure (102)	3.16		-
	Me	$NH.SO_2.C_6H_4.N_3(p)$	Structure (103)	2.70		-
			BW301U		1.74[c]	
Structure shown in Chapter II.1			MTX		-1.85[b]	5.7[f]

[a] Using Hansch values of Log P: Ref. 137 . [b] Octanol/water partion coefficients: Ref. 45 . [c] Octanol/water partion coefficients: Ref. 128 . [d] Determined in water, at 25°C: Ref. 57 . [e] Determined in water, at 20°C: Ref. 45 . [f] See Ref. 138. * Refers to N1 of heterocycle.

BW301U has a degree of lipophilicity it appears to cross the blood brain barrier poorly[135] in marked contrast to the strongly lipophilic pyrimethamine[46,47], etoprine[45] and metoprine[45,47,139]. This could well be due to steric effects. Since the azido group of *m*ZP has approximately the same size and physical properties as a bromo group, and since *m*ZP has a greater lipophilicity than metoprine, it is likely that *m*ZP will cross the blood brain barrier and penetrate fatty tissues. MTX, which is *hydrophilic*, can only be injected directly into the CSF.

Thus, *m*ZP enjoys the great advantages of being lipophilic, of having molecular dimensions similar to those of etoprine, of showing little toxicity in rodents relative to pyrimethamine and etoprine, of possessing pronounced antineoplastic and DHFR inhibitory activity, while at the same time being an agent that is capable of interacting with $^{\gamma}$-irradiation to produce selective anti-tumour effects. From this starting point two plans of action are envisaged: firstly, the potency of *m*ZP as an HMT inhibitor and the plasma half-life must be determined. Secondly, the toxicity of this compound relative to metoprine and BW301U has yet to be determined rigorously.

From the table V.1 it can be seen that the predicted lipophilicity of the *p*-azidobenzenesulphonamido analogue structures (to be prepared) of etoprine (102) and metoprine (103) are high. Kendrew models of these structures have been built and a binding mode has been postulated for *E.coli* DHFR.

ZSQ (14) is predicted to possess greater lipophilicity than ZQ (4). This compound (14), 2-amino-4-hydroxy-6-[4-azidobenzene-sulphonamido]-quinazoline (or its keto tautomer) was found to be a weak

DHFRI (K_I = 90nM) relative to compounds in the pyrimethamine series (K_I in the $<$10nM range), and was also less active than its close structural analogue, 2,4-diamino-6-[4-acetylaminobenzenesulphonamido]-quinazoline (12) (see table III.6). This is probably due to the fact that the 2,4-diaminoheterocycles are more strongly basic than the corresponding 2-amino-4-hydroxy compounds, and are protonated (first) at N1[85, 140]. This effects greatly enhanced binding to DHFR[15,85,140]. Despite its activity as a DHFRI, ZSQ was shown to be significantly active in the W3129 screen at WELLCOME. It would therefore be interesting to synthesise the 2,4-diamino compound (structure 13) in order to ascertain its activity both against DHFR and against the W3129 myeloma. Almost certainly this compound (13) may be expected to be a DHFRI with a K_I of an order of magnitude lower than that of ZQ ($K_I \simeq$ 56nM) since the *p*-nitrobenzenesulphonamido (10) and the *p*-acetylaminobenzenesulphonamido (12) analogues bind an order of magnitude more strongly than the nitro (2) and amino (3) analogues, respectively.

It is interesting to note that in the dihydrotriazine series, the Dimroth-rearrangement product (50) while showing no DHFR inhibition, showed antitumour activity in the W256 screen of the NCI. Similarly, (64, 66, 68 and 70) showed negligible inhibition of L1210 DHFR but possessed very marked activity in the NCI W256 screen. *m*ZG (39) and *p*ZG (40) were equipotent DHFRI but only *m*ZG showed antineoplastic activity (in the W256 screen of the NCI). ZQ (4) showed no activity against the W3129 myeloma system despite its low K_I for L1210 DHFR of about 55nM. No conclusions can be drawn from the single result from NCI (part IV). The aryl-*s*-triazines tested were neither potent DHFRI nor active in any of the NCI screens.

Hyperbaric O_2 and irradiation is selective probably because tumour cells but not normal cells are deficient in $O_2^{\cdot}$ radical scavengers, SOD[113,141]. However, this selectivity is not high although normal tissues can be further protected by SOD injections[142].

Misonidazole (MIS) and irradiation is selective due to the hypoxic nature of tumour cells, allowing increased production of free radical $MIS^{\cdot}$ which is *also* less well scavenged, although this advantage is not always present. Metastases are not necessarily hypoxic and there is a further drawback in the neurotoxicity of the large doses of nitroimidazoles which have to be given for maximal radiosensitisation[3]. Single oral doses of 140 mg/kg of misonidazole have been tolerated before toxic effects became unacceptable[143]. In contrast, the activity of DHFR-targeted azides is not dependent on the absence of oxygen. From the results in part IV.2 it appears that the combined activity of γ-irradiation and *m*ZP is only expressed in tumour cells, and therefore the intriguing possibility exists that whole body irradiation combined with *m*ZP treatment may afford a means of selectively destroying metastases. It is hoped that this will be tested in whole-body irradiation experiments.

"Photochemotherapy" is not a new concept, and psoriasis has been treated with the combination of long-wave u.v. light (320-400nm) and naturally occurring furocoumarin photosensitisers (e.g. methoxsalen)[144]. Everett *et al*[145] have shown that in caucasians about 44% of incident u.v. irradiation of 320 nm penetrates into the dermis, and that this proportion increases with wavelength. Therefore, in a similar manner it might be possible to use DHFR-targeted azido compounds as *photoaffinity* reagents in the treatment of psoriasis, especially since antifolate chemotherapy with MTX is widely used, and that compounds such as *m*ZP and ZSQ, being lipophilic, would be percutaneously absorbed.

PART VI

Experimental ; Chemistry

Notes

Unless otherwise stated:-

1. All melting points are uncorrected.

2. "Ethanol" refers to 95% ethanol.
   "Acetic acid" refers to 100% acetic acid.

3. I.R. spectra were recorded on a Pye-Unicam SP200 as KBr discs.

4. U.V. spectra were recorded on a Pye-Unicam SP8000.

5. ^{1}H n.m.r. spectra were recorded on Perkin Elmer R12B and Varian A-60A (60 MHz) and Brücker WP80 (80 MHz) spectrometers.
   All spectra were recorded in deuterated dimethylsulphoxide, i.e. [$^{2}H_{6}$]DMSO. The n.m.r. spectra (aliphatic protons only) of compounds in the dihydrotriazine series are recorded in Part II.3.
   All the signals are assigned in p.p.m. downfield of tetramethylsilane (δ).

6. The mass spectra were recorded on VG 7070, MS 902 and Micromass 12B spectrometers, at ambient temperature.
   The mass spectra of all sulphonamide derivatives are recorded in Part II.6.

7. Microanalyses were carried out by the Butterworth Laboratories Ltd., and by the Department of Physical Chemistry, Wellcome Research Laboratories.

8. torr. Unit of low pressure equal to head of 1 mm mercury.

PART VI : Experimentals

CHAPTER 1

2,4-Diaminoquinazoline (1)

Following the literature method this compound was obtained (75%) as long colourless needles, m.p. 247-249° (lit.**55**, 248-250°); ν_{MAX} 1090, 1295, 1625 br, 1662, 3150 br, 3380, 3490 cm^{-1}.

2,4-Diamino-6-nitroquinazoline (2)

By following the literature[55] method an excellent yield of this compound was obtained. Crystallisation from acetic acid (*ca.* 0.5 g in 100 ml) afforded the *acetate* as small primrose prisms. It was not found possible to crystallise the *free base*. Washing the crude reaction product free of inorganic reagents used in the method with water followed by ethanol afforded the partially purified *base* as an orange powder, m.p. 278-280° (lit.**55**, $\nless$ 360°); u.v. as in literature[55]; ν_{MAX} 1350 cm^{-1} (NO_2); δ ([$^2H_6$] DMSO - ^2H_2O, 100°, 80 MHz spectrum) 7.28 (1 H, d, 8-H_1, J_{87} ~ 9.1 Hz), 8.2 (1 H, q, 7-H_1, J_{78} ~ 9.3, J_{75} ~ 2.5 Hz), 8.92 (1 H, d, 5-H_1, J_{57} ~ 2.2 Hz).

2,4,6-Triaminoquinazoline (3)

Finely powdered 2,4-diamino-6-nitroquinazoline (14.0 g) was added in portions to a vigorously stirred mixture of tin(II) chloride dihydrate (50.6 g) in 10N-hydrochloric acid (150 ml) at such a rate that the temperature was maintained below 30°. The mixture was kept at 4° overnight and the white precipitate of triaminoquinazoline stannichloride complex was collected. The solid was dissolved in boiling water (100 ml), cooled, and the solution was adjusted to pH 12 with 10N-sodium hydroxide.

From the filtered solution a mixture of yellow-green felted needles and amber prisms crystallised (92%) which on recrystallisation from water afforded amber prisms, m.p. 251-253° (lit.[55], 255-258°).

2,4-Diamino-6-azidoquinazoline (4)

2,4,6-Triaminoquinazoline (7.2 g) was dissolved in 2N-hydrochloric acid (70 ml) and diazotised at 0° by the addition of sodium nitrite (2.84 g) in water (15 ml). Sodium azide (2.68 g) in water (25 ml) was slowly added to the stirred ice-cold diazonium chloride suspension and the resultant creamy mixture was vigorously stirred for a further 2 h at 0° and left to stand overnight below 5°. The precipitate was collected, suspended in water and basified with 10% ammonia solution to yield *2,4-diamino-6-azidoquinazoline* (98%) which crystallised in the dark from ethanol to give an amorphous cream precipitate, m.p. indefinitely above 135° with charring; *m/e* 175 (100%); *M*, 201 absent; ν_{MAX} 2140 cm^{-1} (N_3): δ 6.65 (2 H, s, NH_2), 7.96 (s, NH_2), 7.85 (d), 8.40 (t).

Found: C, 47.7; H, 3.4; N, 48.7.

$C_8H_7N_7$ requires C, 47.7; H, 3.5; N, 48.8%

2,4-Diamino-6-azidoquinazoline monohydrochloride (4)

The following procedure was carried out in the dark with all glass apparatus well shielded with tinfoil.

2,4-Diamino-6-azidoquinazoline (3 g) was dissolved with heating in 2N-hydrochloric acid (30 ml), filtered and cooled. Primrose yellow flakes or needles of the *hydrochloride* (80%) formed, m.p. indefinitely above 135° with either charring or explosion (depending on the rate of

heating); ν_{MAX} 2120 cm^{-1} (N_3).

Found: C, 40.4; H, 3.4; N, 41.4.

$C_8H_7N_7$,HCl requires C, 40.4; H, 3.4; N, 41.3%

2,4-Diaminoquinazolin-6-yl-diazonium chloride hydrochloride (5)

2,4,6-Triaminoquinazoline (0.5 g) was dissolved in 6N-hydrochloric acid (20 ml) with gentle heating. On cooling to 0° the amine crystallised as a hydrochloride salt. The addition of sodium nitrite (0.3 g) in water (2 ml) in portions over 1.5 h to the well stirred fine suspension gave a clear yellow solution initially and a delicate yellow precipitate (62%) after further stirring for 1 h at 0°. This was collected and dissolved in DMSO (1 ml) and the addition of chloroform (0.5 ml) followed by ether (1 ml) effected a slow precipitation of *a hydrochloride salt of the diazonium chloride*, m.p. < 160° indefinite (explodes); ν_{MAX} 2300 cm^{-1} (N_2^+); λ_{MAX}(H_2O) 226, 279, 395 nm.

2,4-Diaminoquinazolin-6-yl-diazonium tetrafluoroborate (6)

2,4,6-Triaminoquinazoline (1.1 g) was dissolved in 50% aqueous tetrafluoroboric acid (25 ml) using gentle heating and cooled to below 5°. A solution of sodium nitrite (0.48 g) in water (5 ml) was then added dropwise over 1.5 h, and the yellow precipitate (82%) which formed was collected and shaken with cold acetonitrile (5 ml) to leave a beige insoluble fraction. Filtration and washing with dry ether yielded an amorphous powder, *a diazonium tetrafluoroborate complex*, m.p. 197-200°; ν_{MAX} 2300 cm^{-1}; λ_{MAX}(H_2O) 225, 280, 396 nm.

Found: C, 30.1; H, 2.7; N, 23.9.

$C_8H_7N_6.BF_4$,HBF_4,C_2H_3N requires C, 29.8; H, 2.7; N, 24.3%

2,4-Diamino-6-azo-(2-hydroxynaphth-1-yl) quinazoline (7)

A solution of 2-naphthol (0.5 g) in 1N-potassium hydroxide (25 ml) was mixed with an aqueous solution of 2,4-diaminoquinazolin-6-yl-diazonium tetrafluoroborate (0.54 g in 25 ml), whereupon a deep red precipitate formed. The mixture was kept at 4° overnight and the dye was recrystallised from aqueous ethanol as an amorphous solid (15%), m.p. ~ 300°; ν_{MAX} 1570, 1620, 3400 br (OH) cm^{-1}: $\lambda_{MAX}(H_2O)$ 480 nm.

Found: C, 60.3; H, 4.7; N, 22.9%; M^+, 330.122 902.

$C_{18}H_{14}N_6O.1\frac{1}{2}H_2O$ requires C, 60.5; H, 4.8; N, 23.5%; M , 330.123 310.

2,4,6-Triacetylaminoquinazoline (8)

2,4,6-Triaminoquinazoline (0.2 g) and acetic anhydride (10 ml) were refluxed for 0.5 h. On cooling the mixture afforded amber/green crystals (50%) which were recrystallised from water as amber prisms, showing as a single spot on t.l.c., m.p. 278-280°; ν_{MAX} 1680 cm^{-1} (C=O); *m/e* 301 (35%; M^+), *m/e* 259 (100%), *m/e* 217 (100%), *m/e* 175 (85%); δ ([$^2H_6$] DMSO) 2.10 (3 H, s, Ac), 2.25 (3 H, s, Ac), 2.35 (3 H, s, Ac), 7.78 (1 H, d, 8-H, J_{87} 9.2 Hz), 8.12 (1 H, dd, 7-H, J_{78} 9.2, J_{75} 2.2 Hz), 8.49 (1 H, d, 5-H, J_{57} 2.2 Hz), 10.30 (1 H, s, NHCO), 10.5 br (s, NHCO).

Found: M^+, 301

$C_{14}H_{15}N_5O_3$ requires M , 301

2,4-Diamino-6-[4-nitrobenzenesulphonamido] quinazoline (10)

2,4,6-Triaminoquinazoline (1.75 g) and 4-nitrobenzenesulphonyl chloride (2.22 g) were refluxed in dry pyridine (50 ml) for 0.5 h. Water (100 ml) was added to dissolve the precipitate which had formed

and the solution was then basified with ammonia to pH 9. Following reduction in volume by vacuum distillation and cooling in ice, the resulting precipitate was collected (85%). Crystallisation from aqueous DMF and drying afforded an amorphous amber powder, m.p. 308-310°; *m/e* 360 (M^+), 174; ν_{MAX} 1362, 1540 (NO_2), 1660, 3180, 3440 cm^{-1}; δ 6.22 (2 H, s, NH_2), 7.09 (2 H, s, 7,8-H_2), 7.48 (2 H, s, NH_2), 7.66 (H, s, 5-H), 7.92, 8.32 (4 H, dd, benzene 2-H_2 and 3-H_2, AA'BB', J_{AB} 9 Hz).

Found: C, 46.7; H, 3.4; N, 23.3%; M^+, 360.06407

$C_{14}H_{12}N_6O_4S$ requires C, 46.7; H, 3.3; N, 23.3%; M, 360.06355

2,4-Diamino-6-[4-nitrobenzenesulphonamido] quinazoline monohydrochloride (10)

The nitrobenzenesulphonamide base (1.5 g) was dissolved with heating in a minimal quantity of 2N-hydrochloric acid (about 50 ml) and then cooled. The *monohydrochloride* crystallised (80%) as fragile pink flakes, m.p. 305-312°; ν_{MAX} 1170 ($-SO_2-$), 1358, 1538(NO_2), 1682 cm^{-1}.

Found: C, 42.2; H, 3.2; N, 21.1%; M^+-HCl, 360.06407

$C_{14}H_{12}N_6O_4S.HCl$ requires C, 42.4; H, 3.3; N, 21.2%; M-HCl, 360.06276

2,4-Diamino-6-[4-acetylaminobenzenesulphonamido] quinazoline monohydrochloride (12)

Solutions in dry pyridine of 2,4,6-triaminoquinazoline (1.1 g in 100 ml) and acetylsulphanilyl chloride (1.47 g in 25 ml) were mixed together with triethylamine (0.7 g) and maintained at 85-90° for 6 h. Following subsequent removal of the pyridine by vacuum distillation

the residue was dissolved in a minimal quantity of hot ethanol (about 70 ml), filtered and allowed to cool. The resulting fine amber precipitate (1.5 g; 58%) was collected and dissolved in a minimal quantity of hot water (about 25 ml) and rapidly cooled. This procedure yielded the required sulphonamide derivative as a sandy microcrystalline powder, m.p. 195-198°; *m/e* 372 (*M*-HCl), 175, 174; ν_{MAX} 1160 (-SO_2-), 1540, 1598, 1660 cm^{-1}; δ 2.06 (3 H, s, Ac), 7.00-7.90 (m, ArH), 10.30 (1 H, s, Ac NH).

Found: C, 46.6; H, 4.4; N, 20.5%; M^+-HCl, 372.10045

$C_{16}H_{16}N_6O_3S.HCl$ requires C, 47.0; H, 4.2; N, 20.5%; *M*-HCl, 372.10097

2-Amino-4-hydroxy-6-[4-azidobenzenesulphonamido] quinazoline (14)

2,4-Diamino-6-[4-acetylaminobenzenesulphonamido]-quinazoline (1.17 g) was refluxed in 2N-hydrochloric acid (40 ml) for 1 h. The solution was cooled to below 5° and an aqueous solution of sodium nitrite (0.26 g in 1.5 ml) was added dropwise with stirring over 0.5 h. Following further agitation for 0.5 h sodium azide (0.41 g) dissolved in water (2 ml) was added over 10 min. After continuing stirring for 1.5 h the resulting slurry was neutralised with concentrated aqueous ammonia, collected, washed with water and dried at 100°/720 torr to yield a pale brown solid (0.95 g: 93%). Crystallisation from aqueous DMF afforded either amber prisms or fluffy beige microrosettes, m.p. ~ 200° (indefinite, with slow decomposition); *m/e* 176 (37%); ν_{MAX} 2140 infl, 2160 cm^{-1}(N_3); δ ([$^2H_6$] DMSO) 6.80 br (s, OH), 7.23 (s; 7,8-H_2) 7.62 (d), 7.26, 7.76 (dd, benzene 2-H_2 and 3-H_2, AA'BB', J_{AB} 8.3 Hz); δ ([$^2H_6$] DMSO-^2H_2O) 7.39 (d, 5-H_1), 7.22, 7.76 (dd, AA'BB'): ratio of exchangeable protons:non exchangeable protons was 4:7.

Found: C, 47.2; H, 3.4; N, 26.4.

$C_{14}H_{11}N_7O_3S$,½DMF requires C, 47.2; H, 3.6; N, 26.7%

Found: *m/e* 176.0697

$C_8H_8N_4O^+$ requires *m/e* 176.0698

2-Amino-4-hydroxy-6-[4-azidophenylsulphonamido] quinazoline monohydrochloride (14)

Recrystallisation of the free base (14) from 2N-hydrochloric acid gave cream microsettes of the *monohydrochloride*, m.p. 180-190° (indefinite); ν_{MAX} 2160 cm^{-1} (N_3).

Found: C, 42.8; H, 3.3.

$C_{14}H_{11}N_7O_3S$,HCl requires C, 42.7; H, 3.1%

2-amino-4-hydrazinoquinazoline (21)

2,4-Diaminoquinazoline (1.25 g) was refluxed in hydrazine hydrate (15 ml) for 2 h. On cooling yellow prisms were deposited. Recrystallisation from pyridine gave pale pea-green prisms of the *monohydrazino* compound, m.p. 206-208°; *m/e* 175 (11%), 160 (10%); ν_{MAX} 1620, 1640, 3100 br, 3300, 3490 cm^{-1}.

Found: C, 54.8; H, 5.1; N, 40.6%; M^+, 175.08579

$C_8H_9N_5$ requires C, 54.9; H, 5.1; N, 40.0%; M , 175.08547

2,4-Dihydrazinoquinazoline (22)

2,4-Diaminoquinazoline (1.25 g) was refluxed in hydrazine hydrate (15 ml) for 6 h and cooled. The yellow crystals which formed were collected and recrystallised from ethanol 90% - hydrazine 10% or

from pyridine to afford pea-green prisms of the *dihydrazinoquinazoline*, m.p. 226-228° (lit.[61], 226-227°); *m/e* 190 (M^+; 36%), 175 (16%), 160 (13%); ν_{MAX} 1135, 1360, 1620, 3320 cm^{-1}.

Found: C, 50.6; H, 5.2; N, 44.2%: M^+, 190.096689

$C_8H_{10}N_6$ requires C, 50.5; H, 5.3; N, 44.2%: M, 190.096330

Reaction of 2,4-diamino-6-azidoquinazoline (4) *with hydrazine hydrate*

The azide (4) (0.3 g) was refluxed in hydrazine hydrate (10 ml) for 1.7 h. The crystalline solid which had formed after overnight standing was identical (i.r., t.l.c., m.p.) with an authentic sample of *2-amino-4-hydrazinoquinazoline*.

Attempted synthesis of 2,4-diamino-6-[4-aminobenzenesulphonamido] quinazoline (11)

(i) A suspension of the corresponding 4-nitrobenzenesulphonamido derivative (10) (1.0 g) in ethanol (250 ml) containing Raney nickel (~5 g) was hydrogenated at 50 p.s.i. for 8 h. Following filtration through kieselguhr the ethanol was removed under vacuum to 20 ml. Upon standing at 4° for 2 days small amber prisms were deposited (0.1 g). By passing HCl gas through an ethanolic solution of this crude material a hydrochloride salt was obtained, m.p. 215-218° (eff); ν_{MAX} 700, 842, 1018, 1040, 1125, 1180, 1230, 1410, 1510, 1605, 1630 br, 3200 br, 3350 br cm^{-1}. T.l.c. examination of the filtrate showed the presence of unchanged starting material and product. Evaporation of the filtrate to dryness gave an amber waxy residue. Crystallisation attempts with this residue were unsuccessful.

(ii) A suspension of the corresponding 4-nitrobenzenesulphonamido derivative (10) (1.5 g) in hot ethanol (100 ml) at 55-60° was treated

with Raney nickel (2 g) and hydrazine hydrate (20 ml) over 2 h. The mixture was maintained at 55-65° for a further 1 h, was filtered through a kieselguhr pad and was evaporated under vacuum to afford a waxy residue similar to that obtained in (i) above (i.r., t.l.c.).

Attempted synthesis of N-acetyl-L-(4-hydroxy-3-azo-2',4'-diaminoquinazolin-6'-yl) phenylalanine ethyl ester (20)

No dye was produced when an aqueous solution of 2,4-diaminoquinazolin-6-yl-diazonium tetrafluoroborate (185.8 mg in 8 ml) and an aqueous solution of N-acetyl-L-tyrosine ethyl ester (19) (182.4 mg in 42 ml) adjusted to pH 7 were each filtered and mixed at 0°, or when the resulting mixture was readjusted to pH 7. When the pH had been raised to pH 9 with 5N-NaOH (2 drops) a brown colloidal precipitate was formed whose yield did not increase on standing overnight at 4°. Because solvent extraction attempts were unsuccessful the suspension was neutralised with 5N-hydrochloric acid (2 drops) and after standing 24 h at 4° the precipitate was collected. Recrystallisation attempts using mixtures of DMSO/ethanol and DMSO/acetone failed. Therefore the product was washed in acetone and dried at 100°/720 torr to leave a dark brown powder (96 mg), m.p. $> 375°$; λ_{MAX} (pH 12.5) 360, 450 nm; λ_{MAX} (pH 1.8) 340 nm. Found: C, 44.8; H, 3.9; N, 27.8; O, 23.0%.

Photolysis of 2,4-diamino-6-azidoquinazoline (4)

A solution of the azide (4) (1.33 g) in methanol (1 litre) was photolysed with a 100 watt unfiltered medium-pressure lamp in a Hanovia photochemical reactor for 10 h, during which time N_2 was

evolved. At hourly time intervals during this period the quartz sleeve of the lamp was cleaned free of adhering maroon product so that the photolytic efficiency was minimally attenuated. The presence in the solution of several photoproducts including 2,4,6-triamino-quinazoline (3) was detected with t.l.c. The methanol was removed under vacuum and the residue (0.5 g) was washed three times with hot water, using an MSE centrifuge (20 000 g/0.5 h) to recover the residue from the fine colloidal suspension formed with each wash. Crystallisation was found impossible: the product was sparingly soluble in acetic acid, DMF and diethylene glycol. Sublimation also failed to yield analytical material. This intractable product was dried after the washing at 100°/720 torr as a maroon powder, probably *bis(2,4-diaminoquinazolin-6-yl) diazine* (25), m.p. > 360°;

ν_{MAX} 840, 1140, 1410, 1500, 1550, 1620, 1650, 3180, 3350 cm^{-1};

λ_{MAX} (H_2O) 480 nm (-N=N-); δ (TFA) 8.25, 8.92 (dd, AA'BB', J_{AB} 9.3 Hz), 9.35 (br s).

Found: M^+, 346.

$C_{16}H_{14}N_{10}$ requires M, 346.

Thermolysis of 2,4-diamino-6-azidoquinazoline (4)

(i) A solution of the azide (4) (0.59 g) in nitrobenzene (15 ml) was refluxed for 2 h in a light protected vessel. The solution was then cooled and the precipitate which had formed (0.49 g; 96%) was collected. Washing with ethanol and drying at 100°/720 torr yielded a deep purple microcrystalline solid, m.p. > 360°, the mass spectrum of which showed ions at *m/e* 346 (required *M*, 346) and *m/e* 175, and whose i.r. and u.v. were identical to those of the product formed from the photolysis (above).

When a 10% aqueous solution of sodium dithionite (15 ml) had been added over 30 min to a hot well-stirred fine suspension of this azo compound (0.10 g) in 2-ethoxyethanol/ethanol (15 ml), 2,4,6-triaminoquinazoline (3) was detected (t.l.c.).

(ii) Thermolysis of the azide (4) in refluxing dekalin (8 h) gave 2,4,6-triaminoquinazoline as the major product (t.l.c.).

Photolysis of 2,4,6-triaminoquinazoline (3)

An aqueous solution of 2,4,6-triaminoquinazoline (4 g in 1 litre) was photolysed for 6 h with the Hanovia photochemical reactor previously used for the photolysis of (4). The solid which precipitated was collected (120 mg; 3%), washed three times with hot water to remove starting material, and dried at 100°/720 torr. The product was identical (i.r., n.m.r. and M^{+}) to that obtained by the photolysis of the azide (4).

PART VI CHAPTER 2

Preparation of the dihydrotriazines

For compounds 31, 32, 34 and 35 the three component synthesis involving the condensation under reflux of molar equivalents of the arylamine, hydrochloric acid, dicyandiamide and acetone was used. Similarly prepared were:

1-phenyl-1,2(2H)-2,2-dimethyl-4,6-diamino-1,3,5-triazine monohydrochloride (31) (52%), m.p. 201-203° (lit.63, 200-203°);

1-[3-nitrophenyl]-1,2(2H)-2,2-dimethyl-4,6-diamino-1,3,5-triazine monohydrochloride (32) (62%), m.p. 210-212° (lit.66, 195-196°);

1-[2-chlorophenyl]-1,2(2H)-2,2-dimethyl-4,6-diamino-1,3,5-triazine monohydrochloride (34) (54%), m.p. 217-221° (lit.63, 217-221°);

1-[4-chlorophenyl]-1,2(2H)-2,2-dimethyl-4,6-diamino-1,3,5-triazine monohydrochloride (35) (65%), m.p. 203-205° (lit.63, 210-215°).

1-[4-nitrophenyl]-1,2(2H)-2,2,-dimethyl-4,6-diamino-1,3,5-triazine monohydrochloride (33)

When a mixture of 4-nitroaniline (6.91 g), dicyandiamide (4.20 g), 10N-hydrochloric acid (4.2 ml) and acetone (100 ml) was vigorously stirred in an ice-packed dark-box for 36 h, a microcrystalline precipitate was formed. Washing in cold acetone (in the dark) and drying in a vacuum desiccator gave the required dihydrotriazine as a very pale yellow photolabile powder (61%), m.p. 206-207° (lit. 64, 202-205°); ν_{MAX} 700, 860, 1020, 1352 and 1530 (NO_2), 1600, 1640, 1664, 3000 (aliph. CH) cm^{-1}; no colour reaction was given in solution with tetraminocopper(II) ions.

1-[3-aminophenyl]-1,2(2H)-2,2-dimethyl-4,6-diamino-1,3,5-triazine trihydrochloride, stannicomplex, dihydrate (36)

1-[3-nitrophenyl]-1,2(2H)-2,2-dimethyl-4,6-diamino-1,3,5-triazine monohydrochloride (32) (1.5 g) was added, with stirring and cooling below 30°, to tin(II) chloride dihydrate (3.5 g) in concentrated hydrochloric acid (12 ml). The mixture was stirred for a further 1 h, then allowed to stand overnight at 0°. The white precipitate (3.2 g) was collected and recrystallised from 5N-hydrochloric acid to yield the required *amine stannicomplex* as the *trihydrochloride dihydrate*, m.p. 230°; ν_{MAX} 700, 800, 1622, 1680, 1715, 2600, 2850 br, 3250 br, 3450 br.

Found: C, 21.5; H, 3.4; N, 13.9; Cl, 36.0; O, 5.4.

$C_{11}H_{16}N_6,SnCl_3,3HCl,2H_2O$ requires C, 21.9; H, 3.8; N, 13.9; Cl, 35.3; O, 5.3%

1-[4-aminophenyl]-1,2(2H)-2,2-dimethyl-4,6-diamino-1,3,5-triazine dihydrochloride (37)

To a mixture of dicyandiamide (0.84 g), 4-phenylenediamine dihydrochloride (1.81 g) and acetone (150 ml) was added enough water (50 ml) to effect total solution. After refluxing and cooling the solution the crystalline precipitate (0.37 g; 13.8%) was collected and washed with acetone. A sample was subsequently recrystallised (over 5 days) from aqueous ethanol to afford colourless microcrystals of the *dihydrochloride*, m.p. 296-298° (eff); *m/e* 356 (0.27%), 257 (18%), 232 (3%), 217 (22%), 173 (6%), 158 (14%), 133 (27%), 124 (14%), 117 (8%), 104 (23%), 99 (13%), 91 (22%), 82 (21%), 78 (14%), 67 (44%), 57 (24%), 42 (100%), 38 (31%), 36 (85%), 32 (17%): ν_{MAX} 765, 1020, 1460, 1495, 1535, 1570, 1600, 1642, 1670 infl, 3000, 3180 br, 3320 cm^{-1}: λ_{MAX} (H_2O) 243 nm.

Boiling, cooling and standing for 4 h gave a Dimroth rearrangement product, λ_{MAX} (H_2O, pH 12) 264 nm.

δ (TFA) 1.84 (s, 2 x CH_3).

Found: C, 43.7: H, 6.4.

$C_{11}H_{16}N_6$,2HCl requires C, 43.3: H, 5.9%.

Found: *m/e* 232.1433

$C_{11}H_{16}N_6$ requires *M*, 232.1435

1-[4-aminophenyl]-1,2(2H)-2,2-dimethyl-4,6-diamino-1,3,5-triazine toluene-4-sulphonate (43)

4-Phenylenediamine toluene-4-sulphonate (4.52 g), dicyandiamide (0.84 g), acetone (100 ml) and ethanol (70 ml) were refluxed for 8 h and then cooled. The precipitate that formed (0.5 g: 12.4%) over about 24 h was collected and washed in acetone. Recrystallisation from ethanol and drying afforded the *toluene-4-sulphonate* as an off-white amorphous powder, m.p. 254-255°; *m/e* 272 (6%), 257 (40%), 242 (5%), 232 (1.3%), 217 (13%), 174 (8%), 158 (13%), 149 (9%), 133 (21%), 124 (38%), 107 (16%), 91 (6%), 83 (15%), 67 (16%), 57 (8%), 45 (45%), 42 (100%); ν_{MAX} 690, 830, 1020, 1200 br, 1460, 1500, 1540, 1570, 1620, 1660 br, 3000, 3200 br, 3400 br cm^{-1}; δ 1.40 (6 H, s, 2 x geminal CH_3), 2.30 (3 H, s, tolyl-CH_3), 7.10 and 7.50 (4 H, dd, benzene, AA'BB', J_{AB} 8.3 Hz, 2-H_2), 7.14 and 7.57 (4 H, dd, tolyl CC'DD', J_{CD} 2.7 Hz, 2-H_2).

Found: C, 51.6; H, 5.8.

$C_{11}H_{16}N_6$,$C_7H_8O_3S$,H_2O requires C, 51.2; H, 6.2%

Found: *m/e* 232.143260

$C_{11}H_{16}N_6$ requires *M*, 232.143637

Attempted reduction of 1-[4-nitrophenyl]-1,2(2H)-2,2-dimethyl-4,6-diamino-1,3,5-triazine (33)

(i) To a solution of tin(II) chloride (5.7 g) in 10N-hydrochloric acid (20 ml) held at 30° was added, with vigorous stirring in portions over 1 h, the nitro compound (33) (2.62 g). The mixture was allowed to stand for 4 h and was then filtered. A solution of the solid residue (4.9 g) in water (25 ml) was carefully neutralised with sodium hydroxide at 50° and cooled. T.l.c. examination of both this neutralised solution and of the initial filtrate revealed the presence of 4-nitrophenylbiguanide and two other unidentified products.

(ii) To a solution of the nitro compound (33) (1.9 g) in ethanol (20 ml) at 50° was added Raney nickel (5 g), followed by hydrazine hydrate (20 ml) in portions over 1 h. After 1.5 h the solution was cooled and the white crystalline precipitate which formed was collected, washed in acetone and dried (0.6 g). The i.r. spectra of the crude product and the material produced by recrystallisation from ethanol or 2N-hydrochloric acid differed from each other and from a sample of the 4-aminophenyldihydrotriazine (37) prepared earlier. No further examination of the products was undertaken.

(iii) Hydrogenation, at room temperature and pressure over 6 h, of the nitro compound (2.62 g) in either water (30 ml) or ethanol (30 ml), using 10% Palladium/carbon catalyst (0.3 g) afforded, after filtration, a colourless solution which gave a red dye upon diazotisation and coupling with 2-naphthol. However, no base-induced (Dimroth rearrangement) shift in the u.v. maximum (240 nm) was observed. The i.r. spectrum of the crude solid material (0.9 g) was similar to a sample of the amine (37) previously prepared. It melted at 255-260°.

1-[2-azidophenyl]-1,2(2H)-2,2-dimethyl-4,6-diamino-1,3,5-triazine monohydrochloride (38)

To a hot stirred mixture of dicyandiamide (0.84 g) and acetone (50 ml) was added 10N-hydrochloric acid (1 ml) followed by 2-azidoaniline (1.34 g). The mixture was refluxed and as soon as a precipitate began to form (after about 1.2 h) the reaction mixture was filtered to remove an oily solid which had earlier appeared. Continued reflux of the mixture for 2 h, cooling and standing overnight afforded a clean (oil-free) precipitate. This was collected (0.81 g: 28%), washed with acetone and recrystallised in the dark from ethanol to yield cream microprisms of the required *dihydrotriazine monohydrochloride*, m.p. 198-200° (eff.); ν_{MAX} 765, 1642, 1668, 2130 (N_3) cm^{-1}; λ_{MAX} (H_2O) 206, 244 nm.

Found: C, 44.7; H, 5.0; N, 38.1.

$C_{11}H_{14}N_8$,HCl requires C, 44.8; H, 5.1; N, 38.0%.

1-[3-azidophenyl]-1,2(2H)-2,2-dimethyl-4,6-diamino-1,3,5-triazine monohydrochloride (39)

To a solution of 3-azidoaniline (4.6 g) in acetone (50 ml) was added a solution of dicyandiamide (2.9 g) in acetone (50 ml) containing 10N-hydrochloric acid. The mixture was refluxed for 3 h and cooled with stirring for 0.5 h. The precipitate was collected (6.5 g: 64%) and washed with acetone. Recrystallisation from ethanol in the dark afforded the required azide *monohydrochloride* as a fine off-white amorphous solid, m.p. 210° (eff.); ν_{MAX} 700, 770, 1600, 1640, 1668, 2160 (N_3) cm^{-1}.

Found: C, 44.9; H, 5.1; N, 38.2.

$C_{11}H_{14}N_8$,HCl requires C, 44.8; H, 5.1; N, 38.0%.

1-[4-azidophenyl]-1,2(2H)-2,2-dimethyl-4,6-diamino-1,3,5-triazine monohydrochloride (40)

To a hot slurry of 4-azidoaniline (1.34 g), dicyandiamide (0.84 g), acetone (50 ml) and 10N-hydrochloric acid (1 ml) was added ethanol (20 ml). The resulting solution was refluxed for 5 h. Upon cooling a cream precipitate (1.4 g: 47.5%) was deposited. Recrystallisation from ethanol gave an amorphous off-white precipitate of the *4-azidophenyldihydrotriazine monohydrochloride*, m.p. 248-250° (eff.); ν_{MAX} 765, 1602, 1642, 1668, 2110 and 2130 (N_3) cm^{-1}; λ_{MAX} (H_2O) 206, 254 nm.

Found: C, 45.3; H, 5.4; N, 38.2; Cl, 11.9.

$C_{11}H_{14}N_8$,HCl requires C, 44.8; H, 5.1; N, 38.0; Cl, 12.1%.

Attempted synthesis of 1-[4-acetylaminophenyl]-1,2(2H)-2,2-dimethyl-4,6-diamino-1,3,5-triazine (41)

When a mixture of 4-acetylaminoaniline (1.5 g), dicyandiamide (0.84 g), 10N-hydrochloric acid (1 ml), acetone (300 ml) and ethanol (100 ml) had been refluxed for 3 h, t.l.c. examination showed that the starting materials had not reacted to any significant extent: traces of two products were present. Extended reflux for 48 h did not increase the yield of either of these products.

4-Nitrophenylbiguanide (42)

When 4-nitroaniline (6.9 g), dicyandiamide (4.2 g), 10N-hydrochloric acid (4.2 ml) and acetone (100 ml) were refluxed for 5 h and cooled, a yellow crystalline mass was formed (8 g: 62%). Recrystallisation from aqueous ethanol gave the biguanide as golden needles, m.p. 273-275° (lit. 63, 268-270°); ν_{MAX} 750 br, 860, 1120, 1270, 1350 and 1550 (NO_2), 1620, 1655, 3200, 3300 cm^{-1}.

Synthesis of the azidoanilines (48)

2-, 3- and 4-nitrophenylphthalimide

These compounds were most efficiently prepared by direct fusion at 160-180° for 1.5 h of phthalic anhydride and the corresponding nitroaniline.

N-(2-aminophenyl) phthalimide

To a solution of N-(2-nitrophenyl) phthalimide (22 g) in acetone (1400 ml) was added acetic acid (200 ml), iron powder (50 g) and water (900 ml). This mixture was refluxed for 1.5 h. The hot solution was filtered rapidly through double thickness filter paper which was renewed during the process as necessary, and was then rewarmed. This solution was very cautiously neutralised, with vigorous swirling, with a hot saturated aqueous solution of sodium carbonate (*ca.* 180 g in 500 ml required). Two clearly defined layers formed: the upper (acetone) was bright yellow and contained the required amine, and the lower (aqueous) was dark green and contained the inorganic ions. The upper layer was separated and poured onto ice (2 kg). The lower layer was swirled and reheated with acetone (500 ml), and the resulting upper layer was separated and added to the bulk extract. After 1 h the required amine precipitated as golden prisms (17 g; 84%), m.p. 184-186° (lit.146, 184-186°); ν_{MAX} 1700 (CO), 3380s and 3960s (NH_2) cm^{-1}, and it was not purified further.

N-(3-aminophenyl) phthalimide and *N-(4-aminophenyl) phthalimide*

These compounds were obtained in 95% yield from the corresponding nitro compounds, using an exactly similar method. The *meta* isomer was

recrystallised from ethanol to give delicate pale yellow flakes, m.p. 185-187° (lit.146, 188-189°); ν_{MAX} 1700 (CO), 3320s and 3400s (NH_2) cm^{-1}.

The *para* isomer was recrystallised from methanol to afford a bright yellow amorphous mass, m.p. 249-251° (lit.146, 250°); ν_{MAX} 1702 (CO), 3360s and 3460s (NH_2) cm^{-1}.

N-(2-azidophenyl) phthalimide

N-(2-aminophenyl) phthalimide (18 g) was dissolved in warm acetic acid (1210 ml). 10N-hydrochloric acid (121 ml), water (151 ml) and ice (10 g) were added with vigorous stirring and cooling to 0°. A delicate mass of pale yellow microneedles was formed. To this suspension was added, dropwise and with stirring, an aqueous solution of sodium nitrite (5.74 g in 45 ml), whereupon a clear yellow solution was formed. After 1 h, an aqueous solution of sodium azide (9.83 g in 40 ml) was added over 0.5 h, and after a further 1 h of continuous agitation the precipitate which had formed was collected. Washing with water gave the required azide (18.32 g: 92%), m.p. 183-185° (eff.) (lit.67, 191°); ν_{MAX} 2110 and 2150 (N_3) cm^{-1}.

N-(3-azidophenyl) phthalimide

N-(3-aminophenyl) phthalimide (2.38 g) was dissolved at room temperature in 2N-hydrochloric acid (100 ml) and cooled to below 5°. An aqueous solution of sodium nitrite (0.76 g in 2 ml) was added dropwise with stirring over 20 min and the mixture was left for 0.5 h. Sodium azide (1.3 g) in water (3 ml) was stirred into the solution over a further 15 min. 1 h later, the precipitate was collected. Washing

with water and drying in a vacuum desiccator gave a clean white powder, m.p. 138-139° (eff.) (lit. **67**, 145-146°), ν_{MAX} 2150 cm^{-1} (N_3).

N-(4-azidophenyl) phthalimide

Dissolution of N-(4-aminophenyl) phthalimide (0.6 g) in acetic acid (40 ml) was effected with heating. When the solution was cooled to below 5° ice (*ca.* 3 g) was added to prevent solidification. Standard diazotisation, reaction with sodium azide and washing with water gave the required product (0.62 g: 94%) as a white solid, m.p. 179-180° (lit.**67**, 190°); ν_{MAX} 1720 (CO), 2150 infl. and 2160 (N_3) cm^{-1}.

2-, 3-, and *4-azidoanilines*

The literature [67] methods were followed to give:

2-azidoaniline (26%) as a white solid, m.p. 61-63° (eff.) (lit.**67**, 63-63.5°); ν_{MAX} 2180 cm^{-1} (N_3).

3-azidoaniline (46%) as a brown oil, ν_{MAX} (thin film) 2170 cm^{-1} (N_3).

4-azidoaniline (36%) as a white solid, m.p. 65° (eff.) (lit.**67**, 65-66°); ν_{MAX} (nujol) 2080s and 2110s (N_3), 3220, 3330 and 3400 (NH_2) cm^{-1}.

Synthesis of anilinodihydrotriazines

The following Dimroth rearrangement products were prepared using the method of Modest [63]. Both were recrystallised from ethanol.

1,2-dihydro-2,2-dimethyl-4-amino-6-[4-chloroanilino]-1,3,5-triazine (49): colourless prisms (80%), m.p. 134-136° (lit.63, 130-133°).

1,2,-dihydro-2,2-dimethyl-4-amino-6-[3-nitroanilino]-1,3,5-triazine hydrate (50) : yellow prisms (75%), m.p. 127-129°

ν_{MAX} 1350 and 1530 (NO_2) cm^{-1}.

Found: C, 47.4: H, 6.0: N, 29.7.

$C_{11}H_{16}N_6O_2,H_2O$ requires C, 47.1: H, 5.7: N, 30.0%

PART VI CHAPTER 3

Syntheses in the aryl-*s*-triazine series

The following compounds were synthesised by using published methods:

2,4-diamino-6-(2-aminophenyl)-1,3,5-triazine (60),
m.p. 179-181° (lit.**147**,165-168°)

2,4-diamino-6-(4-aminophenyl)-1,3,5-triazine (61),
m.p. 206-208° (lit.**147**,185-187°)

2,4-diamino-6-(2-azidophenyl)-1,3,5-triazine (62),
m.p. ~165° (dec) (lit.**54**,165-170° with effervescence, resolidification, and final melting at 310-313°)

2,4-diamino-6-(4-azidophenyl)-1,3,5-triazine (63),
m.p. 210-212° (eff.) (lit.**54** , 212-214° eff.)

2,4-diamino-6-(2-nitrophenyl)-1,3,5-triazine (71),
m.p. 233-239° (lit.**148**, 234-236°)

2,4-diamino-6-(4-nitrophenyl)-1,3,5-triazine (72),
m.p. 342-344° (lit.**148**, 330-331°)

2,4-diamino-6-phenyl-1,3,5-triazine (73),
m.p. 222-225° (lit.**76**, 222°)

4-amino-2(2H)-imino-s-triazino[1,2-c][1,2,3] benzotriazine (74),
m.p. > 200° (eff.) (lit.**54**, ~200° indef.)

2,4-Diamino-6-[2-(4-nitrobenzenesulphonamido) phenyl]-1,3,5-triazine (64)

2,4-Diamino-6-(2-aminophenyl)-*s*-triazine (60) (2.02 g) and 4-nitrobenzenesulphonyl chloride (2.22 g) were boiled in dry pyridine (10 ml) for 3 h. The solution was cooled and trituration with excess water (about 100 ml) caused the precipitation of a buff gum which, when stirred and allowed to stand, metamorphosed to give white crystals (3.5 g; 90%). Recrystallisation from acetone afforded colourless prisms of the required *anhydrous base*, m.p. 252-255°; *m/e* 387 (M^+); ν_{MAX} 1165 ($-SO_2-$), 1350 and 1535 (NO_2), 1620 cm^{-1}; δ 7.10 (s, 2 x NH_2), 7.00-8.40 (m, ArH), 7.95 and 8.32 (dd, benzene 2-H_2 and 3-H_2, AA'BB', J_{AB} 9.3 Hz), 13.10 (s, SO_2NH).

Found: C, 46.5; H, 3.4; N, 25.4.

$C_{15}H_{13}N_7O_4S$ requires C, 46.5; H, 3.4; N, 25.3%

2,4-Diamino-6-[2-(4-acetylaminobenzenesulphonamido) phenyl]-1,3,5-triazine (70)

A method similar to that used for the preparation of the nitrobenzenesulphonamide (64), was employed for the synthesis of the acetylaminobenzenesulphonamide from 4-acetylaminobenzenesulphonyl chloride with similar yield. The compound was recrystallised from 90% aqueous ethanol as *an ethanolic hydrate of the base*, m.p. 238-240°; δ 2.05 (3 H, s, $COCH_3$), 7.07 (4 H, br s, 2 x NH_2), 6.95-8.30 (m, Ar H), 7.55 and 8.30 (dd, benzene 2-H_2 and 3-H_2, AA'BB', $J_{AB} \sim 8$ Hz), 10.34 (1 H, s, AcNH), 12.95 (1 H, s, SO_2NH); 1.10, 3.40, 4.3 (t, q, br s: CH_3CH_2OH of crystallisation, calculated as being 0.5 mol. equiv.).

Found: C, 50.2; H, 4.4; N, 22.4.

$C_{17}H_{17}N_7O_3S,\frac{1}{2}C_2H_6O,\frac{1}{2}H_2O$ requires C, 50.1; H, 4.9; N, 22.7%

This solvate was then dried at 100°/750 torr for 3 h to give the *anhydrous base*:

Found: C, 51.3; H, 4.3; N, 24.2%; M^+, 399.11134

$C_{17}H_{17}N_7O_3S$ requires C, 51.1; H, 4.3; N, 24.6%; M, 399.11076

The compound was also recrystallised as a second *acetate solvate*, whose i.r. spectrum was almost identical to that of the anhydrous base: ν_{MAX} 1100, 1160 ($-SO_2-$), 1260 br, 1320, 1535, 1585, 1630 (br), 1680 (CO; acetone of crystallisation), 3200 br, 3400 br cm^{-1}.

Found: C, 51.5; H, 4.7; N, 23.6.

$C_{17}H_{17}N_7O_3S,\frac{1}{2}C_3H_6O$ requires C, 51.9; H, 4.7; N, 22.9%

2,4-Diamino-6-[4-(4-nitrobenzenesulphonamido) phenyl]-1,3,5-triazine (65)

This compound was prepared in 72% yield from 4-nitrobenzenesulphonyl chloride and 2,4-diamino-6-(4-aminophenyl)-1,3,5-triazine (61) using a method similar to that used in the synthesis of its isomer, (64). Recrystallisation of the crude product from absolute ethanol afforded the *anhydrous base*, m.p. 256-257°; ν_{MAX} 740, 822, 863, 915, 1095, 1160 ($-SO_2-$), 1355 and 1540 br (NO_2), 1400, 1460, 1610, 3200 br, 3300-3400, 3500 s cm^{-1}; δ 6.75 (4 H, s, 2 x NH_2), 7.28 and 8.20 (4 H, dd, AA'BB', triazinyl-C_6H_4: 2-H_2 and 3-H_2, J_{AB} 8.5 Hz), 8.10 and 8.47 (4H, dd, AA'BB', 4-nitrobenzene: 2-H_2 and 3-H_2, J_{AB} 9.3 Hz).

Found: C, 46.5; H, 3.5; N, 25.3%; M^+, 387.07496.

$C_{15}H_{13}N_7O_4S$ requires C, 46.5; H, 3.4; N, 25.3%; M, 387.07464

Recrystallisation from 70% aqueous ethanol gave the *base, hemihydrate*.

Found: C, 45.4; H, 4.0; N, 25.0.

$C_{15}H_{13}N_7O_4S,\frac{1}{2}H_2O$ requires C, 45.5; H, 3.5; N, 24.7%

2,4-Diamino-6-[2-(4-aminobenzenesulphonamido) phenyl]-1,3,5-triazine (66)

An ethanolic solution of 2,4-diamino-6-[2-(4-nitrobenzene-sulphonamido)-phenyl]-1,3,5-triazine (64)(4.0 g in 400 ml) at 55-60° was treated with Raney nickel (*ca.* 5 g), followed by hydrazine hydrate (30 ml) added in small quantities over 2 h. The mixture was maintained at 55-65° for a further 1 h and filtered through kieselguhr. The filtrate was evaporated to dryness and the residue was triturated with water, whereupon white crystals were formed which, on recrystallisation from ethanol gave the *amino* compound as colourless prisms (56%), m.p. 239-241°; *m/e* 387 (47%; M^+); ν_{MAX} 1100, 1160 ($-SO_2-$), 1280, 1320, 1400, 1560, 1605, 1640 cm^{-1}.

Found: C, 50.3; H, 4.3; N, 27.4.

$C_{15}H_{15}N_7O_2S$ requires C, 50.4; H, 4.2; N, 27.5%

2,4-Diamino-6-[4-(4-aminobenzenesulphonamido) phenyl]-1,3,5-triazine (67)

This compound was obtained in 55% yield from the corresponding nitro compound (65) by following the method used in the preparation of the amine (66). Recrystallisation from 100% ethanol afforded colourless needles of the *anhydrous base*, m.p. 235-237°; *m/e* 357 (77%; M^+), 202 (100%), 156 (57%); ν_{MAX} 1090, 1150 ($-SO_2-$), 1310, 1400,

1540 br, 1595, 1620 br, 3360, 3490 cm^{-1}; δ 6.04 (2 H, s, anilino NH_2), 6.75 (4 H, s, 2 x NH_2), 6.65 and 7.53 (4 H, dd, AA'BB', 4-aminobenzene: 2-H_2 and 3-H_2, J_{AB} 8.9 Hz), 7.22 and 8.15 (4 H, dd, AA'BB', triazinyl-C_6H_4: 2-H_2 and 3-H_2, J_{AB} 8.3 Hz). [See figure II.4-1]

Found: C, 50.4; H, 4.2; N, 27.4.

$C_{15}H_{15}N_7O_2S$ requires C, 50.4; H, 4.2; N, 27.5%

2,4-Diamino-6-[4-(4-aminobenzenesulphonamido) phenyl]-1,3,5-triazine dihydrochloride (67)

To a concentrated ethanolic solution of the amine base (*ca.* 1 g in 30 ml) was added 10N-hydrochloric acid (*ca.* 2 ml), whereupon a pale yellow product precipitated. Recrystallisation from 70% aqueous ethanol gave primrose needles of the *dihydrochloride dihydrate*, m.p. $\sim$ 275° (dec. with eff.); ν_{MAX} 1090, 1160 ($-SO_2-$), 1300, 1360, 1520, 1580, 1610 br, 1615 br, 1695, 2900 br, 3200 br, 3400 br.

Found: C, 37.9; H, 4.5; N, 20.7.

$C_{15}H_{15}N_7O_2S,2HCl,2\frac{1}{2}H_2O$ requires C, 37.9; H, 4.6; N, 20.7%

This compound was dried at 100°/750 torr to yield a fine powder of the *dihydrochloride*.

Found: C, 41.4; H, 4.4; N, 23.2.

$C_{15}H_{15}N_7O_2S,2HCl$ requires C, 41.9; H, 4.0; N, 22.8%

2,4-Diamino-6-[4-(4-azidobenzenesulphonamido) phenyl]-1,3,5-triazine monohydrochloride monohydrate (69)

A solution of 2,4-diamino-6-[4-(4-aminobenzenesulphonamido)-phenyl]-1,3,5-triazine dihydrochloride (67) in water (500 mg in 100 ml) was cooled to below 5° and acidified with N-hydrochloric acid (3 ml).

Vigorous stirring and the low temperature were maintained throughout the following reaction steps. Over 0.5 h were instilled separately an aqueous solution of sodium nitrite (133 mg in 2 ml) and supplementary N-hydrochloric acid (3 ml). After a further 0.5 h sodium azide (340 mg) was added in small portions over 15 min. The resultant mixture was stirred for a further 1 h at 0°. The precipitate was collected and crystallised from 2N-hydrochloric acid to give pale yellow microrosettes of the *monohydrochloride monohydrate* (420 mg; 83%), m.p. 200-205° (eff.); ν_{MAX} 840, 935, 1100, 1160 ($-SO_2-$), 1285, 1360, 1610, 1650, 1690, 2140 and 2160 (N_3), 3200 br, 3330 br cm^{-1}: δ([$^2H_6$] DMSO-^2H_2O) 7.31 and 7.97 (dd, AA'BB', 4-azidobenzene: 2-H_2 and 3-H_2, J_{AB} 8.9 Hz), 7.38 and 8.21 (dd, AA'BB', triazinyl-C_6H_4: 2-H_2 and 3-H_2, J_{AB} 8.7 Hz).

Found: C, 41.2; H, 3.6; N, 29.9%; M^+, 383.29199.

$C_{15}H_{13}N_9O_2S,HCl,H_2O$ requires C, 41.1; H, 3.7; N, 29.8%; M, 383.29199

2,4-Diamino-6-[2-(4-azidobenzenesulphonamido) phenyl]-1,3,5-triazine (68)

A solution of 2,4-diamino-6-[2-(4-acetylaminobenzenesulphonamido)-phenyl]-1,3,5-triazine (70) in 2N-hydrochloric acid (1.15 g in 40 ml) was refluxed for 2 h and then cooled to below 5°. An aqueous solution of sodium nitrite (207 mg in 2 ml) was introduced, dropwise, over 0.5 h and the solution was stirred for a further 1 h. The ice-cooled mixture was treated with sodium azide (325 mg) over 15 min, stirred for 1.5 h, and finally basified with aqueous ammonia to pH 8. The precipitate was collected (860 mg: 90%) and crystallised from ethanol to afford

colourless prisms of the *free base*, m.p. 200-202° (dec.);

ν_{MAX} 1100, 1175 ($-SO_2-$), 1295, 1340, 1550, 1590, 1640, 2170 (N_3), 3200 br, 3450 br cm^{-1}.

Found: C, 46.9; H, 3.4; N, 33.1.

$C_{15}H_{13}N_9O_2S$ requires C, 47.0; H, 3.4; N. 32.9%

The base had an i.r. spectrum identical with that of the compound prepared from the aminobenzenesulphonamide (66), *via* a straightforward diazotisation and reaction with sodium azide.

PART VI CHAPTER 4

Syntheses in the pyrimethamine series

2,4-Diamino-5-[3-nitro-4-chlorophenyl]-6-ethylpyrimidine (91)

To a stirred mixture of concentrated sulphuric acid (15 ml) and concentrated nitric acid (15 ml: 1.42 D) maintained below 15°, pyrimethamine (5 g) was added in small portions over 1 h. The mixture was left to stand at room temperature for 1 h, heated to 50° for 2 h, cooled, poured onto ice (*ca.* 200 g) and basified by the slow addition of concentrated aqueous ammonia. The cream solid that precipitated (4.1 g) was collected and recrystallised from aqueous ethanol to afford bright yellow rosettes, m.p. 203-205° ; ν_{MAX} 1340 and 1560 br (NO_2), 1630 br, 3120 br, 3300 and 3450 (NH_2) cm^{-1}; δ 1.00 (t, CH_3), 2.15 (q, CH_2), 5.93 (s, NH_2), 6.03 (s, NH_2), 7.51 (q, benzene 6'-H_1, $J_{62} \sim 3$ Hz, $J_{65} \sim 9.4$ Hz), 7.81 (ss, benzene 2',5'-H_2).

2,4-Diamino-5-[3-amino-4-chlorophenyl]-6-ethylpyrimidine (92)

The nitro analogue (91) (16 g) was added in small portions with stirring over 15 min to a solution of tin(II) chloride dihydrate (38 g) in 10N-hydrochloric acid (160 ml) that was maintained at 5-10°. The mixture was stirred thoroughly for 1.5 h (using a mortar) and allowed to stand overnight. The white stannicomplex that was produced was collected, dissolved in hot water and basified to pH 14 with 10N-sodium hydroxide. The resulting white precipitate was collected and crystallised from 100% ethanol to yield small amber prisms of the *anhydrous base* (9.5 g), m.p. 215-217°; ν_{MAX} 1622 br, 3180 br, 3320 and 3480 (NH_2) cm^{-1}; δ 1.00 (3 H, t, CH_3), 2.18 (2 H, t, CH_2),

5.35 (2 H, s, 3'-NH_2), 5.69 (2 H, s, NH_2), 5.90 (2 H, s, NH_2), 6.65 (1 H, d, benzene 2'-H_1), 6.38 (1 H, q, benzene 6'-H_1, J_{26} 1.7 Hz, J_{65} 8 Hz), 7.25 (1 H, d, benzene 5'-H_1).

Found: C, 54.4; H, 5.1; N, 26.4.

$C_{12}H_{14}N_5Cl$ requires C, 54.6; H, 5.3; N, 26.7%

A sample of the crude white precipitate was also crystallised from 50% aqueous ethanol to afford colourless needles of the *amine monohydrate*, m.p. 215-217°, *m/e* 265 (31%), 263 (96%; M^+), 247 (100%); ν_{MAX} 1602, 3180 br, 3330, 3480 (NH_2) cm^{-1}.

Found: C, 50.0; H, 5.4; N, 24.9.

$C_{12}H_{14}N_5Cl,1\frac{1}{4}H_2O$ requires C, 50.4; H, 5.8; N, 24.5%

2,4-Diamino-5-[3-azido-4-chlorophenyl]-6-ethylpyrimidine (93)

A fine suspension of the 3'-amino compound (93) (1.84 g) in 5N-hydrochloric acid (60 ml) was diazotised at 0° by the addition of sodium nitrite (0.6 g) dissolved in water (2 ml), with stirring, over 15 min. When the resulting suspension had been stirred for 1 h, sodium azide (1.8 g) was added in small portions over 2 h, the agitation being maintained. Following basification to pH 9 with concentrated aqueous ammonia, and stirring for 0.5 h, the solid product was collected (1.82 g; 90%) and crystallised from ethanol to yield photosensitive cream microprisms of the *azide base*, m.p. 185-188° (eff.); *m/e* 291 (15%), 289 (41%; M^+), 263 (21%), 262 (39%), 226 (66%), 65 (100%); ν_{MAX} 1450, 1564 br, 1639 br, 2150 (N_3), 3140 br, 3300 and 3460 (NH_2) cm^{-1}; δ 1.00 (3 H, t, CH_3), 2.18 (2 H, q, CH_2), 5.84 (2 H, s, NH_2), 5.96 (2 H, s, NH_2), 7.00 (1 H, q, benzene 6'-H_1, J_{62} 2 Hz, J_{65} 8.4 Hz), 7.19 (1 H, d, benzene 2'-H_1), 7.55 (1 H, d, benzene 5'-H_1).

Found: C, 49.9; H, 4.2; N, 33.3.

$C_{12}H_{12}N_7Cl$ requires C, 49.7; H, 4.2; N, 33.8%

2,4-Diamino-5-[3-azido-4-chlorophenyl]-6-ethylpyrimidine salts

The azide base (93) (1.0 g) was dissolved in a minimal quantity of acetic acid (about 8 ml). Following the addition of 10N-hydrochloric acid (2 ml) and standing for 48 h at 4° in the dark, white microprisms of the *azide monohydrochloride* (0.5 g), m.p.* 220-225°, were deposited; ν_{MAX} 1542, 1575, 1600, 1642, 1660 infl., 2180 (N_3) cm^{-1}; δ 1.08 (3 H, t, CH_3), 2.30 (q, CH_2), 7.13 (1 H, q, 6'-H_1), 7.39 (1 H, d, 2'-H_1), 7.48 (exchangeable, s), 7.68 (1 H, d, 5'-H_1).

Found: C, 44.3; H, 4.0; N, 30.3.

$C_{12}H_{12}N_7Cl,HCl$ requires C, 44.2; H, 4.0; N, 30.0%

Upon crystallisation in the dark of the azide base (93) from acetic acid (8 days at 0°) the *azide diacetate* was formed as a colourless precipitate. This was dried at 100°/750 torr for 3 h to give a white powder, m.p.* 190-195°; ν_{MAX} 1320, 1542 infl., 1575, 1645, 1660 br, 2180 (N_3); no band 1700-1720 cm^{-1} (therefore acetic acid of crystallisation absent); δ 1.02 (3 H, t, CH_3), 1.93 (1.5 H, s, CH_3COO), 2.20 (q, CH_2), 6.21 (2 H, br s, NH_2), 6.50 (2 H, br s, NH_2), 7.15 (1 H, q, benzene 6'-H_1), 7.22 (1 H, d, 2'-H_1), 7.58 (1 H, 5'-H_1), 9.40 (br s, $\overset{+}{N}H$).

Found: C, 46.5; H, 4.6.

$C_{12}H_{14}N_7Cl,2C_2H_3O_2$ requires C, 46.9; H, 4.9%

* chars

2,4-Diamino-5-[3-yl-4-chlorophenyl]-6-ethylpyrimidinediazonium tetrafluoroborate (99)

2,4-Diamino-5-[3-amino-4-chlorophenyl]-6-ethylpyrimidine (92) (1.713 g) was diazotised over 0.5 h, at 0° with stirring, in 50% aqueous tetrafluoroboric acid (30 ml) with an aqueous solution of sodium nitrite (0.47 g in 1 ml). The reaction mixture was left to stand at 4° in the dark overnight. The precipitate which had formed (1.6 g; 68%) was collected and dissolved in a minimal quantity of acetonitrile (about 3 ml). The addition of filtered diethylether caused a gelatinous precipitate which metamorphosed to form golden oily droplets and later fine pale orange shining needles of the *tetrafluoroborate, hydrotetrafluoroborate hemihydrate*, m.p. 165° (eff.); ν_{MAX} 1020-1120 (BF_4^-), 2290 (N_2^+) cm^{-1}.

Found: C, 31.1; H, 3.1; N, 18.5.

$C_{12}H_{12}N_6Cl^+,BF_4^-,HBF_4,\frac{1}{2}H_2O$ requires C, 31.4; H, 3.0; N, 18.3%

A second sample was crystallised from ethanol to give white needles of the *tetrafluoroborate, hydrotetrafluoroborate, monohydrate*, of identical m.p., and i.r. spectrum.

Found: C, 30.8; H, 3.0; N, 17.9.

$C_{12}H_{12}N_6Cl^+,BF_4^-,HBF_4,H_2O$ requires C, 30.8; H, 3.2; N, 17.9%

2,4-Diamino-5-[3-azo-(2-naphthol-1-yl)-4-chlorophenyl]-6-ethylpyrimidine (100)

A 95% yield of this azo dye was obtained when a solution of 2-naphthol (300 mg) in 2N-potassium hydroxide (5 ml) was mixed with an aqueous solution of the diazonium salt (99) (920 mg in 3 ml) at 5°. The precipitated dye (795 mg) was collected, washed with ethanol and

and crystallised from DMF as red needles of the *base solvate*, m.p. 300-302°; ν_{MAX} 1200, 1502, ~1570 br (N=N ?), 1620 br, 3180, 3330 br, 3490 cm^{-1}; λ_{MAX} 485 nm (azo); δ (TFA) 1.35 (t, CH_3), 2.70 (q, CH_2), 6.97-8.47 (m, aryl).

Found: C, 60.9; H, 5.3; N, 20.3.

$C_{22}H_{19}N_6O\,Cl, C_3H_7NO$ requires C, 61.0; H, 5.3; N, 19.9%

Found: M^+, 418.13088

$C_{22}H_{19}N_6O\,Cl^{35}$ requires M, 418.13080

2,4-Diamino-5-[3-acetylamino-4-chlorophenyl]-6-ethylpyrimidine (94)

A mixture of 2,4-diamino-5-[3-amino-4-chlorophenyl]-6-ethylpyrimidine (92) (2.64 g), acetyl chloride (1 ml) and pyridine (15 ml) was heated on a steam bath for 3 h, cooled and allowed to stand for 8 h. The addition of concentrated aqueous ammonia (10 ml) caused a cloudy precipitate which, on standing for 8 h, gave amber crystals (1.6 g: 52%). Washing with water and recrystallisation from ethanol gave amber microprisms of the *3'-acetylamino* compound, m.p. 257-259°; *m/e* 307 (35%), 305 (100%), 270 (21%); ν_{MAX} 1280, 1450, 1578, 1618, 1640, 1670, 2950, 3180, 3330, 3450 cm^{-1}; δ 0.98 (3 H, t, CH_3), 2.18 (2 H, q, CH_2), 2.12 (3 H, s, Ac), 5.70 (2 H, br s, NH_2), 5.94 (2 H, br s, NH_2), 7.00 (1 H, q, aryl 6'-H_1), 7.54 (1 H, d, aryl 5'-H_1, J_{56} 8.7 Hz), 7.66 (1 H, d, aryl 2'-H_1, J_{26} ~2 Hz), 9.50 (1 H, br s, NHCO).

Found: C, 54.8; H, 5.2; N, 23.2.

$C_{14}H_{16}N_5O\,Cl$ requires C, 55.0; H, 5.2; N, 22.9%

Found: M^+, 305.10433

$C_{14}H_{16}N_5O\,Cl^{35}$ requires M, 305.10380

Diacetylated 2,4-diamino-5-[3-amino-4-chlorophenyl]-6-ethylpyrimidine (95)

A mixture of the 3-amino derivative of pyrimethamine (92)(2.64 g), acetic anhydride (15 ml) and acetic acid (15 ml) was refluxed on a waterbath for 1.5 h and then cooled. Water (40 ml) was added and after about 2 h the crystals which had formed were collected (1.9 g; 55%). Recrystallisation from ethanol yielded colourless prisms of the *diacetylated* derivative, m.p. 243-244°; *m/e* 349 (32%), 347 (100%; M^+), 304 (61%): 262 (16%); ν_{MAX} 1320, 1575 br, 1660 br, $\sim$3300 br cm^{-1}; δ 1.02 (3 H, t, CH_3), 2.14 and 2.28 (8 H, ss + q, 2 x $CO.CH_3$ + CH_2), 6.18 (2 H, br s, NH_2), 7.04 (1 H, q, aryl 6'-H_1), 7.58 (1 H, d, J_{56} 8.3 Hz, aryl 5'-H_1), 7.71 (1 H, d, J_{26} $\sim$ 2 Hz, aryl 2'-H_1), 9.50 (1 H, s, CONH), 9.76 (1 H, s, CONH).

Found: C, 55.2; H, 5.1; N, 20.2.

$C_{16}H_{18}N_5O_2Cl$ requires C, 55.3; H, 5.2; N, 20.1%

Found: M^+, 347.11489

$C_{16}H_{18}N_5O_2Cl^{35}$ requires M, 347.11449

Triacetylated 2,4-diamino-5-[3-amino-4-chlorophenyl]-6-ethylpyrimidine (96)

A solution of the amine (92)(1.0 g) in acetic anhydride (20 ml) was refluxed for 1 h, cooled, shaken with water (20 ml) and neutralised with aqueous ammonia. Following extraction of the solution with chloroform, drying with sodium sulphate and evaporation, a gum was produced. Trituration with ice-water and standing for 12 h at 4° afforded colourless crystals (0.7 g; 47%). Recrystallisation from ethanol afforded an analytical sample of *2,4-diacetylamino-5-[3-*

acetylamino-4-chlorophenyl]-6-ethylpyrimidine, m.p. 174-176°, *m/e* 391 (12%), 389 (36%; M^+), 346 (41%), 303 (41%), 261 (19%); δ 1.09 (3 H, t, CH_3), 2.10, 2.13, 2.28 (sss, 3 x $CO.CH_3$), 7.08 (1 H, q, aryl 6'-H_1), 7.58 (1 H, d, J_{56} 8.3 Hz), 7.68 (1 H, d, J_{26} ~ 2 Hz, aryl 2'-H_1), 9.18 (1 H, br s, NHCO), 9.53 (1 H, s, NHCO), 10.45 (1 H, s, NHCO).

Found: C, 55.1; H, 5.1; N, 18.1.

$C_{18}H_{20}N_5O_3Cl$ requires C, 55.5; H, 5.1; N, 18.0%

Found: M^+, 389.12557

$C_{18}H_{20}N_5O_3Cl^{35}$ requires *M*, 389.12552

Attempted synthesis of 2,4-diamino-5-[3-(4-acetylaminobenzene-sulphonamido)-4-chlorophenyl]-6-ethylpyrimidine (98)

2,4-diamino-5-[3-amino-4-chlorophenyl]-6-ethylpyrimidine (92) (800 mg) and 4-acetylaminobenzenesulphonyl chloride (800 mg) were refluxed for 8 h in dry pyridine (20 ml). T.l.c. examination of the cooled reaction mixture showed that a small quantity of product had formed. The addition of triethylamine (1 ml) to the mixture and further heating at 85-90° for 2 h did not greatly increase the proportion of product (t.l.c.). When the pyridine was removed under vacuum an amber gum resulted, from which it was not found possible to fractionally crystallise either starting material or product.

PART VI CHAPTER 5

Preparation of 4-azidobenzenesulphonamides

The 4-amino compounds (sulpha drugs) were diazotised and reacted with sodium azide, following the established methods, to produce the following azides. The structures were confirmed by n.m.r. and mass spectroscopy.

2-(4-azidobenzenesulphonamido)-4,6-dimethylpyrimidine (111)

86%; m.p. 128-129° (lit.150, 129-130°).

2-(4-azidobenzenesulphonamido)-4-methylpyrimidine (114)

84%; m.p. 190-192° (lit.150, 191-192°).

2-(4-azidobenzenesulphonamido) pyrimidine (117)

85%; m.p. 215-216° (lit.150, 215-216°).

4-azidobenzenesulphonamide (120)

80%; m.p. 113-114° (lit.151, 115°).

Preparation of N^4-acetylated-benzenesulphonamides

The following five compounds were similarly prepared by refluxing the appropriate aminobenzenesulphonamide and either acetic anhydride or 50% acetic anhydride in acetic acid, as indicated, for 0.5 h. All melting points correspond to literature values and all structures were confirmed by n.m.r. and mass spectroscopy.

N^4-acetylsulphadimidine (112), acetic anhydride;

67%; m.p. 248-250°.

N^4-acetylsulphamerazine (115), 50% acetic anhydride in acetic acid;

76%; m.p. 247-250°.

N^4-acetylsulphadiazine (118), 50% acetic anhydride in acetic acid; 95%; m.p. 258-260°.

N^4-acetylsulphanilamide (121), 50% acetic anhydride in acetic acid; 90%; m.p. 215-216°.

N^4-acetylsulphamerazine (123), acetic anhydride; 70%; m.p. 216-219°.

PART VI

Experimental : Biology

Notes

1. For manual kinetic assays not involving any of the automated system components a Pye-Unicam SP1800 ultraviolet spectrophotometer was used incorporating a temperature controlled cell holder connected to an external Churchill pump set to circulate water at any desired temperature, and an AR25 chart recorder.

2. The kinetic equation I was fitted to the data using a non-linear iterative computer programme (GENFIT) based on an algorithm due to Powell[156]. The function minimised (ϕ) was the sum of the squares of the weighted residuals between calculated and observed rate (v) values:

$$\phi = \sum_{i=o}^{n} \left[\frac{v_i - v_i'}{v_i + v_i'} \right]^2$$

where v_i = calculated v_i

v_i' = observed v_i

The method of Ottaway[157] was used for the weighting. Standard errors for the parameters were estimated numerically from the rate of change of this function with respect to each parameter.

3. The slopes and intercepts of the Dixon-plot lines ($\frac{1}{v}$ versus [I]) were calculated using a simple least-squares linear regression programme, and the point of intersection (abscissa coordinate) was also computed.

4. The computer used was a Digital PDP 8/E.

PART VI

CHAPTER 6

The *in vitro* studies

Sources of Reagents and Enzymes

DHFR from L1210 cells and from *E.coli* (RT500, form I) was obtained from Protein Chemistry, Beckenham.

Tris buffer pH 7.5 was used for both enzymes in this study.

FH_2, Tris, DTT, EDTA and BSA were purchased from Sigma and used without further purification as was NADPH from Boehringer.

Activity testing of DHFR inhibitors

The kinetic assay reaction mixture contained in 1.5 ml total volume: Tris (100 mM), KCl (100 mM), EDTA (1 mM), DTT (2.2 mM), BSA (4.5 µg), NADPH (150 µM), FH_2 (5 µM), DHFR (20 nM) and Compound at the required concentration (indicated in Results table III.6-1). The DHFR concentration was estimated from a knowledge of the total protein content of the enzyme preparation and the approximate molecular weights: that of L1210 DHFR was taken as 21500 Daltons[152] and that of *E.coli* DHFR as 17989 Daltons[153].

K_I determinations of ten DHFRI

The assay reaction mixture was identical to that used for the activity testing except that FH_2 was fixed at either 5 µM or 10 µM. The compound concentrations used are indicated in table VII.1.

Table VI

Compound Number	Ref.	Experimental Run	Final concentrations used (nM)				
4	ZQ	I	200	300	400	500	
		II	200	400	600	800	
14	ZSQ	I	300	600	900	1800	
		II	600	700	800	900	1000
32	*m*NG	I	10	20	50	75	100
		II	25	50	75	100	
39	*m*ZG	I	50	100	150	200	
		II	20	50	75	100	125
40	*p*ZG	I	50	100	150	200	
		II	20	50	75	100	125
90	Pyrimeth-amine	I	50	75	100	125	
		II	10	50	75	100	125
91	*m*NP	I	1	5	10	20	30
92	*m*AP	I	10	50	75	100	125
		II	50	75	100	150	
93	*m*ZP	I	10	20	30	40	
		II	5	10	20	30	40
105	Etoprine	I	5	10	15	20	
		II	1	5	10	20	30

Automated assay system : processing times

In the activity testing the mixing delay was fixed at 10 sec and two integrations of 15 sec were taken. By fixing these intervals the automated system turnover time per sample was (10 sec + 15 sec + 15 sec) + 20 sec processing time. The incubation time per sample was a constant 10 minutes.

In the tests with the *E.coli* enzyme the total incubation time was 10 minutes and the delay and 'twin' integrations were set at 5 sec and 10 sec respectively for optimal response, i.e.

(5 sec + 10 sec + 10 sec) + 20 sec.

Appendices

Abbreviations Used

e	Total enzyme concentration
k	Rate constant
K	Equilibrium constant
K_A	Dissociation constant of A from binary complex EA.
$K_{A'}$	Dissociation constant of A from ternary complex EAB.
$K_{B''}$	Dissociation constant of B from ternary complex EIB.
K_i	Dissociation constant of inhibitor from binary complex EI.
$K_{i'} \equiv K_i$	Dissociation constant of inhibitor from ternary complex EIB.
I_{50}	Inhibitor concentration effecting ½ maximal velocity.
v_o	Uninhibited initial velocity.
V_i	Inhibited initial velocity.
E	refers to Enzyme, i.e. DHFR
A and B	refer to substrates FH_2 and NADPH
P and Q	refer to products FH_4 and NADPH

Appendix I

Kinetic model and assumptions used

The rate equations for both the uninhibited and inhibited DHFR reactions have been derived by using the rapid-equilibrium random-order model. This rests on the crucial first Michaelis-Menten assumption that in the (effectively) rapid-equilibrium below $k_1 = k_2$, and that k_3 is insignificant.

$$E + A + B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EAB \xrightarrow{k_3} EPQ$$

The alternative (general case) assumption of Briggs and Haldane[154] is that k_3 is *significant*. Theoretically, if the step EAB $\longrightarrow$ EPQ is *not* solely rate limiting (Briggs-Haldane), the rate equation is complex. Reciprocal plots are straight only if one substrate is saturating, and product inhibition is always noncompetitive[155]. However, if the centre step *is* rate limiting (rapid-equilibrium), the rate equation is simple, and product inhibition by either product against *either* substrate is competitive[155]

With L1210 DHFR product inhibition has been shown[82] to be *competitive* for FH_4 with respect to FH_2 and NADPH, and NADPH with respect to $NADP^+$, but *noncompetitive* for $NADP^+$ with respect to FH_2[82].

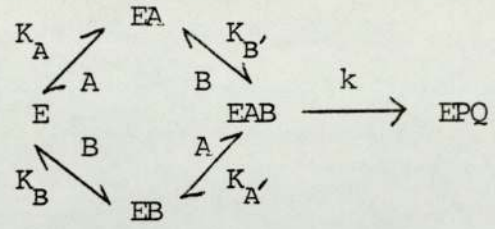
The situation is therefore complex. In the simple scheme above, if $k_3 \eqsim k_2$ then $K_m = k_2 + k_{3/k_1}$. However, if $k_3 \ll k_2$ then $K_m = k_{2/k_1}$, i.e. K_m is the dissociation constant of the substrate from the ternary complex. Although some evidence for the first case has been presented for chicken liver by Kaufman and Gardiner[105], current feeling is in general favour of the rapid-equilibrium model (e.g. see Ref.82).

Two other assumptions are:- (a) The concentration of enzyme is very small compared to the substrate concentrations, so that the formation of

the binary or ternary complexes does not significantly deplete the unbound substrate concentrations.

(b) The concentration of products is effectively zero. This implies that the amounts of products formed in the time required for a rate measurement are too small to give rise to a significant reverse reaction.

The random bi bi mechanism at initial steady state: inhibitor absent



Scheme VII.1

Except [EPQ], concentrations are related by dissociation constants rather than rate constants since the reaction is here regarded as in rapid equilibrium. Since the rate limiting step is given by equation (i) it is is simplest to express the other concentrations in terms of [EAB]. The initial velocity, v, is expressed as

$$v = k[EAB] \qquad \text{(i)}$$

$$\therefore \frac{v}{e} = \frac{k[EAB]}{e}$$, where e is the concentration of total enzyme.

$$\therefore v = \frac{ke}{e/[EAB]}$$

Since $V = ke$

$$\therefore v = \frac{V}{e/[EAB]} \qquad \text{(ii)}$$

Now, $e = [E] + [EA] + [EB] + [EAB]$

$$\therefore v = \frac{V}{\frac{[E]}{[EAB]} + \frac{[EA]}{[EAB]} + \frac{[EB]}{[EAB]} + \frac{[EAB]}{[EAB]}} \qquad \text{(iii)}$$

Each of the denominator terms may be reached by considering the separate dissociations:

(a) $$K_{B'} = \frac{[EA][B]}{[EAB]} \qquad \therefore \frac{[EA]}{[EAB]} = \frac{K_{B'}}{[B]}$$

(b) $$K_{A'} = \frac{[EB][A]}{[EAB]} \qquad \therefore \frac{[EB]}{[EAB]} = \frac{K_{A'}}{[A]}$$

(c) $$K_A = \frac{[E][A]}{[EA]} \qquad \therefore [E] = \frac{[EA]K_A}{[A]}$$

$$= \frac{[EAB].K_{B'}K_A}{[B][A]}$$

$$\frac{[E]}{[EAB]} = \frac{K_{B'}K_A}{[B][A]}$$

Similarly, by considering K_B it can be shown that $\frac{[E]}{[EAB]} = \frac{K_{A'}K_B}{[A][B]}$

Substituting into equation (iii),

$$v = \frac{V}{\frac{K_{B'}}{B} + \frac{K_{A'}}{A} + \frac{K_{B'}K_A}{BA} + 1}$$

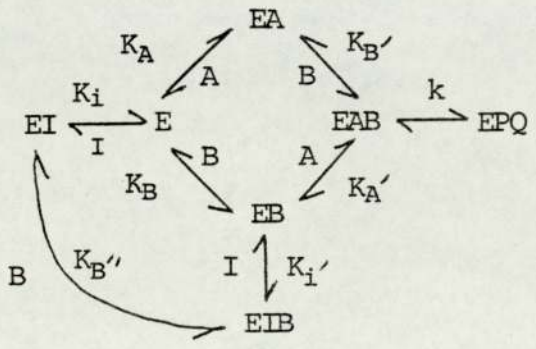
where A and B denote the separate concentrations [A] and [B] respectively.

$$\therefore \quad v = \frac{VAB}{AK_{B'} + BK_{A'} + K_{B'}K_A + AB} \qquad \text{(iv)}$$

When $B \longrightarrow \infty$ the equation simplifies to the pseudo first order equation

$$v = \frac{VA}{K_{A'} + A} \qquad \text{(v)}$$

The mechanism with inhibitor that can bind to E and/or EB



Scheme VII.2

The contribution of EI may or may not be important depending on the degree of cooperativity and [B].

As with no inhibitor present the rate is defined by equation (ii) above. In the presence of inhibitor which competes with the enzyme site for A the enzyme conservation equation is given by

$$e = [E] + [EA] + [EB] + [EAB] + [EI] + [EIB]$$

and the rate by

$$v = \frac{V}{\frac{[E]}{[EAB]} + \frac{[EA]}{[EAB]} + \frac{[EB]}{[EAB]} + \frac{[EAB]}{[EAB]} + \frac{[EI]}{[EAB]} + \frac{[EIB]}{[EAB]}} \qquad \text{(vi)}$$

Each of the denominator terms may be reached by considering the separate dissociations:-

(a) $K_{B'} = \frac{[EA][B]}{[EAB]}$ $\quad \therefore \frac{[EA]}{[EAB]} = \frac{K_{B'}}{[B]}$

(b) $K_{A'} = \frac{[EB][A]}{[EAB]}$ $\quad \therefore \frac{[EB]}{[EAB]} = \frac{K_{A'}}{[A]}$

(c) $K_A = \frac{[E][A]}{[EA]}$ $\quad \therefore [E] = [EA]\frac{K_A}{[A]} = [EAB].\frac{K_{B'}}{[B]}.\frac{K_A}{[A]}$

$$\therefore \frac{[E]}{[EAB]} = \frac{K_{B'}K_A}{[B][A]}$$

(d) $K_{i'} = \frac{[EB][I]}{[EIB]}$ $\quad \therefore \frac{[EIB]}{[EB]} = \frac{[I]}{K_{i'}}$

Also $\frac{[EIB]}{[EAB]} = \frac{[EIB]}{[EB]} \cdot \frac{[EB]}{[EAB]} = \frac{[I]}{K_{i'}} \cdot \frac{K_{A'}}{A}$

(e) $K_i = \frac{[E][I]}{[EI]}$ $\quad \therefore [EI] = \frac{[E][I]}{K_i}$

$$\therefore \frac{[EI]}{[EAB]} = \frac{[E]}{[EAB]} \cdot \frac{[I]}{K_i} = \frac{[I]}{K_i} \cdot \frac{K_{B'}K_A}{[B][A]}$$

$\therefore$ substituting the above terms from (a) to (e) into equation (vi),

$$v = \frac{V}{\frac{K_B' K_A}{[B][A]} + \frac{K_B'}{[B]} + \frac{K_A'}{[A]} + 1 + \frac{[I]}{K_i}.\frac{K_B' K_A}{[B][A]} + \frac{[I]}{K_i'}.\frac{K_A'}{[A]}}$$

$$\therefore v = \frac{VAB}{K_B' K_A + AK_B' + BK_A' + AB + \frac{I}{K_i}.K_B' K_A + \frac{I}{K_i}.BK_A'}$$

where A, B and I denote the separate concentrations [A], [B] and [I] respectively.

$$\therefore v = \frac{VAB}{K_B' K_A\left(1 + \frac{I}{K_i}\right) + BK_A'\left(1 + \frac{I}{K_i'}\right) + AK_B' + AB} \qquad \text{(vii)}$$

When $B \longrightarrow \infty$ this equation simplifies to the pseudo first-order equation

$$v = \frac{VA}{K_A'\left(1 + \frac{I}{K_i'}\right) + A} \qquad \text{(viii)}$$

Appendix II

Relationship between K_I and I_{50}

The I_{50} of a compound is its concentration required to inhibit the enzyme activity by 50%, i.e. $v_i = v_o/2$ where v_i and v_o are inhibited and uninhibited rates respectively.

Hence,
$$\frac{2VA}{K_A'\left(1 + \frac{I}{K_i'}\right) + A} = \frac{VA}{K_A' + A}$$

where $I = I_{50}$ and $K_A' = K_m$:

$$\therefore \quad 2 = \frac{K_A'\left(1 + \frac{I}{K_i'}\right) + A}{K_A' + A}$$

$$\therefore \quad 2 = 1 + \frac{K_A' I}{(K_A' + A)K_i'}$$

$$\therefore \quad 1 = \frac{K_A' I}{(K_A' + A)K_i'}$$

$$\therefore \quad I = K_i'\left(1 + \frac{A}{K_A'}\right)$$

$$\text{i.e.} \quad I_{50} = K_I\left(1 + \frac{A}{K_m}\right) \qquad \text{(ix)}$$

$\therefore$ I_{50} depends on [A] used in the experiment.

1. H.B. Stone in "Biochemical and Clinical aspects of Oxygen", ed. W.S. Caughey, Acad.Press, P 811 (1979)

2. J.W. Harris in "Modification of Radiosensitivity of Biological Systems", International Atomic Energy Agency, Vienna, p 11 (1976)

3. K. Hellmann, *Antibiotics Chemother.*, 23, 58 (1978)

4. G.W.J. Fleet, R.R. Porter and J.R. Knowles, *Nature*, 244(5218), 511 (1969)

5. J.R. Knowles, *Accounts Chem. Res.*, 5, 155 (1972)

6. E.H.F. Escher, T. Mai Dung Nguyen, G. Guillemette and D.C. Regoli, *Nature*, 275, 145 (1978)

7. J. Katzenellenbogen, K. Carlson, H. Johnson and H.N. Myers, *Biochemistry*, 15, 1970 (1977)

8. W.E. White and K.L. Yielding, *Biochem. Biophys. Res. Comm.*, 52. 1129 (1973)

9. H. Kiefer, J. Lindstrom, E.S. Lennox and S.J. Singer, *Proc. Nat. Acad. Sci. U.S.A.*, 67, 1688 (1970)

10. T.B. Rogers and M. Lazdunski, *Biochemistry*, 18(1), 135 (1979)

11. G.A.M. Butchart, Ph.D. Thesis, University of Aston in Birmingham (1974)

12. C.K. Wong, Ph.D. Thesis, University of Aston in Birmingham (1980)

13. A.C. Mair and M.F.G. Stevens, *J.C.S. Perkin I*, 161 (1972)

14. J.R. Bertino, *Cancer Res.*, 39, 293 (1979)

15. B.R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors", Wiley, N.Y., p 192 (1967)

16. R.L. Blakley, "The Biochemistry of Folic Acid and Related Pteridines", North Holland Res. Monographs, Frontiers of Biology, Vol.13, Gen. eds. A. Neuberger and E.L. Tatum, p 139 (1969)

17. G.C.K. Roberts in "Drug Action at the Molecular Level", ed. G.C.K. Roberts, Macmillan, p 127 (1977)

18. J.J. McCormack in "The Chemistry and Biology of Pteridines", W. de Gruyter, ed. W. Pfleiderer, p 125 (1975)

19. C. Hansch, J.Y. Fukunaga and P.Y.C. Jow, *J. Med. Chem.*, 20(1), 96 (1977)

20. J.Y. Fukunaga, C. Hansch and E.E. Steller, *J. Med. Chem.*, 19(5), 605 (1976)

21. J.J. McCormack and J.J. Jaffe, *J. Med. Chem.*, 12, 662 (1969)

22. F.M. Sirotnak, P.L. Chello, J.R. Piper, J.A. Montgomery and J.I. Degraw in "Chemistry and Biology of Pteridines", eds. Kisliuk/Brown, Elsevier North Holland, p 597 (1979)

23. B.S. Hurlbert, R. Ferone, T.A. Herrmann, G.H. Hitchings, M. Barnett and S.R.M. Bushby, *J. Med. Chem.* 11, 711 (1968)

24. W.E. Richter, Jr. and J.J. McCormack, *J. Med. Chem.*, 17(9), 943 (1974)

25. G.H. Hitchings and J.J. Burchall, *Adv. Enzymol.*, 27, 417 (1965)

26. G.H. Hitchings, *Postgrad. Med. J.*, 45(suppl), 7 (1969)

27. W.M. Hryniuk, L.W. Brox, J.F. Henderson and T. Tamaoki, *Cancer Res.*, 35, 1427 (1975)

28. W.M. Hryniuk, *Cancer Res.*, 32, 1506 (1972)

29. I.D. Goldman, *Cancer Chemother. Rept.*, 6(part 3), 51 (1975)

30. D. Kirk, U. Mittwoch, A.B. Stone and D. Wilkie, *Biochem. Pharmacol.*, 25, 681 (1976)

31. R.C. Jackson, L.I. Hart and K.R. Harrap, *Cancer Res.*, 36, 1991 (1976)

32. J.H. Fendler and A. Romero, *Life Sci.*, 20, 1109 (1977)

33. B. Rerat, *Acta Cryst.*, B25, 1392 (1969)

34. E. Shefter, *Cryst. Struct. Comm.*, 5, 847 (1976)

35. C.H. Koo, H.S. Kim, Y.K. Yoon and I.H. Suh, *J. Korean Phys. Soc.*, 8(1), 37 (1975)

36. D.G. Johns, R.L. Capizzi, A. Nahas, A.R. Cashmore and J.R. Bertino, *Biochem. Pharmacol.*, 19, 1528 (1970)

37. J. Davoll, A.M. Johnson, H.J. Davies, O.D. Bird, J. Clarke and E.F. Elslager, *J. Med. Chem.* 15(8), 812 (1972)

38. E.F. Elslager, J. Clarke, L.M. Werbel, D.F. Worth and J. Davoll, *J. Med. Chem.*, 15(8), 827 (1972)

39. J.B. Hynes, W.T. Ashton, D. Bryansmith and J.H. Freisheim, *J. Med. Chem.*, 17(9), 1023 (1974)

40. J.B. Hynes and W.T. Ashton, *J. Med. Chem.*, 18(3), 263 (1975)

41. E.F. Elslager, M.P. Hutt, P. Jacob, J. Johnson, B. Temporelli, L.M. Werbel, D.F. Worth and L. Rane, *J. Med. Chem.*, 22(10), 1247 (1979)

42. E.F. Elslager, P. Jacob, J. Johnson, L.M. Werbel and D. Worth, 167th Nat. Meet. Amer. Chem. Soc., Abstr.55 (1974)

43. J.J. Johnson, E.F. Elslager and L.M. Werbel, *J. Heterocyclic Chem.*, 14, 1209 (1977)

44. R.T. Skeel, W.L. Sawiki, A.R. Cashmore, J.R. Bertino, *Cancer Res.*, 33, 2972 (1973)

45. C.A. Nichol, J.C. Cavallito, J.L. Wooley and C.W. Sigel, *Cancer Treat. Rep.*, 61(4), 559 (1977)

46. G.F. Geils, C.W. Scott, Jr., C.M. Baugh and C.E. Butterworth, Jr., *Blood*, 38(2), 131 (1971)

47. D.R. Stickney, W.S. Simmons, R.L. De Angelis, R.W. Rundles and C.A. Nichol, *Proc. Am. Assoc. Cancer Res.*, 14, 52 (1973)

48. K. Ganeshaguru and A.V. Hoffbrand, *Biochem. Pharmacol.*, 26, 543 (1977)

49. W.D. Sedwick, M. Kutler, T. Frazer, O.E. Brown and J. Laszlo, *Cancer Res.*, 39, 3612 (1979)

50. J. Laszlo, M.J. Fyfe, D. Sedwick, L. Lee and O. Brown, *Cancer Treat. Rep.*, 62, 341 (1978)

51. S. Farber, G. Foley, V. Downing, R. Appleton and J. King, *Proc. Am. Assoc. Cancer Res.*, 1, 15 (1953)

52. I.D.C. Douglas, M.Y. Gordon, G.H.G. Prentice, B.T. Hill and L.A. Price, *Lancet*, 2, 607 (1977)

53. B.R. Baker and B.T. Ho, *J. Heterocyclic Chem.*, 2, 340 (1965)

54. S.M. Mackenzie and M.F.G. Stevens, *J. Chem. Soc.(C)*, 2298 (1970)

55. J. Davoll and A.M. Johnson, *J. Chem. Soc.(C)*, 997 (1970)

56. E.H. Northey, "The Sulphonamides and Allied Compounds", (Am. Chem. Soc. Monograph series No. 106), Reinhold, N.Y., 1948

57. M.F.G. Stevens, Personal Communication (1979)

58. N.A. Lange and F.E. Sheibley, *J. Amer. Chem. Soc.*, 55, 1188 (1933)

59. N.A. Lange, W.E. Roush and H.J. Asbeck, *J. Amer. Chem. Soc.*, 52, 3696 (1930)

60. F.J. Wolf, R.H. Beutel and J.R. Stevens, *J. Amer. Chem. Soc.*, 70, 4264 (1948)

61. M. Claesen and H. Vanderhaeghe, *Bull. Soc. chim. belg.*, 68, 220 (1959)

62. A. Reiser and H.M. Wagner in "The Chemistry of the Azido Group" ed. S. Patai, Interscience, London, p 441 (1971)

63. E.J. Modest, *J. Org. Chem.*, 21(1), 1 (1956)

64. E.J. Modest and P. Levine, *ibid*, 21(1), 14 (1956)

65. B. Roth, R.B. Burrows and G.H. Hitchings, *J. Amer. Chem. Soc.*, 6, 370 (1963)

66. R.J.A. Walsh, K.R.H. Woolridge, D. Jackson and J. Gilmour, *Eur. J. Med. Chem., Chimica Therapeutica*, 12(6), 495 (1977)

67. P.A.S. Smith, J.H. Hall and R.O. Kan, *J. Amer. Chem. Soc.*, 84, 485 (1962)

68. H.C. Carrington, A.F. Crowther and G.J. Stacey, *J. Chem. Soc.*, 1017 (1954)

69. L.D. Colebrook, H.G. Giles, A. Rosowsky, W.E. Bentz and J.R. Fehlner, *Canad. J. Chem.*, 54, 3757 (1975)

70. C.H. Schwalbe and W.E. Hunt, *J.C.S. Chem. Comm.*, 188 (1978)

71. W.E. Hunt, Ph.D. Thesis, University of Aston in Birmingham (1979)

72. M.W. Fisher and L. Doub, *Biochem. Pharmacol.*, 3, 10 (1959)

73. B.R. Baker and B.T. Ho, *J. Pharm. Sci.*, 53(9), 1137 (1964)

74. B.R. Baker and B.T. Ho, *ibid*, 55(5), 470 (1966)

75. B.R. Baker and B.T. Ho, *J. Heterocyclic chem.*, 1, 79 (1965)

76. U.S. Patent 2,302,162 (1942)

77. W. Brunner and E. Bertsch, *Monatsch.*, 79, 106 (1948)

78. J.E. Gurst in "The Chemistry of the Azido Group", ed. S. Patai, Interscience, London p 191 (1971)

79. G. Spiteller and R. Kaschnitz, *Monatsch.*, 94, 964 (1963)

80. E. Dynesen, S.O. Lawesson, G. Schroll, J.H. Bowie and R.G. Cooks, *J. Chem. Soc.* (B), 15 (1968)

81. P.N. Preston, W. Steedman, M.H. Palmer, S.M. Mackenzie and M.F.G. Stevens, *Org. Mass Spectrometry*, 3, 863 (1970)

82. J.L. McCullough, P.F. Nixon and J.R. Bertino, *Ann. N.Y. Acad. Sci.*, 186, 131 (1971)

83. D.A. Matthews, R.A. Alden, J.T. Bolin, S.T. Freer, R. Hamlin, N. Xuong, J. Kraut, M. Poe, M. Williams and K. Hoogsteen, *Science*, 197, 452 (1977)

84. D.A. Matthews, R.A. Alden, S.T. Freer, N. Xuong and J. Kraut, *J. Biol. Chem.*, 254(10), 4144 (1979)

85. D.A. Matthews, R.A. Alden, J.T. Bolin, D.J. Filman, S.T. Freer, R. Hamlin, W.G.J. Hol, R.L. Kisliuk, E.J. Pastore, L.T. Plante, N. Xuong and J. Kraut, *J. Biol. Chem.*, 253(19), 6946 (1978)

86. B. Birdsall, A.S.V. Burgen, J.R. de Miranda and G.C.K. Roberts, *Biochemistry*, 17(11), 2102 (1978)

87. R.L. Blakley, M. Schrock, K. Sommer and P.F. Nixon, *Ann. N.Y. Acad. Sci.*, 186, 119 (1971)

88. A.V. Reddy, W.D. Behnke and J.H. Freisheim, *Biochim. Biophys. Acta*, 533, 415 (1978)

89. J.J. Burchall and G.H. Hitchings, *Mol. Pharmacol.*, 1, 126 (1965)

90. J. Burchall in "Chem. Biol. of Pteridines", Proc. 4th Int. Symp. on Pteridines, Toba; Int. Academic Printing Co. Ltd, Tokyo; p 326 (1970)

91. D. Baccanari, A. Phillips, S. Smith, D. Sinski and J. Burchall, *Biochemistry*, 14, 5267 (1975)

92. M. Poe, A.S. Breeze, J.K. Wu, C.R. Short, Jr. and K. Hoogsteen, *J. Biol. Chem.*, 254(6), 1799 (1979)

93. F.A.M. Fennell and N.G.L. Harding, *Biochem. Soc. Trans.*, 2, 341 (1974)

94. T.J. Williams, T.K. Lee and R.B. Dunlap, *Arch. Biochem. Biophys.*, 181, 569 (1977)

95. L. Lee, Personal Communication (1979)

96. M. Dixon, *Biochem. J.*, 55, 170 (1953)

97. D. Baccanari, Personal Communication (1978)

98. S. Smith, Personal Communication (1978)

99. R.L. Blakley, *Nature*, 188, 231 (1960)

100. C.K. Matthews and F.M. Huennekens, *J. Biol. Chem.*, 238, 3436 (1963)

101. D.P. Baccanari, *Arch. Biochem. Biophys.*, 191(1), 351 (1978)

102. B.L. Hillcoat, P.F. Nixon and R.L. Blakley, *Analyt. Biochemistry*, 21, 178 (1967)

103. M.N. Williams and C.D. Bennett, *J. Biol. Chem.*, 252(19), 6871 (1977)

104. P. Reyes and F.M. Huennekens, *Biochemistry*, 6(11), 3519 (1967)

105. B.T. Kaufman, *J. Biol. Chem.*, 243(22), 6001 (1968)

106. J.R. Bertino, *Biochim. Biophys. Acta*, 58, 377 (1962)

107. M.F. Argus, J.C. Arcos, J. Mathison and J.A. Bemis, *Arzneimittel Forschung*, 16, 740 (1966)

108. R. Freudenthal and P. Hebborn, *Internat. J. Biochem.*, 1(1), 121 (1970)

109. L. Pauling, *Science*, 134, 15 (1961)

110. NCI Instruction Manual 14 (1978)

111. A.J. Ryan, J.J. Ashley and R.A. Upton, *J. Pharm. Sci.*, 67(3), 306 (1978)

112. T.A. Connors and B.J. Phillips, *Biochem. Pharmacol.*, 24, 2217 (1975)

113. G.M. Bartoli, T. Galeotti and A. Azzi, *Biochim. Biophys. Acta*, 497, 622 (1977)

114. E.J. Cone, *Xenobiotica*, 8(5), 301 (1978)

115. J. Knoll, G. Zsilla and S. Makleit, *Medical Biology*, 53, 501 (1975)

116. M.E.C. Biffin, J. Miller and D.B. Paul in "The Chemistry of the Azido Group" ed. S. Patai, Interscience, London, p 203 (1971)

117. A. Treinin in "The Chemistry of the Azido Group", ed. S. Patai, Interscience, London p 1 (1971)

118. F.M. Huennekens, K.S. Vitols and G.B. Henderson, *Adv. Enzymol.*, 47, 313 (1978)

119. G.A. Fischer, *Biochem. Pharmacol.*, 11, 1233 (1962)

120. B.T. Hill and L.A. Price, *Cancer Topics*, 1(8), 2 (1977)

121. B.T. Hill, L.A. Price and J.H. Goldie, *Eur. J. Cancer*, 11, 545 (1975)

122. B.T. Hill, L.A. Price, S.I. Harrison and J.H. Goldie, *Biochem. Pharmacol.*, 24, 535 (1975)

123. R.H. Denlinger, C.A. Nichol, J.C. Cavallito and C.W. Sigel, *Proc. Am. Assoc. Cancer Res.*, 17, 95 (1976)

124. B.T. Hill, J.H. Goldie and L.A. Price, *Brit. J. Cancer*, 28, 263 (1973)

125. D.S. Duch, S.W. Bowers and C.A. Nichol, *Biochem. Pharmacol.*, 27, 1507 (1978)

126. D.S. Duch, M.P. Edelstein and C.A. Nichol, *Pharmacologist*, 21, 266 (1979)

127. C.A. Nichol, *Cancer Topics*, 1(9), in Report on Zurich Chemotherapy Conference (1977)

128. E.M. Grivsky, S. Lee, C.W. Sigel, D.S. Duch and C.A. Nichol, *J. Med. Chem.*, 23, 327 (1980)

129. L.A. Price, J.H. Goldie and B.T. Hill, *Brit. Med. J.*, 2, 20 (1975)

130. L.A. Price and B.T. Hill in "Chemotherapy - Proc. IXth Int. Congr. Chemother.", ed. J.D. Williams, Plenum Press, N.Y., 8, 481 (1976)

131. B.T. Hill, L.A. Price, S.I. Harrison and J.H. Goldie, *Eur. J. Cancer*, 13, 861 (1977)

132. L.A. Price, B.T. Hill and J.H. Goldie, *Clinical Oncology*, 3, 281 (1977)

133. B.T. Hill, *Eur. J. Cancer*, 16, 147 (1980)

134. D.S. Duch, S.W. Bowers, M.P. Edelstein and C.A. Nichol, Proc. 11th Int. Congr. Chemotherap., Abstr. 1016 (1979)

135. C.A. Nichol, S.W. Bowers, C.W. Sigel, J.C. Cavallito, F.G. Foss and D.S. Duch, *ibid*, Abstr. 1015 (1979)

136. D.S. Duch, D.D. Bigner, S.W. Bowers and C.A. Nichol, *Cancer Res.*, 39, 487 (1979)

137. Parameter and Log P listings, Medicinal Chemistry Project, Pomona College, California, 14 and 15 (1979)

138. M. Poe, *J. Biol. Chem.* 252(11), 3724 (1977)

139. D.S. Miller, R.W. Rundles, C.A. Nichol, J.L. Wooley and C.W. Sigel, *Proc. Am. Soc. Clin. Oncol.*, 17, 263 (1976)

140. A.M. Perault and B. Pullman, *Biochim. Biophys. Acta*, 52, 266 (1961)

141. A. Bozzi, I. Mavelli, A. Finazzi Agrò, R. Strom, A.M. Wolf, B. Mondovi and G. Rotilio, *Molec. and Cellular Biochem.*, 10(1), 11 (1976)

142. H. Marberger, G. Bartsch, W. Huber, K.B. Menander and T.L. Schulte, *Current Therapeutic Research*, 18(3), 466 (1975)

143. I.E. Smith, *Cancer Topics*, 1(11), 2 (1978)

144. J.A. Parrish, T.B. Fitzpatrick, L. Tanenbaum and M.A. Pathak, *New Engl. J. Med.*, 291(23), 1207 (1974)

145. M.A. Everett, E. Yeargers, R.M. Sayre and R.L. Olson, *Photochem. Photobiol.*, 5, 533 (1966)

146. R. Meyer and J. Maier, *Ann*, 327, 51 (1903)

147. B.P. 908,301 (1962)

148. V.A. Titkov and I.D. Pletnev, *Zhur. obschei Khim.*, 33(6), 1983 (1963): *Chem. Abs.*, 59, 15408f

149. R.L. DeAngelis, W.S. Simmons, C.A. Nichol, *J. Chromatog.* 106, 41 (1975)

150. T.B. Brown, Ph.D. Thesis, Heriot-Watt University, Edinburgh (1973)

151. H. Bretschneider and H. Rager, *Monatsch.*, 81, 970 (1950)

152. D. Stone and A.W. Phillips, *FEBS Lett.*, 74, 85 (1977)

153. D. Stone, A.W. Phillips and J.J. Burchall, *Eur. J. Biochem.*, 72, 613 (1977)

154. G.E. Briggs and J.B.S. Haldane, *Biochem. J.*, 19, 338 (1925)

155. K.M. Plowman, "Enzyme Kinetics", McGraw Hill, p 143 (1972)

156. M.J.D. Powell in "Numerical Methods for Non-Linear Algebraic Equations", ed. P. Rabinowitz, Gordon and Breach, London, pp 87-161 (1970)

157. J.B. Ottaway, *Biochem. J.*, 134, 729 (1973)