SELECTIVE TOXICITY AND THE COVALENT BOND

BY

GRAHAM ALEXANDER MILNE BUTCHART

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Department of Pharmacy
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SUMMARY

The interactions of organic molecules with bio-macromolecules are reviewed in Chapter 1. The use of 'masked' reactive intermediates is a novel approach to the labelling of biologically-significant macromolecules (Chapter 2). The involvement of a covalent bond is the central theme of these reactions.

The photochemistry and radiochemistry of 2,4-diamino-6-(2-azidoaryl)-s-triazines was examined. The only products isolated from these reactions were 2,4-diamino-s-triazino [1,2-b] indazoles. The formation of these heterocyclic compounds possibly involves a nitrene intermediate. The mass spectral fragmentations of 2,4-diamino-6-(2-azidoaryl)-s-triazines has been worked out. The possible use of 2,4-diamino-6-(2-azidoaryl)-s-triazines as irreversible inhibitors of dihydrofolate reductase was examined. Although these azides decomposed to evolve reactive intermediates on γ-radiolysis, the dose levels were intolerable for biological tissues.

The synthesis and spectroscopic properties of 9-anilinoacridines and 9-azido and 9-chloro-10-methyl-acridinium methosulphate was studied. The pKa values of the anilinoacridines were calculated by the spectroscopic method of Albert and Serjeant, or predicted from the Hammett Equation.

A correlation between pKa and anti-tumour activity for the anilinoacridines was discerned. Those anilinoacridines with a +I substituent in the phenyl ring showed marginal activity with a slight increase in

basicity compared to the unsubstituted 9-anilinoacridine. Those anilinoacridines with a +M substituent in the para-position of the phenyl group showed pronounced anti-tumour activity and had the highest pKa values. The one exception was 9-(4-acetoxyanilino)acridine with a pKa value considerably lower than the other active anilinoacridines. This derivative may be hydrolysed in vivo to the active 9-(4-hydroxyanilino)-acridine.

9-Azido and 9-chloro-10-methylacridinium methosulphate were shown to have anticholinergic activity and were compared with physostigmine. The extreme toxicity shown by these salts may be partly due to their anti-cholinergic activity.

2,4-Dinitrochlorobenzene and 9-chloroacridine were used to study the arylation of pyridine, 2-3- and 4-aminopyridines.

2,4-Dinitrophenylpyridinium chloride on treatment with triethylamine in ethanol ring-opened to form 5-(2,4-dinitroanilino)-2,4-pentadienal. Similar treatment of the related salts of 3-and 4- aminopyridine resulted in formation of 2,4-dinitrophenyl ethers.

Attempted arylation of adenine with 2,4-dinitro-chlorobenzene in dimethylacetamide resulted in formation of 6-acetamidopurine and 1-dimethylamino-2,4-dinitrobenzene This implies that initial reaction involved N(1) of adenine.

The chemistry of 2,4-dinitrophenylaziridine and the related tumour-inhibitory compound (CB 1954) is described. Previous work showed that the predominant

products are acyclic formed by nucleophile-promoted ring cleavage. These results have been confirmed and the aziridine undergoes ring-opening with acids, bases, alcohols and upon photolysis. The significance of these reactions in relation to the intracellular reaction mechanism is discussed.

ACKNOWLEDGEMENTS

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U.V. - Pye Unicam S.P. 8000
I.R. - Pye Unicam S.P. 200
N.M.R. - Varian H-A100D

ABBREVIATIONS

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
t.l.c.	Thin layer chromatography
i.r.	Infra red
u.v.	Ultra violet
n.m.r.	Nuclear magnetic resonance
e.s.r.	Electron spin resonance
DMSO	Dimethysulphoxide
DMSO-d_6	Hexadeuterated dimethylsulphoxide
$CDCl_3$	Deuterochloroform
CH_2Cl_2	Dichloromethane
DMAC	Dimethylacetamide
DMF	Dimethylformamide

PART 1.

CHAPTER 1.

PART I.

CHAPTER 1.

Introduction.

Selective toxicity was defined by Albert[1] as; "the injury of one kind of living matter without harming some other kind, with which the first is in intimate contact." When dealing with chemotherapy, this implies that any potential drug, whilst toxic to the invading body (e.g. tumour), is not toxic to the host. Most compounds however, are non-selective and show a wide range of biological activity other than reaction with the invading body.

When a drug reacts *in vivo* it does so with areas known as biological receptors. These receptors can be enzymes, proteins, nucleic acids or other types of biological macromolecules. There are several ways by which a drug can combine with these biological receptors. The main types of weak bonds important in biological systems are van der Waals bonds, hydrogen bonds, hydrophobic bonds and ionic bonds. Of these bonds the former are the weakest and arise from a non-specific attraction originating when two atoms come within close proximity of each other. Van der Waals bonds are an effective binding force under physiological conditions only when several atoms in a given molecule are bound to several atoms in another. The strongest type of van der Waals bonds arise when a molecule contains a cavity

exactly complementary in shape to a protruding group on another molecule. This is the type of situation thought to exist between an antigen and its specific antibody.[2]

In enzymology, hydrophobic bonding must also be considered and will occur along with van der Waals bonding in enzyme-inhibitor or enzyme-substrate complexes. The term hydrophobic bonding is a misnomer, in that, the phenomenon it seeks to emphasise is the absence, not the presence of bonds. The bonds that tend to form between non-polar groups are due to van der Waals attractive forces; the term hydrophobic bond is used to illustrate the fact that non-polar groups will try to arrange themselves so that they are not in contact with water molecules.[2] The result of these forces is the formation of a reversible complex, since the strength of the bond between enzyme and substrate/inhibitor is still relatively weak.

Hydrogen bonding arises when a hydrogen atom serves as a bridge between two electonegative atoms, holding one by a covalent bond and the other by purely electrostatic forces. The electronegative atoms can either be in the same molecule, (hence intramolecular hydrogen bonding) or in a different molecule, (intermolecular hydrogen bonding). For hydrogen bonds to be important both electronegative elements must come from the group : N, O, S, P, F. Hydrogen bonds are much more specific (and considerably stronger) than van der Waals bonds, since they demand the existence of complementary acceptor hydrogen and donor electronegative groups. The

biologically most important hydrogen bonds are those involving hydrogen atoms covalently bound to oxygen or nitrogen. The stability of the double helical arrangement of DNA is due to hydrogen bonds between the adfacent heterocyclic bases.[3]

Ionic bonds are formed between ions of opposite charge and, although these bonds are stronger than the other short range forces, their biological lifetimes are extremely short even if the bond is reinforced. Some ionic bonds are in effect hydrogen bonds. For example, both the carboxylate and ammonium groups of amino acids are held together by hydrogen bonds. These hydrogen bonds are stronger than those that involve groups with less than a unit of charge, and are correspondingly shorter.

These weak forces play an important role in many biological processes such as the folding of proteins and enzyme-substrate interactions. In order for these bonds to be broken very little energy is needed: in fact, these bonds are constantly broken and reformed within a living cell. For additional reading on the role of weak bonds in biological processes, see references 1,2, 4 and 5.

Covalent bonds differ from the above in that electrons are shared between two atoms and such bonds are extremely strong. The chemistry of compounds potentially capable of covalent reactivity with bio-macromolecules is the central theme of this thesis. Covalent bonds can only be broken by heat, powerful chemicals, (both intolerable in a biological environment) or certain enzymes. Thus drugs that induce their biological activity by formation

of a covalent bond, tend to form irreversible complexes with biological macromolecules. Such drugs are usually electrophilic species and hence, react only with nucleophilic centres on the macromolecule: alternatively, they can be reactive intermediate precursors which can react indiscriminately. This latter type will be discussed in Chapter 2. The target macromolecules of greatest interest, in terms of covalent interactions, are proteins and nucleic acids.

A. PROTEINS

Structure and Mode of Action of Enzyme Inhibitors

When designing enzyme inhibitors, it is necessary to simulate the natural situation with a reagent that structurally mimics the natural ligand and preserves the specificity of the interaction. If the reagent is not to label an irrelevant receptor, (i.e. be non-selective) then these specificity requirements must be satisfied. In contrast to the necessary specificity of the initial binding to the enzyme, the subsequent intracomplex reaction should be indiscriminate as possible.

In enzymology such a reagent has been called an "active-site-directed irreversible inhibitor,"[5] and its design is based on the following criteria:-

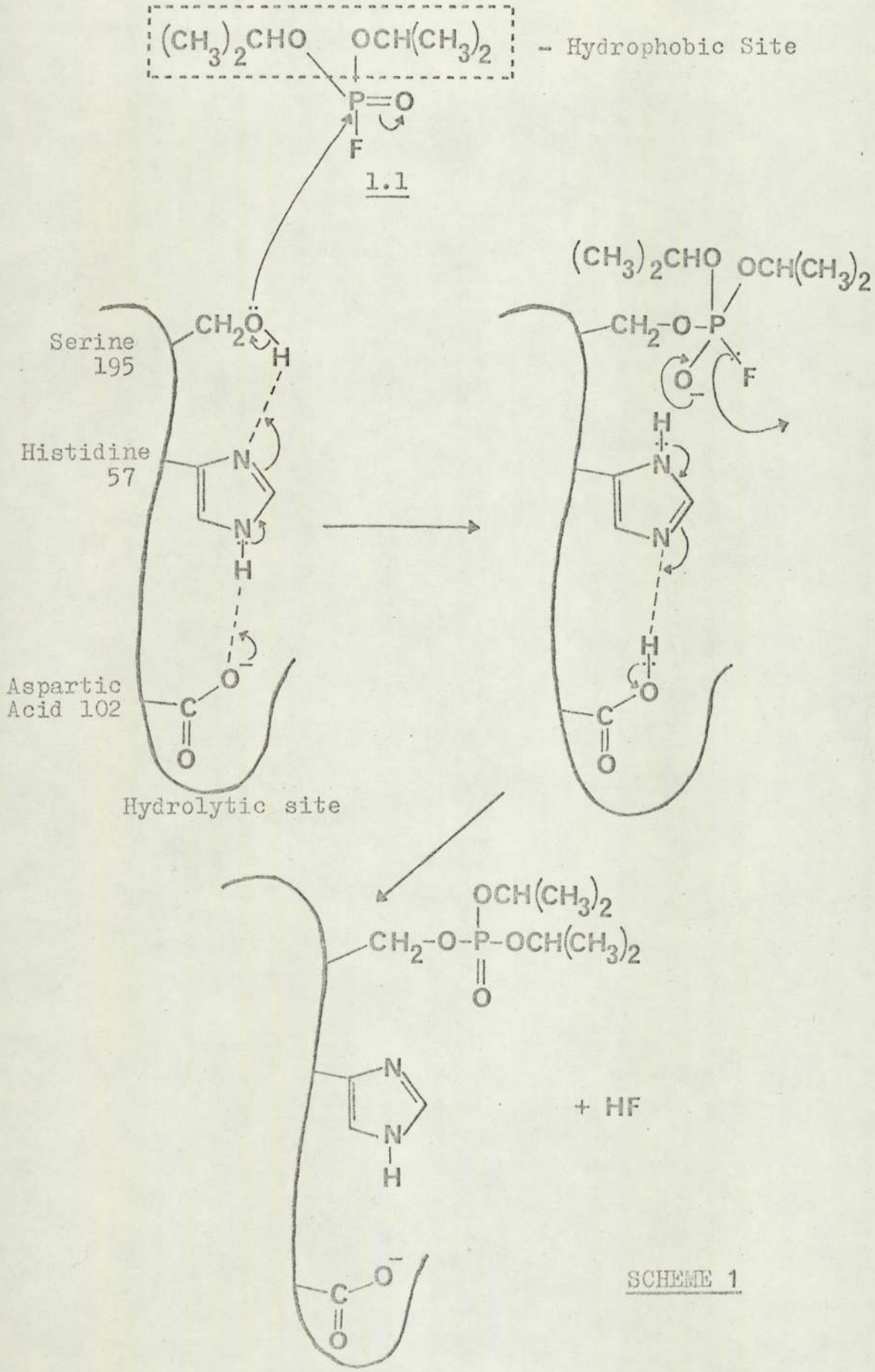
1. The reagent must contain the structural elements required for binding at the active site. It may be that groups at the active site have by the nature of their environment enhanced chemical reactivities, but even if they do not, the high local concentration of reagent at the binding site should ensure preferential reaction.
2. A reagent subject to rapid hydrolytic breakdown is not useful, nor is one that reacts so indiscriminately with amino acid groups that the effect of local concentration is nullified.
3. If possible the reagent should be designed in the knowledge of the enzyme's probable mechanism.

A major milestone in the study of irreversible enzyme inhibitors was the discovery [6-8] that diisopropylphosphofluoridate (Dyflos) (1.1) was a specific reagent for the active site of some esterases and proteolytic enzymes, e.g. α-Chymotrypsin. Dyflos (1.1) reacts by initial complexation with the active site of the enzyme,[9,10] (thought to be a hydrophobic area within the active site) followed by a facile neighbouring group reaction within the enzyme-inhibitor complex (Scheme 1). The active-site of α-Chymotrypsin is thought to contain three crucial areas; namely a hydrogen bonding site, a hydrophobic site and a hydrolytic site.[5] It is this latter site that is ultimately labelled by Dyflos. Within this site a charge-relay system is thought to exist involving the three amino acids, serine 195, histidine 57, and aspartic acid 102 (see Scheme 1).[10]

Of over twenty serine residues of α-chymotrypsin only one reacts with Dyflos,[11] thus indicating the unique character of serine 195. This uniqueness is due to the increased nucleophilicity of the serine molecule as a result of its involvement in the charge relay system.

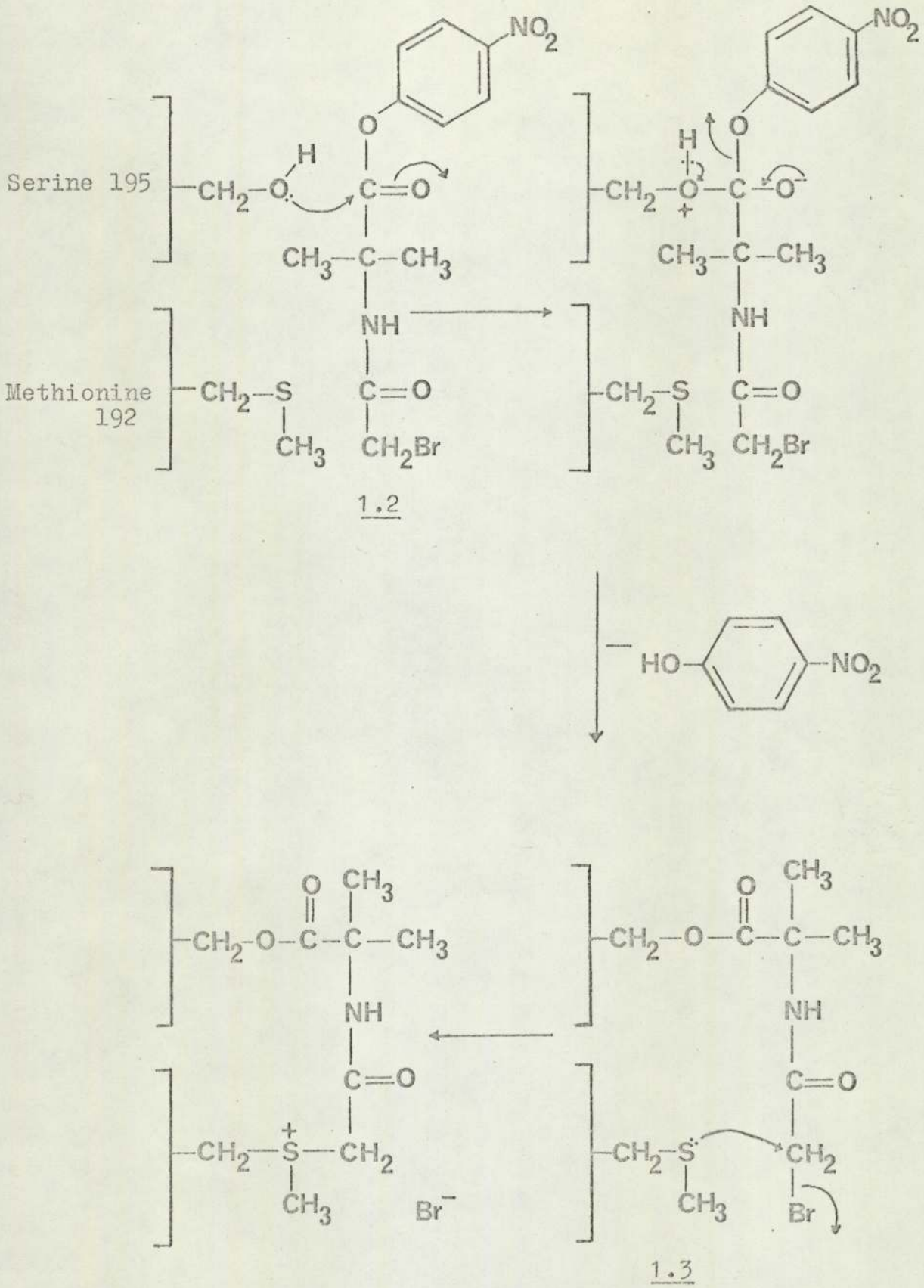
The acylation of the reactive serine residue as the initial covalent attachment of a substrate has been utilised to design a number of irreversible inhibitors of serine esterases.[5] In an elegant study Lawson and Schramm[12] selected 4-nitrophenyl-N-bromoacetyl -α-aminoisobutyrate (1.2) as a bifunctional agent for α-chymotrypsin (Scheme 2). Once the substrate is

Reaction of α-Chymotrypsin with Dyflos



SCHEME 1

Irreversible Enzyme-inhibitor complex.



1.2

1.3

SCHEME 2.

fixed in position by acylation of serine 195 a secondary reaction occurs with methionine 192 (1.3). Many other alkylating bromo compounds[13] have been shown to attack methionine 192. The bifunctional agents have been used as irreversible inhibitors of a number of proteolytic and esteratic enzymes, e.g. trypsin[14]. Baker[5] has reviewed work on the design of active-site-directed inhibitors; some examples are given in Table 1.

Penicillins (1.4) are classical examples of enzyme inhibitors evoking their activity by acylation of a nucleophilic amino acid (Scheme 3). It is commonly supposed that penicillins irreversibly acylate some enzyme playing a key role in cell wall synthesis.[1,19] Penicillins are one of the most widely used irreversible inhibitors in clinical medicine and their antibacterial activity is modified by the nature of the side chain (R) (1.4). In a recent review O'Grady [20] summarised the relative activities of the commonly used penicillins. Resistant bacteria synthesise the enzyme penicillinase in response to attack of the penicillin. This enzyme also hydrolyses the β-lactam ring in the general manner illustrated in Scheme 3.

Inhibition of Dihydrofolate Reductase.

In mammalian cells, the enzyme dihydrofolate reductase catalyses the reduction of the vitamin, folic acid, to 7,8-dihydrofolate. Further reduction by the same enzyme produces the cofactor form of the vitamin, tetrahydrofolate which is then involved in seventeen enzyme reactions used for one-carbon transfer.[21] In most cases

TABLE 1.

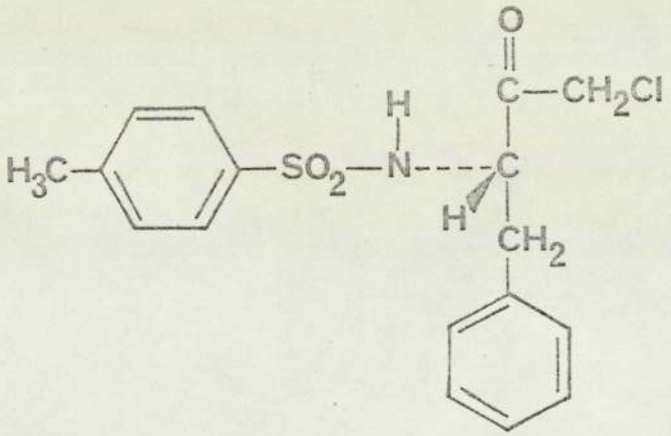
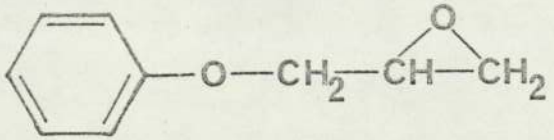
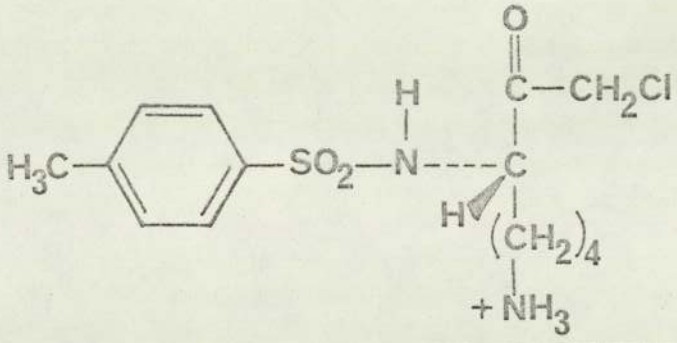
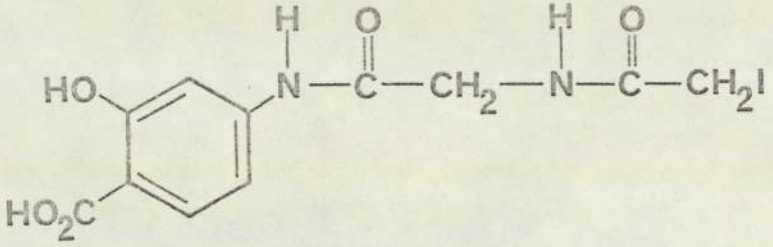
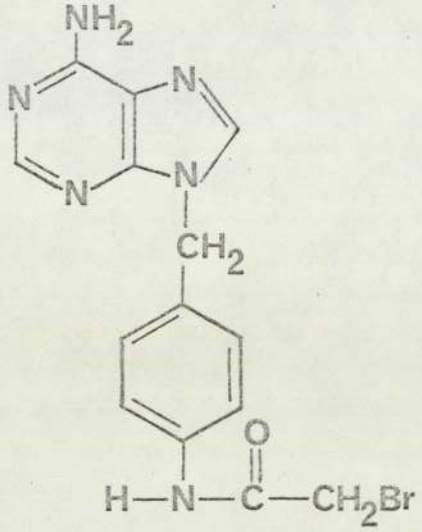
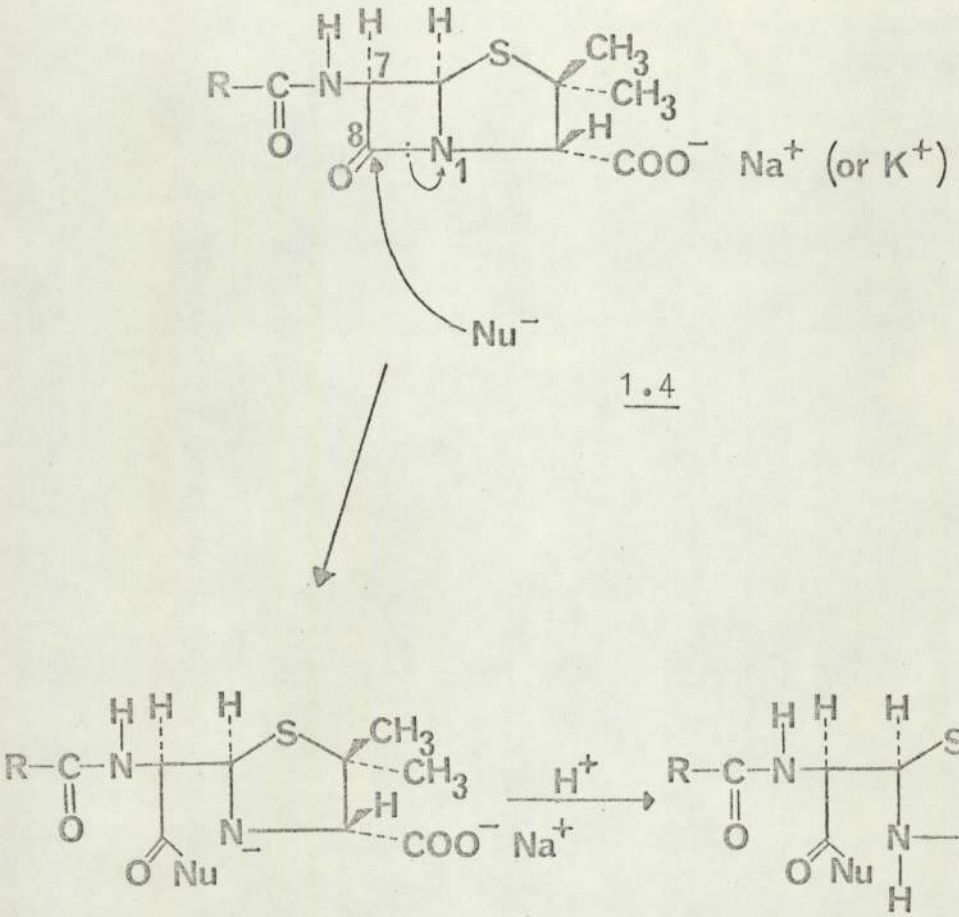
ENZYME	INHIBITOR	AMINO ACIDS ATTACKED	REFERENCE
α-Chymotrypsin		Serine 195 Histidine 57	15
"		Methionine 192	16
Trypsin		Serine 195 Histidine ?	14

TABLE 1 (Continued)

ENZYME	INHIBITOR	AMINO ACIDS ATTACKED	REFERENCE
Lactic and glutamic dehydrogenases		Histidine ?	17
Adenosine deaminase		Amino acid within hydrophobic area of active site.	18



1.4

R			
	a:	benzyl	Benzylpencillin
	b:	phenoxymethyl	Phenoxymethylpenicillin
	c:	2,6-dimethoxyphenyl	Methacillin
	d:	5-methyl-3-o chloro-phenyl-4- isooxazoyl	Cloxacillin
	e:	α-aminobenzyl	Ampicillin
	f:	α-carboxybenzyl	Carbenicillin

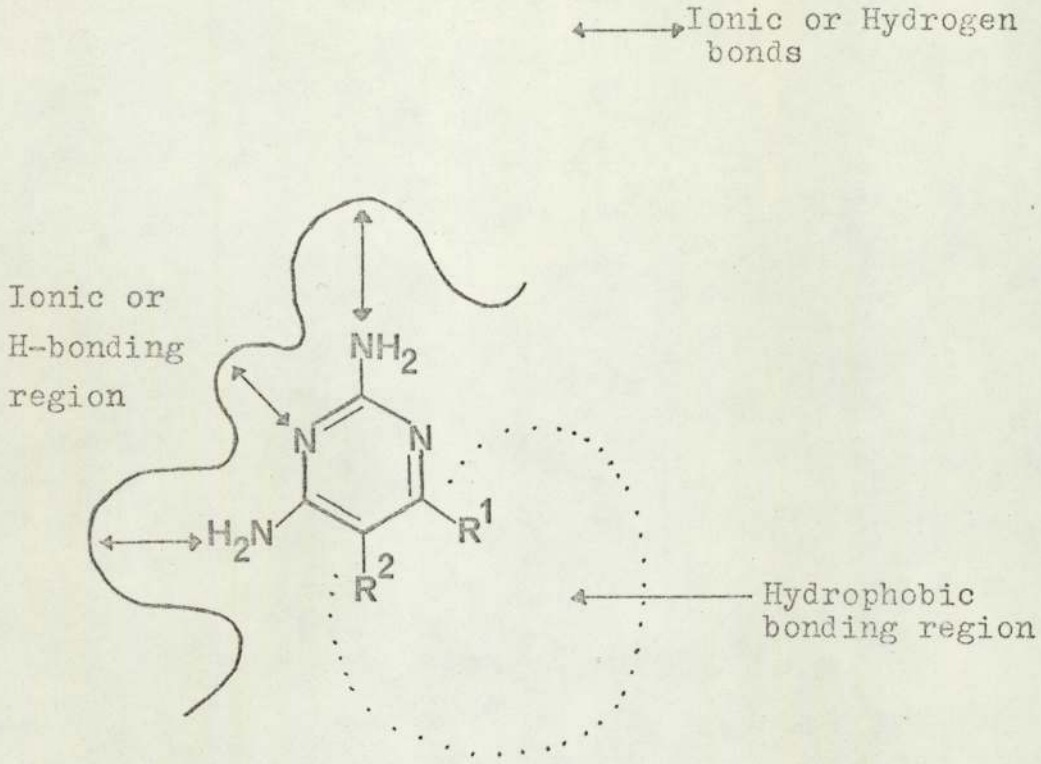
SCHEME 3

the blockade of tetrahydrofolate formation leads to cell death, due to the key involvement of tetrahydrofolate in nucleic acid synthesis.

Since it was discovered that there is a relationship between folic acid and cancer, in that folic acid activity is considerably higher in cancerous cells,[22, 23] a great deal of work has been done into research of anticancer drugs based on inhibition of dihydrofolate reductase. Initially reagents were designed based on the structure of folic acid; e.g. Aminopterin and Methotrexate. These reagents are extremely potent competitive inhibitors of dihydrofolate reductase.[24] Methotrexate is one of the drugs used in leukaemia therapy for children. These folic acid derivatives inhibit dihydrofolate reductase by forming a reversible enzyme complex with the active site of the enzyme. These reagents, however, suffer from two drawbacks:-

1. They show little selectivity for inhibiting the enzyme from different species or between normal enzyme and the enzyme of cancerous cells.
2. The development of resistance.

Alteration of the pteridine nucleus of the folic acid analogues to a 2, 4 - diamino-heterocyle leads to an increase in the selectivity of such compounds - however, the extent of inhibition is reduced. For example, Pyrimethamine (1.5) is an inhibitor of the enzyme and is a useful antimalarial,[24] and Trimethoprim (1.6) is a good inhibitor of bacterial dihydrofolate reductase but is inactive towards the mammalian enzyme.[24] Again both



R^1 = Et, R^2 = p - chlorophenyl (1.5)

R^1 = H, R^2 = 3,4,5-trimethoxybenzyl (1.6)

FIGURE 1.

these reagents form reversible complexes with the enzyme.

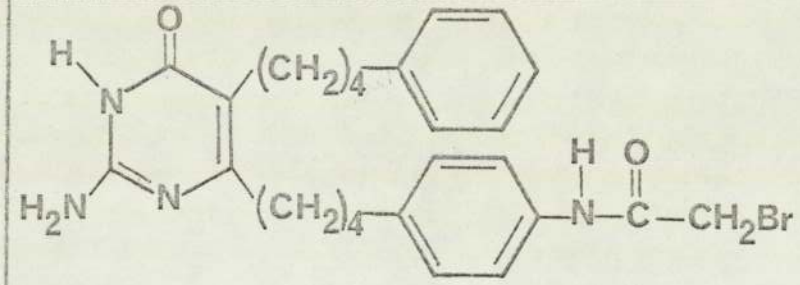
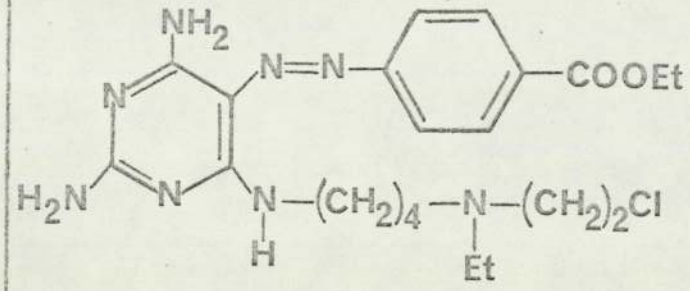
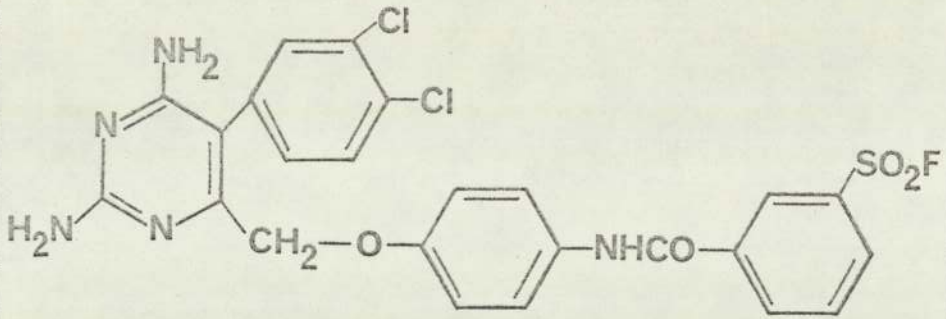
The first active-site-directed irreversible inhibitors of dihydrofolate reductase were the 2-aminopyrimidin-4(3H)-ones.[25] However, since 2,4-diaminopyrimidines show a greater affinity for the active site, this moiety has played a more important role. Examples of both types are shown in Table 2. The two criteria necessary for the successful design of an active-site-directed irreversible inhibitor of the enzyme are as follows:

1. The molecule must possess a 2,4-diaminopyrimidino fragment or a comparable moiety (see Figure 1) capable of complexing with the ionic bonding region of the active site.
2. It must contain a group capable of reacting covalently with the hydrophilic region of the active site.

The compounds illustrated in Table 2 fulfil both these criteria. They have structural groups that satisfy the first condition. The second criteria is fulfilled by an haloalkyl fragment or a sulphonyl fluoride group - both capable of reacting covalently with a hydrophilic site in the enzyme's active site. All of these compounds are irreversible enzyme inhibitors and are selective in their activity to some leukaemic cells.[25-27]

The 2,4-diamino-s-triazines have also been shown[28] to exhibit selective toxicity to micro-organisms and proliferating cells, and this reflects their ability to complex reversibly with dihydrofolate reductase.

TABLE 2

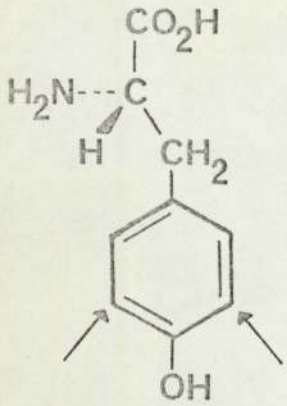
ENZYME	INHIBITOR	AMINO ACID ATTACKED	REFERENCES
Dihydrofolate reductase		Amino acid in hydrophilic area of active site	25
		"	26
		"	27

Reaction of Diazonium Compounds with Proteins.

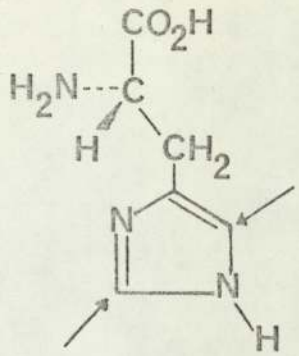
The high reactivity of diazonium compounds and their concomitant ability to react covalently with a wide range of nucleophilic and non-nucleophilic substrates makes them ideal probes for chemically reactive centres on biologically significant macromolecules. Pauly[29] found that diazotised sulphanilic acid coupled with L-tyrosine (1.7) and L-histidine (1.8) to give coloured C-azo compounds. Howard and Wild[30] studied the reactions of diazotised aniline with amino acids in alkaline media and found that when the amino group is terminal and in the α-position high yields of pentazenes were formed [with the exception of glycine (1.9a) and glycylglycine (1.10)] e.g. β-alanine (1.9b) and γ-aminobutyric acid (1.9c). An important amino acid in the labelling studies of proteins is lysine which also forms a pentazene derivative (1.11). From these examples the possible sites of attack within the active site of an enzyme or antibody are suggested.

In the last decade, attention has been focused on exploiting the reactivity of diazonium compounds to elicit information about the nature of the active sites of enzymes. In many of these studies specific amino acids have been identified and have been shown to have a role in either promoting the enzyme-substrate complex or being involved in the molecular events in catalytic activity. Examples of some of the enzymes that have been investigated are outlined in Table 3.

Landsteiner[37] utilised the diazo-protein reaction in his pioneering studies of the specificity of antigen-



1.7

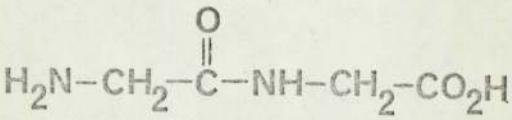


1.8

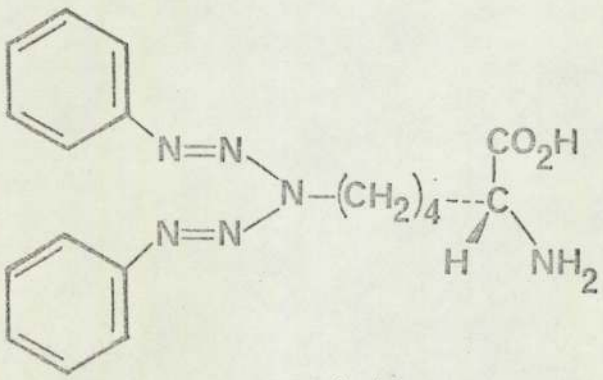
⟶ site of azo coupling with diazotised sulphanilic acid

$H_2N-(CH_2)_n-CO_2H$

1.9 a: n = 1 glycine
b: n = 2 β-alanine
c: n = 3 γ-aminobutyric acid



1.10

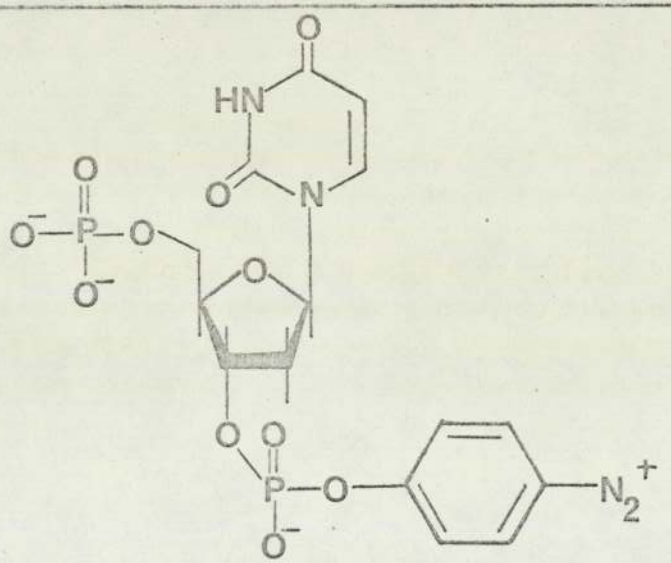
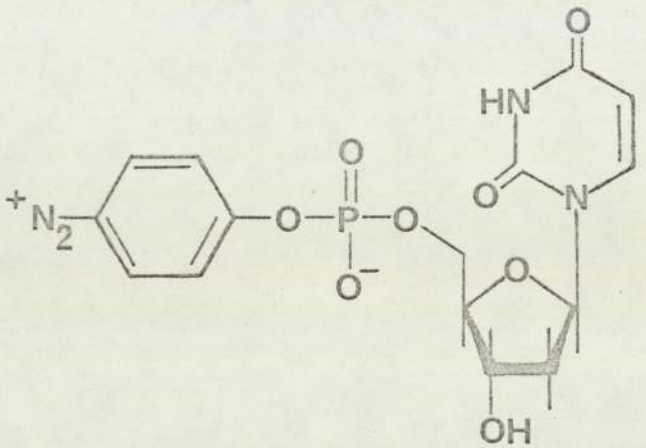


1.11

TABLE 3

ENZYME	REAGENT	LABELLED AMINO ACID	REFERENCE
Trypsin	Diazotised sulphanilic acid	Tyrosine Histidine Lysine	31
Fructose diphosphate	"	Tyrosine	32
α-Chymotrypsin	Diazotised p-nitroaniline	Lysine	33
Carboxypeptidase	Diazotised arsanilic acid	Tyrosine Lysine	34
Bovine pancreatic ribonuclease	Diazotised 5-aminotetrazole	Histidine	35

TABLE 3 (Continued)

ENZYME	REAGENT	LABELLED AMINO ACID	REFERENCE
Staphylococcal nuclease		Tryptophan Histidine	36
"		Tyrosine	36

antibody reactions. More recently,[38,39] these investigations have been directed towards an examination of the chemical nature of the active site of antibodies. The active sites or binding regions of antibody molecules would not be expected to contain uniquely reactive groups, since unlike enzymes they exert no catalytic activity. To overcome this difficulty the "affinity labelling" method was introduced by Singer and Doolittle[40] who utilised a 4-nitrophenyldiazonium salt (1.12) in their original paper. (Scheme 4). The mechanism of affinity labelling can be described in four steps:-

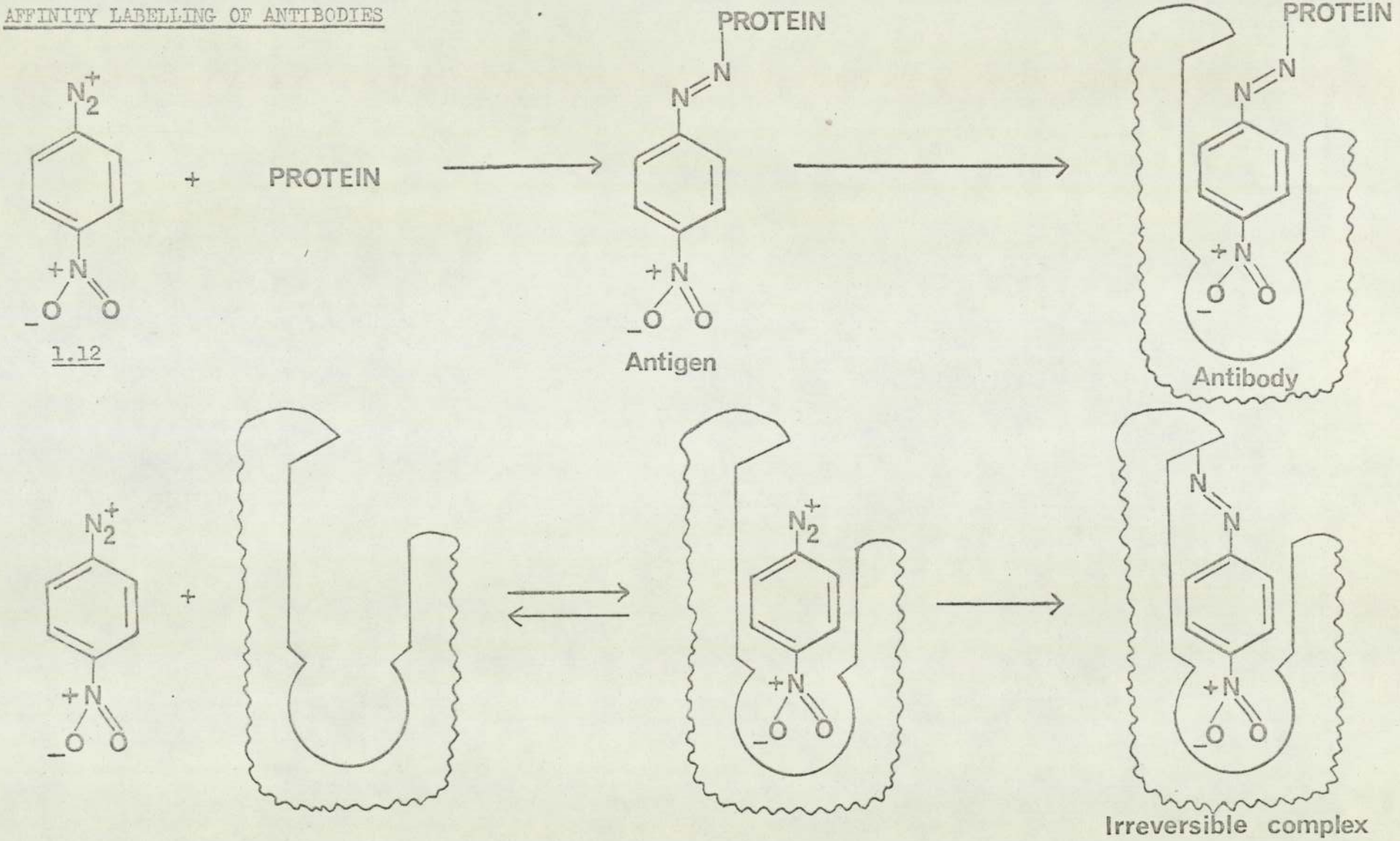
1. The diazonium compound is reacted with a protein to form an azo compound (as described previously). This derivative acts as the antigen.
2. The antigen is then injected into an animal, which builds up antibodies specific to the "azo" antigen, and capable of recognising the nitrophenyl fragment.
3. The specific antibody is then isolated and reacted with the original diazonium compound to form the antigen-antibody complex.
4. The diazonium compound reacts covalently with the antibody to form an irreversible complex.

From this complex the identity of the covalently bound amino acid within the antibody molecule can be recognised by hydrolysis of the labelled antibody.

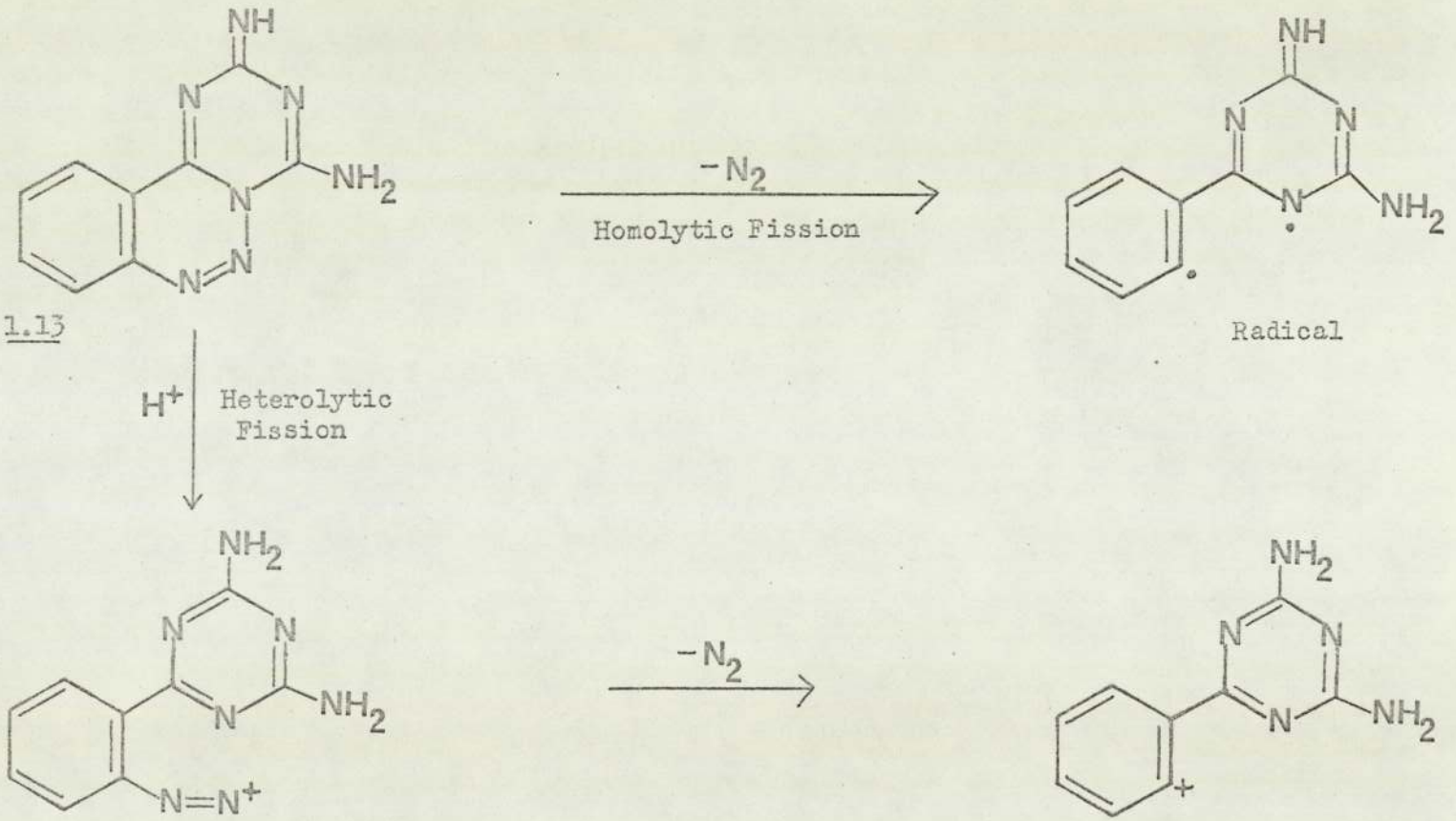
Diazonium compounds are potentially of little use as selective chemotherapeutic agents due to their great chemical reactivity and hence instability. Even if

SCHEME 4

AFFINITY LABELLING OF ANTIBODIES



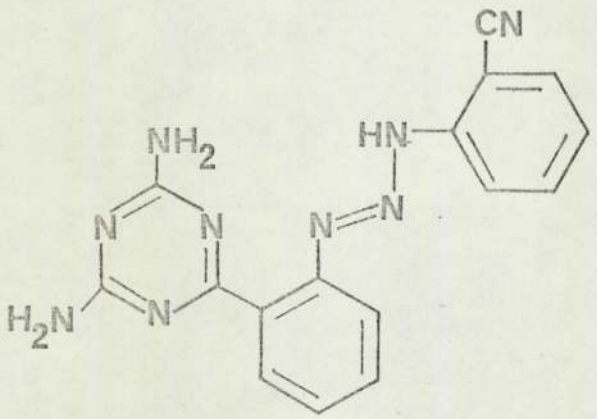
1.12


NH
N
N
NH2
N
N=N
1.13
−N2
Homolytic Fission
Radical
H+
Heterolytic
Fission
NH2
N
N
NH2
N=N+
Diazonium ion
−N2
NH2
N
N
NH2
+
Carbonium ion

SCHEME 5.

specific activity could be achieved, formulation of a diazonium compound into an acceptable pharmaceutical preparation would present formidable problems. To overcome these problems the biological activity of "masked" diazonium compounds was examined.[41] For example, derivatives of 1,2,3-benzotriazine and 1,3-disubstituted triazenes behave chemically as typical diazonium compounds and decompose homolytically or heterolytically depending on the nature of the reacting substrate.[42,43] An example of this type of compound is 4-amino-2(2H)-imino-s-triazino [1,2-c][1,2,3] benzotriazine (1.13) which was synthesised as a potential inhibitor of dihydrofolate reductase.[41] The mechanisms whereby a reactive intermediate could form with regard to this compound are outlined in Scheme 5.

Of the many benzotriazine and triazene compounds prepared by Mackenzie and Stevens only one compound (1.14) has shown reproducible anti-leukaemic activity.[44] The mode of action is unknown, but it is possibly acting as an irreversible inhibitor of dihydrofolate reductase.



1.14

B. NUCLEIC ACIDS

Nucleophilic Sites within the Nucleic Acid Macromolecule and their Chemical Reactivity.

Chemicals which react with nucleic acids *in vivo* usually produce cytotoxic effects in biological systems.[45] The purine bases, adenine and guanine are the most sensitive targets in nucleic acids for electrophilic reagents, and an understanding of the basic strengths of these components is important.[45]

It was shown conclusively by X-ray crystallography[46,47] that adenine residues protonate exclusively on N (1). Adenine can exist as a mixture of the 7 (H) and 9 (H) tautomers. The 9 (H) tautomer directs electrophilic attack to N (1) and the 7 (H) tautomer to N (3). Substituted adenine derivatives behave similarly. N (7) and N (9) of guanine residues are equally basic, and the site of protonation depends on the availability of these sites.[47] Thus, in the case of guanosine where N (9) is substituted, N (7) protonation occurs. The overall basicity is N (7) = N (9) guanine > N (1) adenine > N (7) adenine > N (3) adenine > N (3) guanine. Guanine can exist as a neutral or anionic species depending on pH. Electrophilic attack on the neutral species occurs in the imidazole ring at N (7); in the anion, N (1) is the site of attack.[49]

The most reactive site within the intact nucleic acid is N (7) of guanine[48] irrespective of the environment of the purine residue. Adenine is about one third less

reactive than guanine. In many cases the exact site of attack depends on the nature of the electrophilic reagent, the pH of the medium, and the nature of the nucleic acid (i.e. whether RNA or DNA). For reviews on this subject see references 45, 49-53.

Reagents which Interact with Nucleic Acids.

a. Monofunctional alkylating agents.

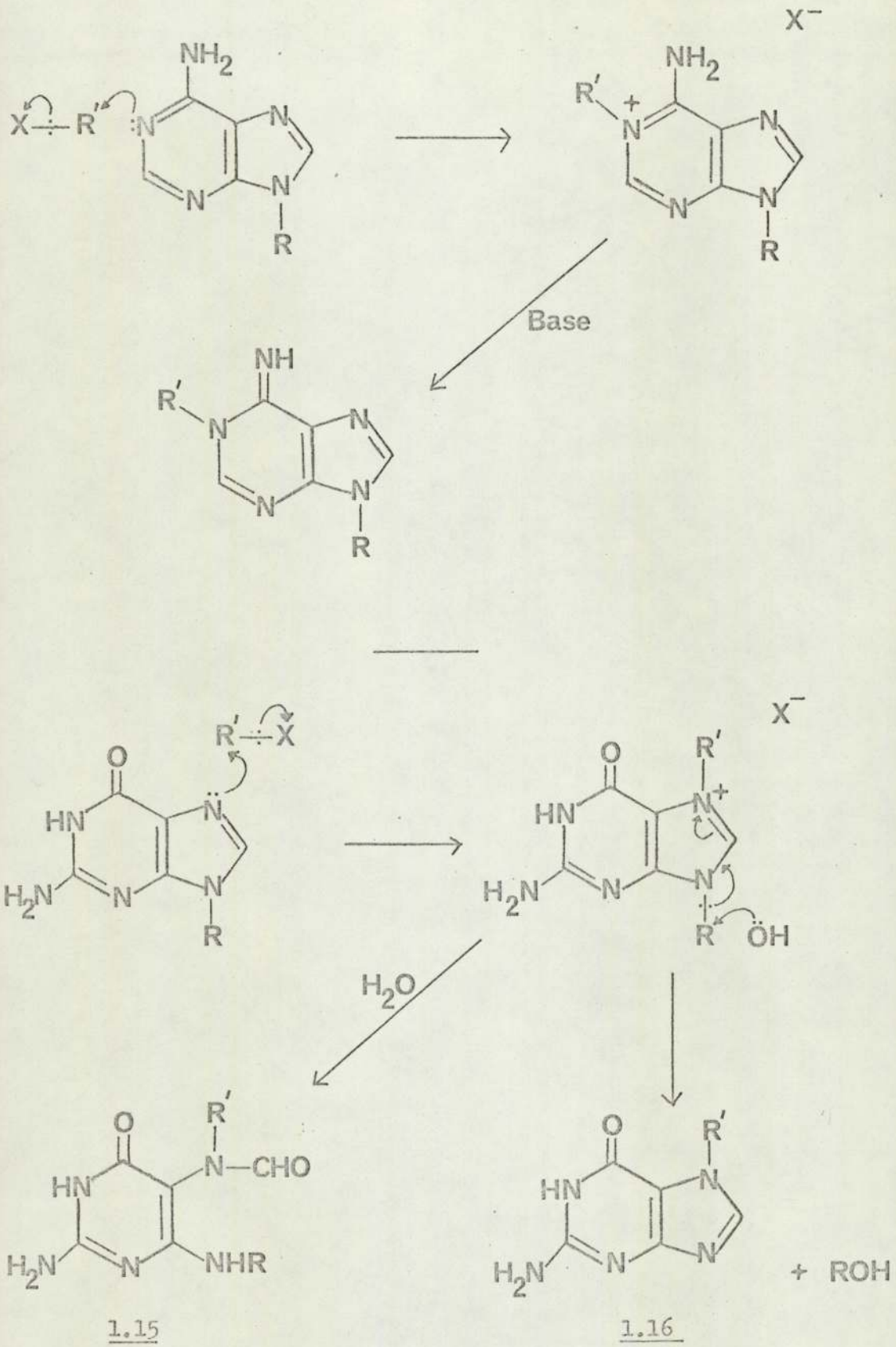
i. Alkyl salts and Alkyl halides.

Reaction of alkyl salts and alkyl halides with purine bases and their nucleosides has been widely studied; examples are shown in Table 4. The key parameter to these reaction is pH. For example, adenine reacts with benzyl chloride at position N (3) - this is due to the preferred existence of this molecule as the 7 (H) tautomer at acidic pH. If the acid released in the reaction is neutralised with carbonate then the 9 (H) tautomer predominates and N (9) alkylation occurs.[54] With the nucleoside adenosine, the preferred site of alkylation is at N (1). The influence of pH is even more important with guanine residues. In acidic or neutral conditions N (7) alkylation occurs, whereas, at alkaline pH N (1) alkylation is preferred.

The alkyl sulphates are more complicated in that reactivity differs according to the alkyl group. Dimethylsulphate reacts with guanosine at pH 9 to form several compounds, the major site of attack being the ribose moiety,[57] whereas diethylsulphate reacts to form mainly an O (6) alkylated product. The reasons for this are as yet

TABLE 4

REAGENT	CONDITIONS	pH	BASE	MAJOR SITES OF ACTIVITY	REFERENCES
Benzyl chloride	DMAC	5 - 6	Adenine	N3	54
Benzyl chloride	DMAC/K_2CO_3	5 - 6	Adenine	N9	54
Benzyl bromide	DMAC	5 - 6	Adenosine Deoxyadenosine	N1	55
Benzyl bromide	DMAC	5 - 6	Guanosine	N7	55
Alkyl halides Alkyl = Methyl, Ethyl, Benzyl, Phenethyl.	DMAC/K_2CO_3	5 - 6	Adenine	N9	56
Methyl iodide	DMAC	7	Guanine	N7/N9 dialkyl	57
Methyl iodide	DMAC	5 - 6	Adenosine	N1	57
Methyl iodide	CH_2Cl_2	7	Guanosine	N7	58
Methyl iodide	CH_2Cl_2/K_2CO_3	7	Guanosine	N1	59
Dimethylsulphate	DMF	7	Adenosine	N1	57
Dimethylsulphate	-	7 9	Guanosine	N7 Ribose Unit	57
Diethylsulphate	-	6 - 7 7 - 8	Adenosine	N1 N6 N1 N6	60 61
Diethylsulphate	-	7 9	Guanosine	N7 06 06 N7	62



SCHEME 6.

R = ribose R′ = alkyl group X = halide or alkylsulphate

unknown, but this observation could explain the differences in the biological effects of the compounds.[60,61] The most important reactions of the alkyl salts and alkyl halides with adenine and guanine derivatives are outlined in Scheme 6. The 7-alkyl guanosine once formed can either ring-open to form a pyrimidine derivative (1.15) or, more significantly, it can lose the ribose unit to form 7-alkyl guanine (1.16). This latter process explains the base deletion which can occur subsequent to attack of guanine by alkylating agents. References 48-53,63 should be consulted for additional information on the reactions of nucleic acids with alkyl halides and alkyl salts.

ii. Aziridines

Another group of monofunctional alkylating agents are the aziridine derivatives; such compounds have profound effects on living cells. The detailed mode of action[64] and metabolism[65] of aziridine-derived alkylating agents is unknown. However, it is reasonably certain that the nucleic acids are the sites of attack, although other cellular components may also be involved. Workers have speculated that the prime site of attack is N (7) of guanine and that DNA is the most susceptible nucleic acid.[66]

An example of such a derivative is 2,4-dinitrophenylaziridine[67] (1.17a). Extension of the studies on 2,4-dinitrophenylaziridines led to the synthesis of a large number of structurally related compounds.[68-70] One of these derivatives, 5-aziridino-2,4-dinitrophenylbenzamide (1.17b) (CB 1954) proved to be an exceptional

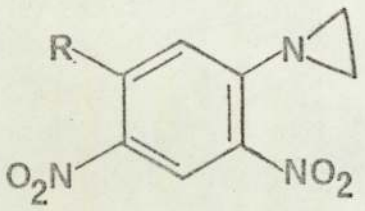
agent selectively toxic to the Walker carcinoma.[69,70] It is inactive, however, against a number of other transplantable tumours, many of which are highly sensitive to bifunctional alkylating agents. Generally monofunctional aziridines, although often just as toxic, rarely have any antitumour properties. CB 1954 is an exception and thoughts on its mechanism of action will be discussed in Chapter 7.

The mechanism of other monofunctional aziridines is believed to proceed through an SN2 reaction with bio-nucleophiles. The polyfunctional aziridines such as Tetramin and TEPA are generally more effective than the monofunctional agents. This suggests a crosslinking effect (cf. Nitrogen mustards) but there are many exceptions A monograph on ethyleneimine derivatives contains a valuable chapter on the biological properties of aziridines.[64]

iii. "Incipient" carbonium ion precursors.

The nitrosoamines[70-79] and alkyltriazenes[70,80-82] fall into this category. Magee[71] summarised the possible mechanisms of the biological activity of the nitroso derivatives and related the results to their mutagenic and carcinogenic activity. The exact nature of the proximate alkylating species is open to doubt.[71] Examples of the various types of nitroso compounds and their proposed reactive intermediates are shown in Table 5.

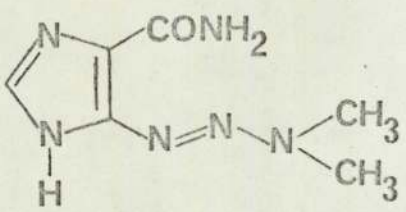
Recent evidence [72,74,78] has indicated that the methyl group of the nitroso compounds is transferred intact and earlier theories that diazomethane is the effective



1.17

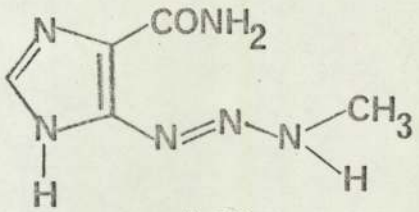
a: R = H

b: R = $CONH_2$

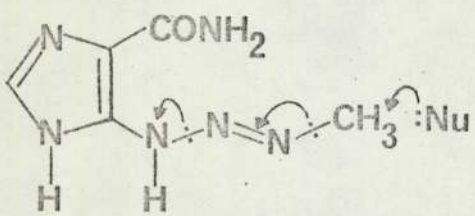


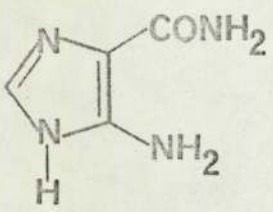
1.18

Enzyme



1.19



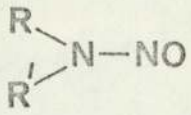


+ N_2 + CH_3Nu^+

1.20

SCHEME 7

TABLE 5



NITROSO DERIVATIVE R	R^1	PROPOSED INTERMEDIATE	REFERENCE
Me	Me	CH_3^+/CH_2:	71
Me	$CONH_2$	CH_3 -N = N-OH	
		$CH_3N_2^+$	72,73
		CH_3^+	
Me	$C(:NH)NH_2$	CH_3^+	74
Me	CO_2Et	CH_2N_2	75
$-(CH_2)_5-$		Unknown	76,77
$-(CH_2)_2-O-(CH_2)_2-$		Unknown	76,77

alkylating agent are shown to be in error. The mechanism of the cyclic nitroso derivatives is as yet unknown; whether these agents do in fact alkylate nucleic acids is controversial.[76,77] Alkylation of nucleic acids with the nitroso compounds leads to the methylation of N (7) of guanine and N (3) of adenine;[79] the extent to which this occurs is extremely high and hence explains the profound cytotoxicity of these compounds.[71]

The nitroso compounds show many points of resemblance to the difunctional alkylating agents in terms of mechanism of action and biological activity. They are effective against various leukaemias and a number of solid tumours.[70] Despite these similarities to the difunctional alkylating agents they have a completely different spectrum of anti-tumour activity. Correlation between the alkylating ability and anti-tumour activity of the nitroso compounds has yet to be fully determined. For a more detailed account of the biological activity of nitroso compounds see references 70-79.

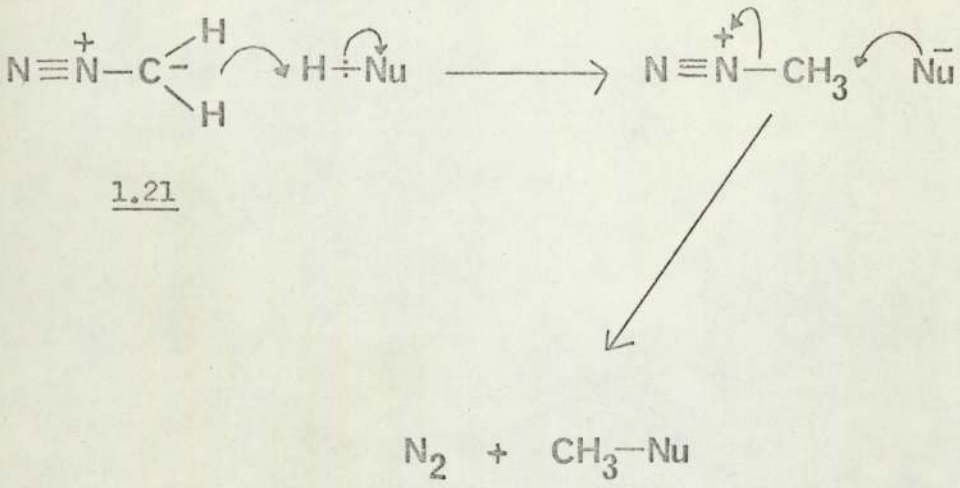
The 1-aryl-3,3-dialkyltriazenes[80] and the imidazotriazene (1.18)[81,82] have significant cytotoxic activity. These dialkyl triazenes are dealkylated *in vivo* to form monoalkyl derivatives which are in fact the cytotoxic agents. Of all the triazenes synthesised, 5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide (1.18) (DIC) proved to be the most active and is used clinically in the treatment of various leukaemias.[70] It is a derivative of the purine precursor 4-aminoimidazole-5-carboxamide (1.20).

The nature of the reactive intermediate responsible for the triazenes ability to alkylate the nucleic acids is again controversial. The methyl carbonium ion, diazomethane, or methylene have all been considered.[70,80-82]

However, a more likely mechanism is that illustrated in Scheme 7 and involves initial dealkylation to the monoalkyltriazene (1.19). After a prototrophic shift the resulting azomethane tautomer would be susceptible to Sn2 attack by a biologically significant nucleophile. The N (7) of guanine has been identified as the participating nucleophile.[80]

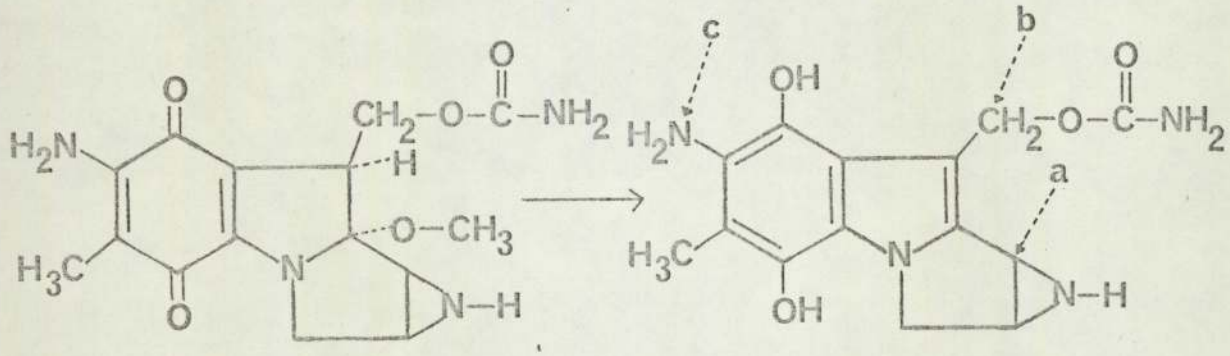
With regard to the triazenes in general, the essential reaction is methylation, analogous ethyl derivatives although equally toxic to cells in vitro inhibit no anti-tumour properties. This equally applies to the nitroso compounds. For further information on the biological activity of triazenes see references[70,80-82]

The biological properties of diazomethane (1.21) have been investigated by a number of workers.[83,84] Haines and his co-workers used diazomethane to methylate various purines, and showed that it could be used to bring about a cytotoxic effect. Reaction with the nucleic acids themselves has been extensively studied;[85-87] these studies have shown that diazomethane acts as a monofunctional alkylating agent. Since diazomethane can only alkylate unionised substrates it is likely that an intermediate methyldiazonium ion is the reactive species (Scheme 8). For reading on the cytotoxic effects of diazomethane, see references 75,89-93.



1.21

SCHEME 8



1.22 Mitomycin C

1.23 Activated form

-----→ Reactive site

b. Difunctional Agents.

i. Mitomycins

Of all the mitomycins only Mitomycin C (1.22) is of significant medicinal importance. It attracted interest because of its therapeutic action against certain tumours;[94] it is also active as an inhibitor of bacterial growth.[95] The ability of Mitomycin C to inhibit DNA selectively, without subsequent inhibition of RNA or protein synthesis, has been observed.[96] Further investigations by Matsumo and Lark[97] showed that the lethal effects of Mitomycin C (on bacterial and mammalian cells) are associated with dramatic changes in the structure of cellular DNA. Exposure of the cells for short periods revealed that Mitomycin C had reacted covalently.[97] This combination occurs in such a way as tc form cross-links between complementary strands of the DNA helix.

Mitomycin C is thought to be transformed to an activated form (1.23) which contains three reactive sites[98] (1.23a,b and c). Iyer and Szybalski[99] suggest that reactive sites (a) and (b) are sufficient to explain the necessary difunctional alkylating properties of the activated molecule. The significance of the third reactive site (c) is uncertain.[98,99]

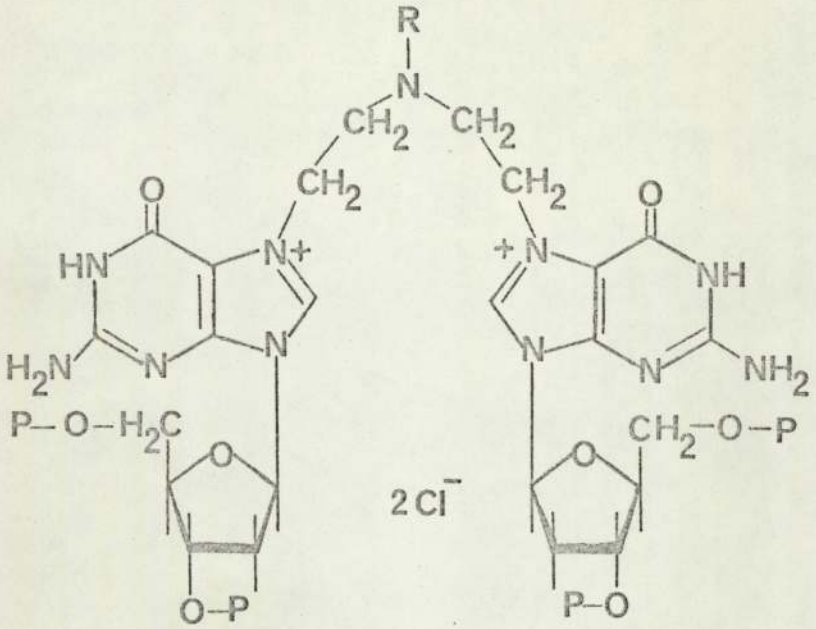
Direct evidence for covalent interaction between Mitomycin C and nucleic acids has been established,[100] N (7) of guanine is one of the nucleophilic centres that is alkylated.[101]

ii. Nitrogen Mustards

The chemical and biological properties of the nitrogen mustards have been extensively reviewed[67,88,102-106] and will be only briefly mentioned. Reaction with nucleic acids can proceed via an SN1 or SN2 reaction, and in all cases N (7) of guanine is the vulnerable target.[102] Reaction with guanine leads to the formation of a N (7) - N (7') linked biguanyl moiety (1.24) responsible for the crosslinking lesion in nucleic acids. These quaternised guanine residues are sensitive to hydrolytic fission at the bond linking the hetero moiety to the sugar residue, leading to base deletion similar to that illustrated in Scheme 6.

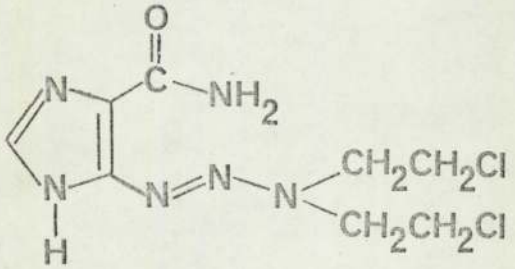
Recently interest has been centred on nitrogen mustards based on monofunctional alkylating compounds i.e. imidazotriazenes and nitroso derivatives. Two such agents with exploitable anti-tumour activity are 5-[3,3-bis-(2-chloroethyl)-1-triazeno]-imidazole-4-carboxamide (1.25) and 1,3-bis-(2-chloroethyl)-1-nitrosourea (1.26). These bifunctional compounds are effective against various leukaemias[107-111] and solid tumours.[112] For further details on the mechanism of action of these "newer" difunctional agents see references 108 and 110.

There are many other alkylating agents which react with nucleic acids, and for an account of some of these reagents see references 102, 113-118.



1.24

P = phosphate R = aliphatic or aromatic group



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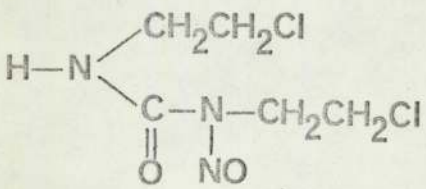


1.26

CHAPTER 2.

CHAPTER 2

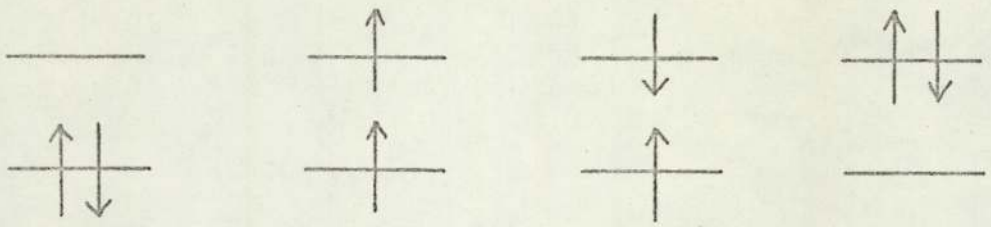
The Interaction of Carbenes with Biological Substrates.

Carbenes are neutral bivalent carbon reactive intermediates in which a carbon atom has two covalent bonds to other groups, and two non-bonding orbitals containing two electrons between them. The possible arrangements of two electrons between two orbitals of different energies are shown in Figure 2. The reactions of carbenes[119-121] include addition to olefines, insertion into CH, NH, SH and OH bonds and formation of transition metal complexes.[122]

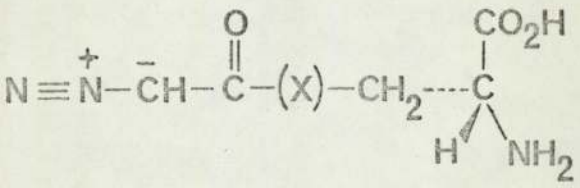
Carbenes generated from a suitable precursor have been used in the specific exploration of biological macromolecules; their particular interest lies in a potential ability to react with both hydrophilic and hydrophobic centres within those molecules. They therefore have an added dimension to their reactivity in comparison to the examples described in Chapter 1 which could only react with hydrophilic species. This equally applies to nitrenes.

The antibiotic L - azaserine (2.1a) was first shown by Buchanan [123] to be an irreversible inhibitor of amide transferase. The related analogue 6-diazo-5-oxo-L-norleucine (2.1b) also irreversibly inhibits this enzyme: only the L stereoisomers are active. These results suggest that the active compounds first form a specific but reversible complex at the active site of the enzyme and then react covalently possibly *via* a carbene inter-

FIGURE 2

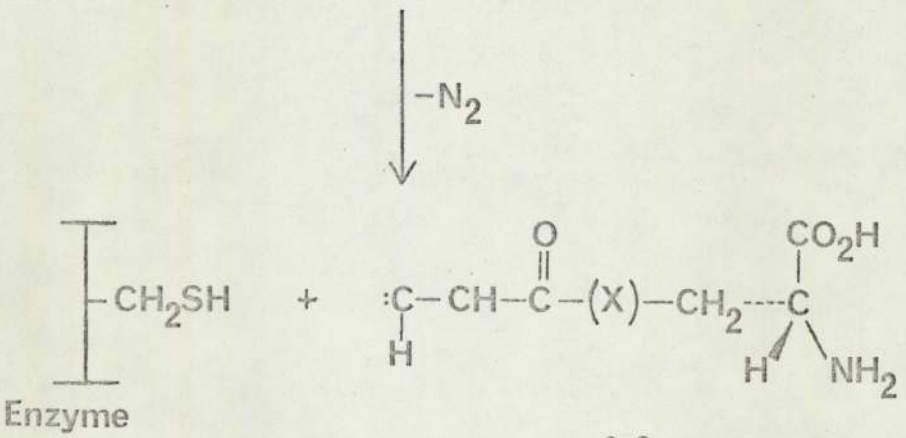


Lowest Singlet Triplet Excited Singlets

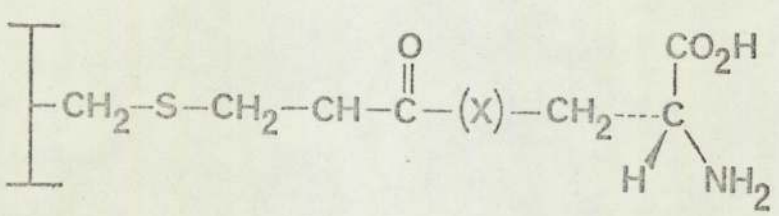


2.1 a: X = O

b: X = CH_2



2.2



SCHEME 9

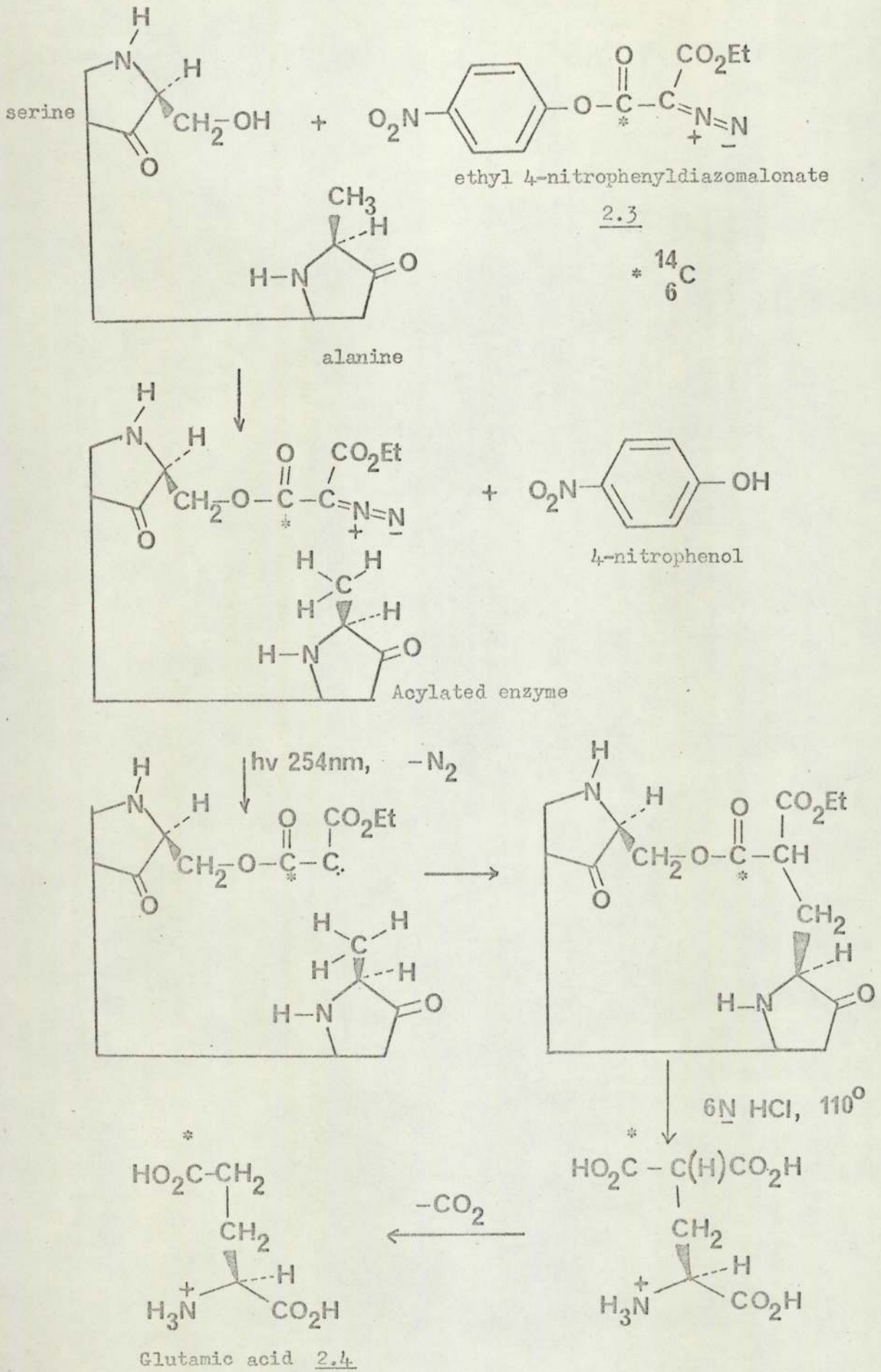
mediate (2.2) with a suitably positioned amino acid residue (Scheme 9). This residue has been recognised as a cysteinyl fragment[124,125] and the stereochemical analogy between the amide group the enzyme transfers and this fragment strongly suggests, that the cysteine molecule is within the enzyme's active-site.

Vaughan and Westheimer[126] described a similar insertion into an alkyl group on the enzyme trypsin, using C^{14}-labelled 4-nitrophenyl-diazomalonate (2.3) as the carbene precursor. This compound forms a reversible enzyme-substrate complex and then a stable acylated enzyme (cf. Scheme 1, Chapter 1). On irradiating this complex a carbene intermediate forms which subsequently reacts covalently with a suitably placed amino acid within the active-site of the enzyme (Scheme 10). On hydrolysis of the covalently labelled enzyme, C^{14}-glutamic acid (2.4) was isolated. This could only have come from carbene insertion into the methyl side chain of an alanine residue and represents the first successful labelling of a hydrophobic amino acid. The yield of insertion product was very small (1-3%) but the feasibility of the photo-generative approach to enzyme labelling was put on firm ground by this work.

For information on other biological macromolecules that have been labelled by carbene precursors see references 127 - 135.

In utilising carbene precursors in biological receptor site labelling three factors have militated against complete success.[136] Firstly, the photogenerated

SCHEME 10


serine
ethyl 4-nitrophenyldiazomalonate
2.3
* $^{14}_{6}C$
alanine
4-nitrophenol
Acylated enzyme
hv 254nm, $-N_2$
6N HCl, 110°
$-CO_2$
Glutamic acid 2.4

carbene was produced on the surface of the macromolecule, and the extraordinarily high reactivity of the carbene leads to a large amount (50 - 60%) of product resulting from reaction with water. Secondly, the intrinsic chemical reactivity of the diazo compounds leads to products arising from chemical (as distinct from photochemical) reactions with neighbouring groups. Thirdly, some of the carbene undergoes a Wolff-type rearrangement to form a ketene. In summary the amount of labelled macromolecule varies from 1 - 2% to about 40% depending on the macromolecule involved.

The Interaction of Nitrenes with Biological Substrates.

a. The Generation and Reactivity of Nitrenes.

Nitrenes are neutral, univalent nitrogen intermediates in which a nitrogen atom has one covalent bond to another group, one lone pair of electrons and two non-bonding orbitals containing two electrons between them. As with carbenes if these two electrons are spin paired then the nitrene is a singlet; if the spins of the electrons are parallel the nitrene is a triplet.

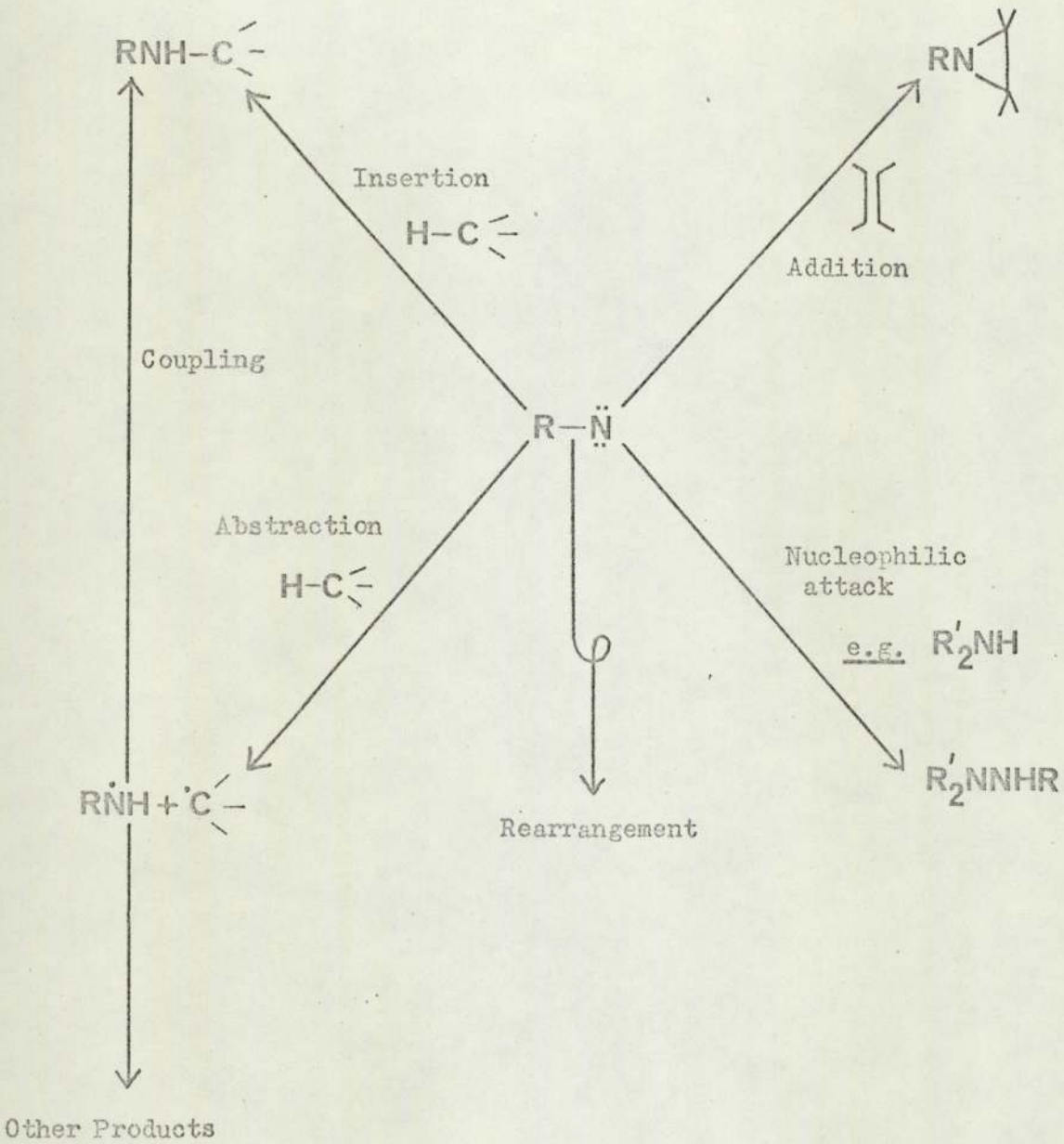
Rose[137] was the first to suggest that nitrene intermediates could be the chemical species produced by bio-transformation of hydroxylamines, amines and amides and be responsible for the cytotoxicity of these compounds. A nitrene may be generated during the metabolic oxidation of the parent amine or amide. If the possible enzymatic formation of nitrenes is conceded then it is not difficult to see how the disorganisation of normal cellular processes might then follow from the interaction

of these labile substances with, for example, the nucleic acids.

Nitrenes may also be produced photolytically by reactions analogous to those forming carbenes, from alkyl, aryl and acyl azides. In general nitrenes are more selective in their activity than carbenes.[138] Some typical nitrene reactions are summarised in Scheme 11. The likely reactions open to nitrenes are abstraction (normally of hydrogen from carbon), cycloaddition, direct insertion (normally C-H bonds) attack by nucleophiles and rearrangement.[121] Abstraction reactions followed by radical coupling will lead to insertion and while direct intermolecular insertions are rare, insertion products are common in intra-molecular reactions. If a nitrene is generated *in situ* at a binding locus, the intra-molecular situation is more applicable. Direct insertion, abstraction, coupling or addition reactions will then result in covalent attachment of the nitrene precursor to biological macromolecules.

Rearrangement reactions can reduce the effectiveness of a reagent designed to label a biological macromolecule. Acyl azides as potential precursors are ruled out since on generation of the nitrene a Curtius rearrangement to the isocyanate results,[139] analogous to the Wolff rearrangement undergone by carbenes. Even in cases stereochemically favoured for intramolecular insertion by acyl nitrene, some 70% of isocyanate results. Aryl nitrenes are much less susceptible to rearrangement and the major rearrangement path results in formation of

SCHEME 11


RNH–C
RN
Insertion
H–C
Addition
Coupling
R–N
Abstraction
H–C
Nucleophilic attack
e.g. R'2NH
R'2NNHR
RNH + C
Rearrangement
Other Products

azepines.[140]

There are three criteria for the utilisation of nitrene precursors as potential labelling reagents. These are:-

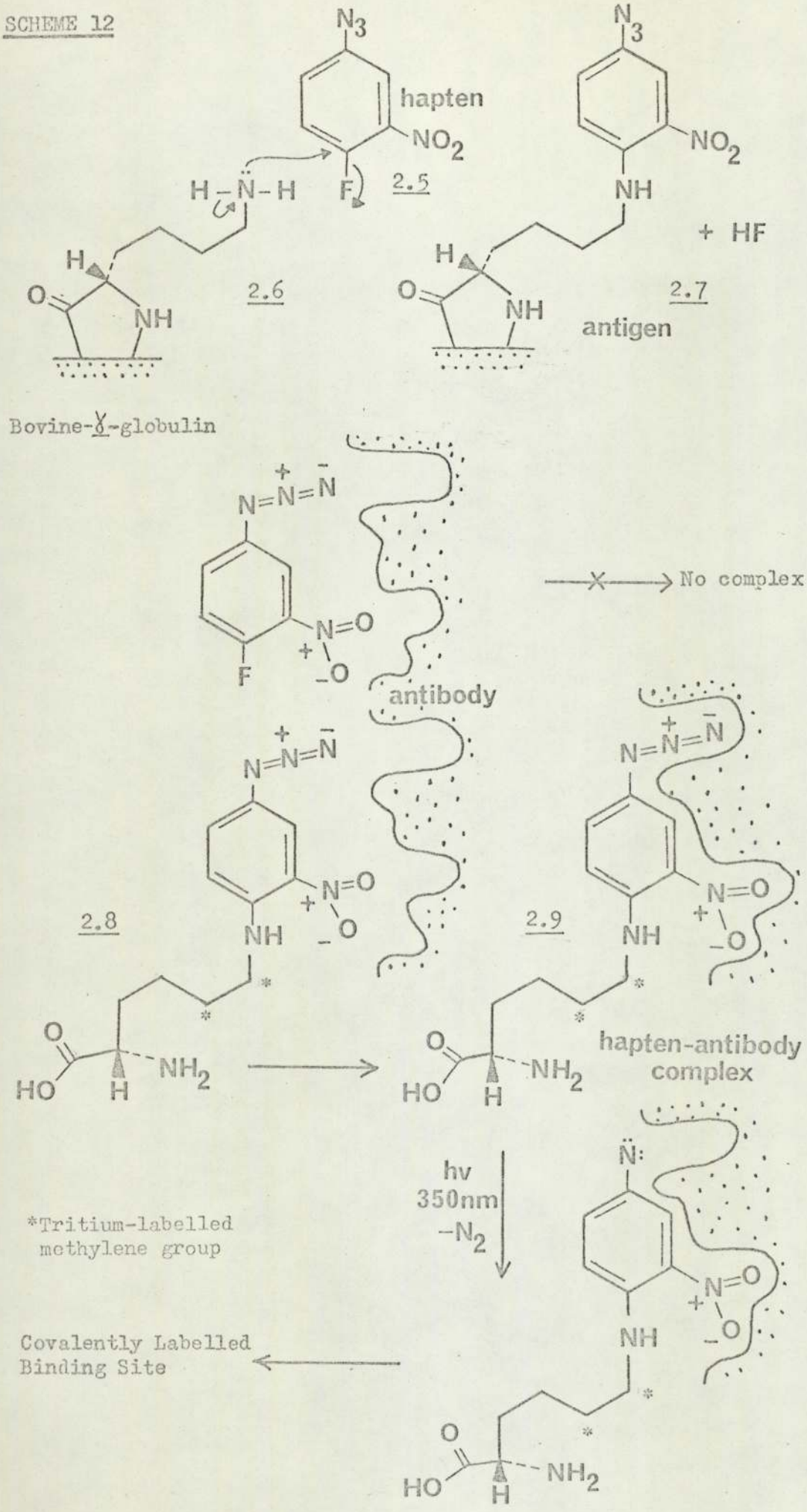
1. They must be chemically inert.
2. They must not be susceptible to rearrangement.
3. The nitrene must be generated at a suitable wavelength not damaging to the biological macromolecule.

The first two criteria are satisfied by some alkyl and most aryl azides. However alkyl azides have absorption maxima around 290nm. and hence it is not possible to photolyse the azide in a biological environment without damaging the target receptor. So we are left with aryl azides, which are chemically stable at 37°C, not drastically susceptible to rearrangement, and which, if appropriately substituted, can be photolysed to the aryl nitrene at wavelengths not damaging to biological macromolecules.

b. The Biological use of aryl nitrenes.

An aryl nitrene has been used as a labelling reagent for a specific antibody.[141] Scheme 12 shows the main steps in the preparation and labelling of rabbit NAP antibody (NAP; 2-nitro-4-azidophenyl). The antigen (2.7) used was formed by combination of the hapten 4-fluoro-3-nitrophenyl azide (2.5) with the $\underline{\varepsilon}$ - amino groups of lysine residues of bovine $\underline{\gamma}$-globulin (2.6). The nitro group was included not only to facilitate

SCHEME 12


hapten
2.5
2.6
2.7
+ HF
antigen
Bovine-γ-globulin
No complex
antibody
2.8
2.9
hapten-antibody
complex
hv
350nm
−N2
*Tritium-labelled
methylene group
Covalently Labelled
Binding Site

preparation of the antigen but for two other reasons. Firstly, the substituent shifts the λ-max of the hapten into the visible region. Secondly, the nitro group decreases the half life (i.e. increases the reactivity) of the nitrene.[142]

The antigen (2.7) was injected periodically over four months until an adequate serum level of antibody was obtained. The antibody combining site was assumed to be specific for the hapten group, 3-nitrophenyl azide, but precipitation and purification of the antibodies followed by reaction with 4-fluoro-3-nitrophenyl azide (2.5) did not give hapten-antibody conjugate. However, the antibody did combine with N-(2-nitro-4-azidophenyl)-L-(4,5-^{3}H) lysine (2.8), and, after irradiation of the (antibody-hapten) complex (2.9), some 65% of the antibody sites were blocked by the specific covalent attachment of the hapten.

This method of affinity labelling is also in widespread use for attachment of covalent labels to the active-sites of other macromolecules. These include the opiate receptor,[143,144] the binding site of the proteins involved in the respiratory chain,[145] glycosidal proteins[146] and many enzymes, e.g. cholinesterases,[147] glutamate dehydrogenase,[148] α-chymotrypsin[149] and dihydrofolate reductase.[41]

This thesis is partly concerned with the potential radiogeneration of nitrenes from suitable aryl azides. The azides chosen were such that the aryl moiety had a known affinity for a biological macromolecule. The "carrier" molecules chosen were the 2,4-diamino-s-triazino nucleus (known to have an affinity for dihydrofolate

reductase) and the acridine nucleus (known to have an affinity for nucleic acids). If after forming the initial reversible complex, a nitrene could be generated in situ this might lead to formation of a covalent bond between the 'carrier' molecule and the biological macromolecule, resulting in an irreversible inhibition of that macromolecule. In view of the involvement of dihydrofolate reductase and nucleic acids with cancerous cells, this irreversible inhibition could result in these azides exhibiting irreversible anti-tumour activity.

PART II

DISCUSSION OF EXPERIMENTAL RESULTS

CHAPTER 3.

PART 2

DISCUSSION OF EXPERIMENTAL RESULTS

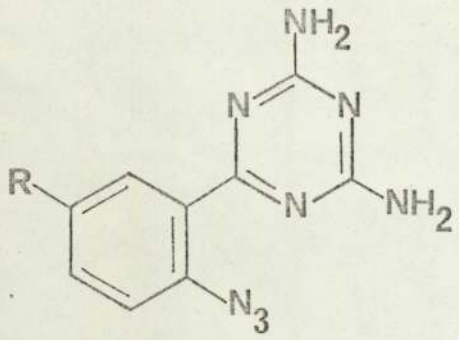
CHAPTER 3

Possible Radiogeneration of Nitrene Intermediates.

The ultimate objective of this work was to design a radiation-sensitive molecule with both affinity for a target site within a tumour cell and bearing a masked reactive group (e.g. an azide) incorporated into the structure.

2,4-Diamino-6-(2-azidoaryl)-s-triazines (3.1) were synthesised by McKenzie and Stevens[41] as potential inhibitors of dihydrofolate reductase. These compounds were known to have an affinity for the active-site of this enzyme and it was hoped that once the enzyme-inhibitor complex formed a nitrene could be generated in situ; then an irreversible inhibition of this enzyme could occur (cf. Chapter 1). These workers examined the thermal stability of these azides; in this work the photochemical and radio-chemical stability was examined.

The X and γ - radiolysis of organic solids is an extremely complicated subject. In general most of the reactions which occur are ordinary thermal reactions, although some involve unusual chemical species. In some cases regions of excess energy - 'hot spots' - may be present and these regions can provide activation energies greater than those available thermally. Aromatic compounds tend to be more stable than aliphatic

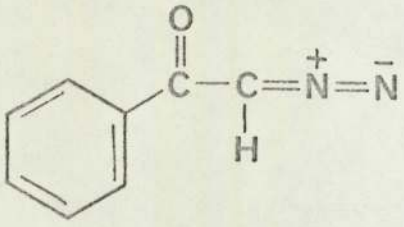


3.1

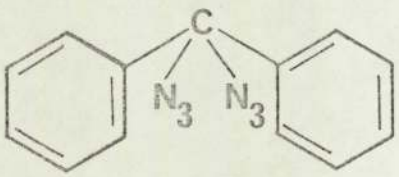
R a : H

b : Br

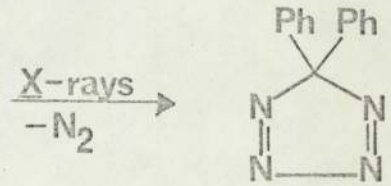
c : Me



3.2



3.3



3.4

compounds. The stability of aromatic compounds does not, however, extend to resistance to attack by free radicals, e.g. those present in water. In recent years a great deal of work has been done on solid phase and solution radiolyses but the isolation and identification of the products has been the main aim; the mechanisms involved are as yet not fully understood.[150-152]

In the radiolysis of aqueous solutions the formation of "relatively" inert species can occur; molecular hydrogen, hydrogen peroxide and hydrogen ions. However, certain highly reactive species can also form, for example, hydrated electrons, hydroxyl radicals and hydrogen atoms. The radio-chemistry of aqueous solutions can be reduced to the chemistry of these highly reactive intermediates.[150-151].

Very little work has been done on the radiolysis of carbene and nitrene precursors. Yukawa and Ibata[153] examined the effect of γ-rays on α-diazo-acetophenone (3.2) but failed to demonstrate the generation of a carbene intermediate. Cronheim[154] and his co-workers examined the effect of X-rays on benzophenonediazide (3.3) and isolated 5,5-diphenyltetrazole (3.4) as the major product. These workers did not appreciate that a nitrene intermediate might be involved.

Photochemical and Thermal Stability of 2,4-Diamino-6-(2-azidoaryl)-s-triazines and their Salts.

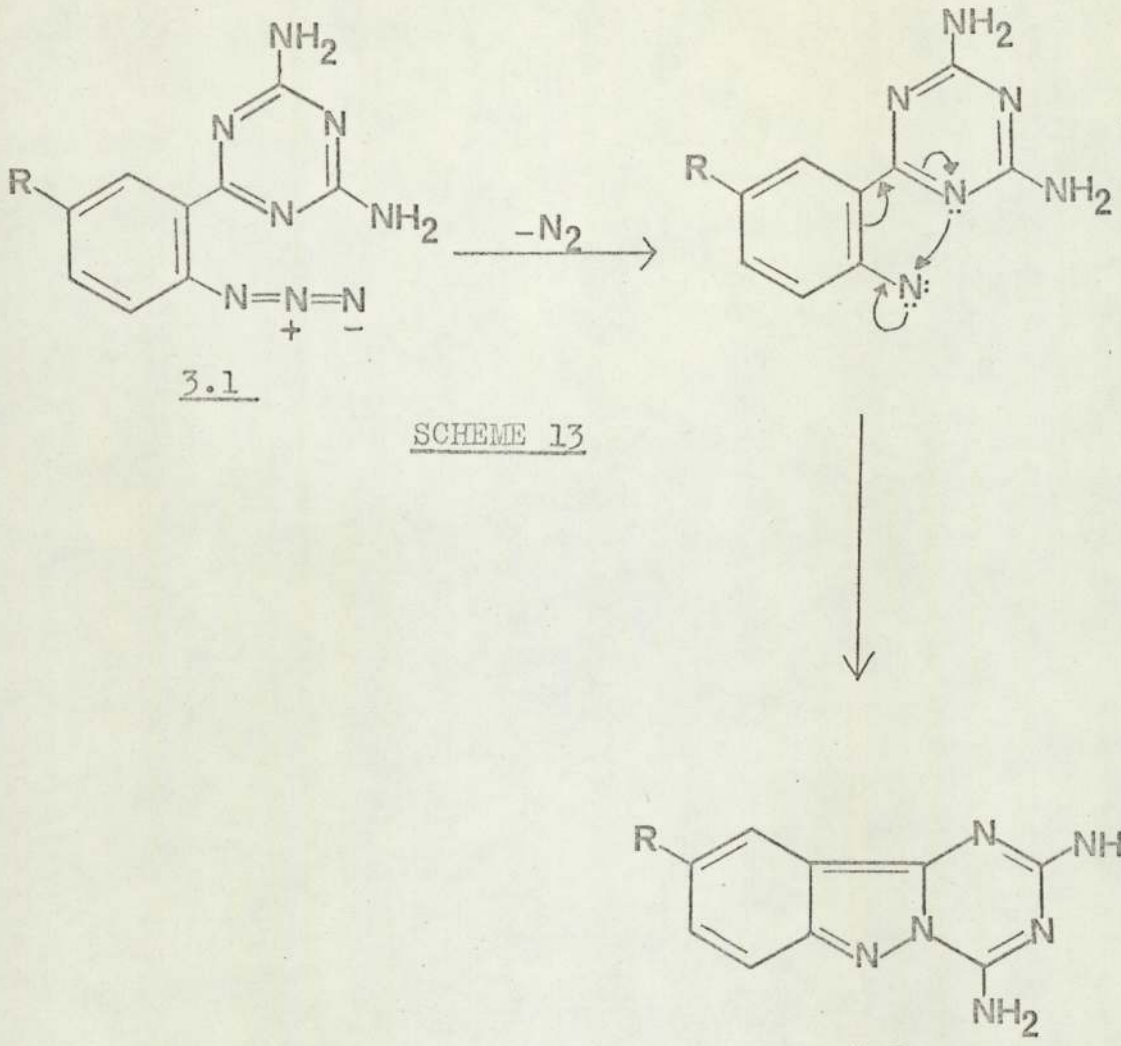
2,4 Diamino-6-(2-azidophenyl)-s-triazine (3.1a) decomposed with evolution of nitrogen within a temperature range between 160° - 170°C to a yellow solid which

finally melted above 300°C. The i.r. spectrum of the product (purified by vacuum sublimation) showed no azide absorption in the region 2200-2000cm^{-1} and was identified as 2,4-diamino-s-triazino [1,2-b] indazole (3.5a). Similar treatment with the bromo derivative yielded the analogous bromo-indazole (3.5b).[41]

Photolysis of (3.1a)(Quartz Filter) in acetone (with a 100 watt medium pressure arc having its output above 313 nm) similarly afforded the triazino-indazole (3.5a) in excellent yield.[155] Photolytic decomposition was more conveniently studied in 95% ethanol by following changes in the electronic absorption spectrum (Figure 3). Initially the spectrum exhibited a peak at 260 nm and was featureless at longer wavelengths. As photolysis proceeded peaks developed at 270, 297, 367, 380 and 400 nm and eventually the spectrum was identical to that of the pure triazinoindazole prepared thermally. No other products were detected in these reactions (t.l.c)

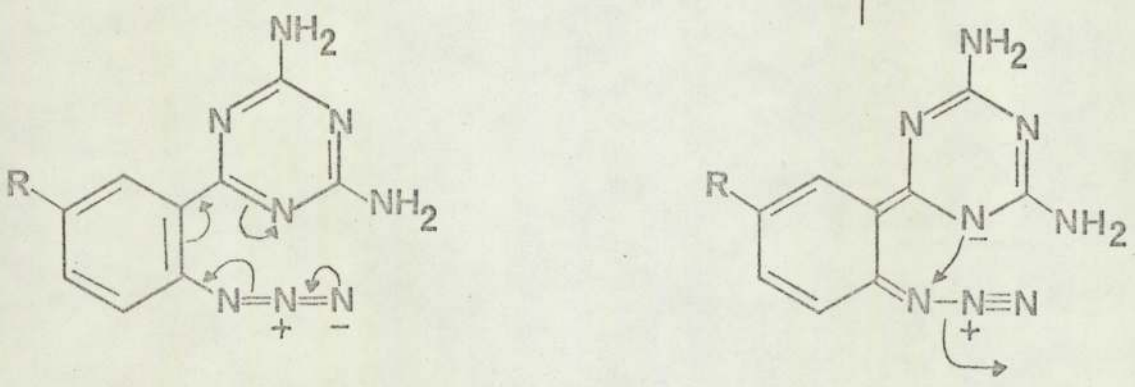
Photolysis of the bromo analogue (3.1b) followed a similar pattern. The reaction was followed in absolute ethanol by observing changes in the electronic absorption spectrum (Figure 4.) As photolysis proceeded peaks developed at 314, 378, 395 and 415 nm and eventually the spectrum was identical to that of a sample of pure bromoindazole.[156]

The mechanism for the decompositions of these azides could involve a nitrene intermediate (Scheme 13) or the product could form by a concerted loss of



3.1

SCHEME 13



SCHEME 14.

R = a : H

b : Br

c : Me

Photolysis of 2,4-Diamino-6-(2-azidophenyl)-s-triazine in ethanol

FIGURE 3.

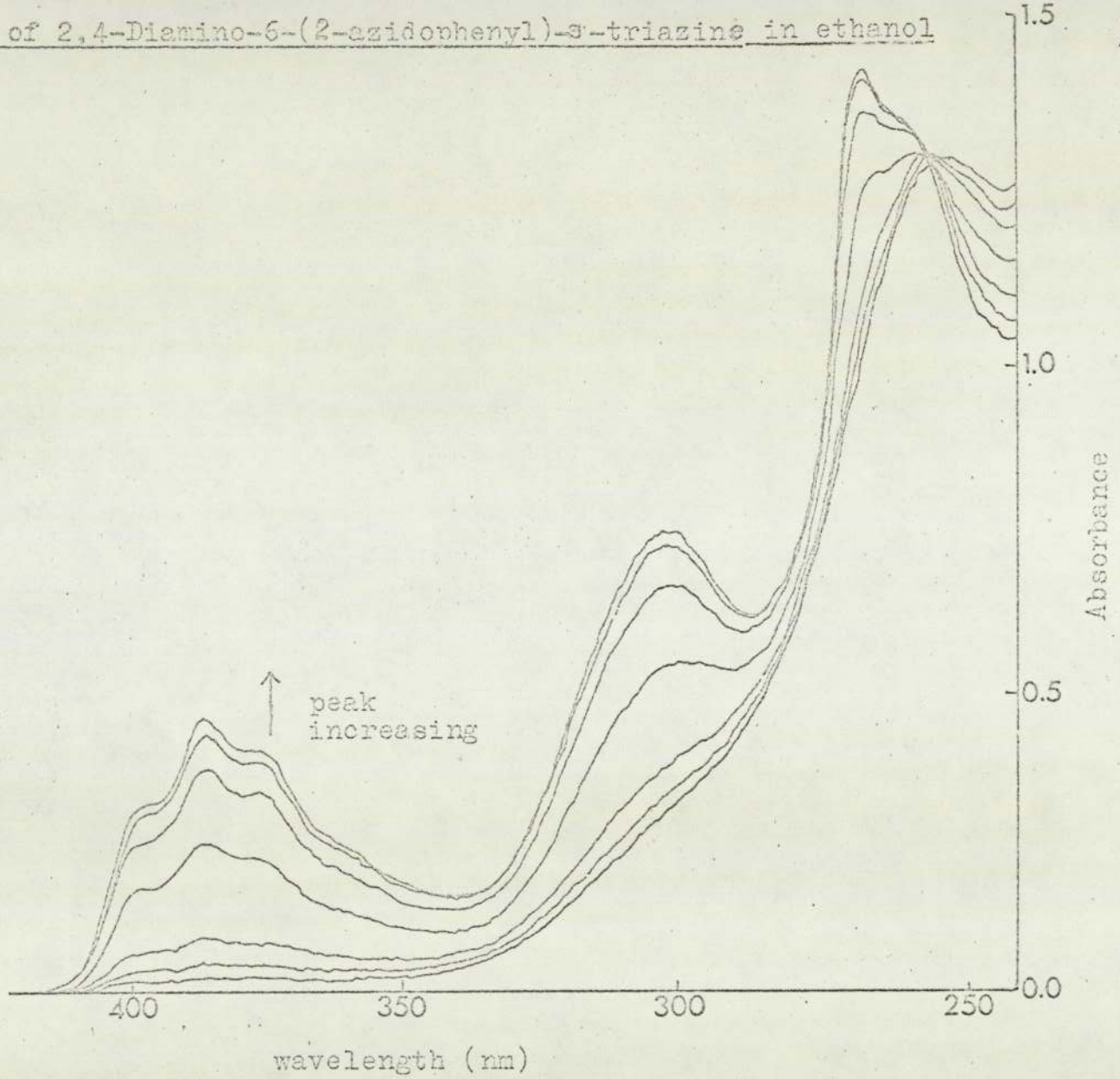
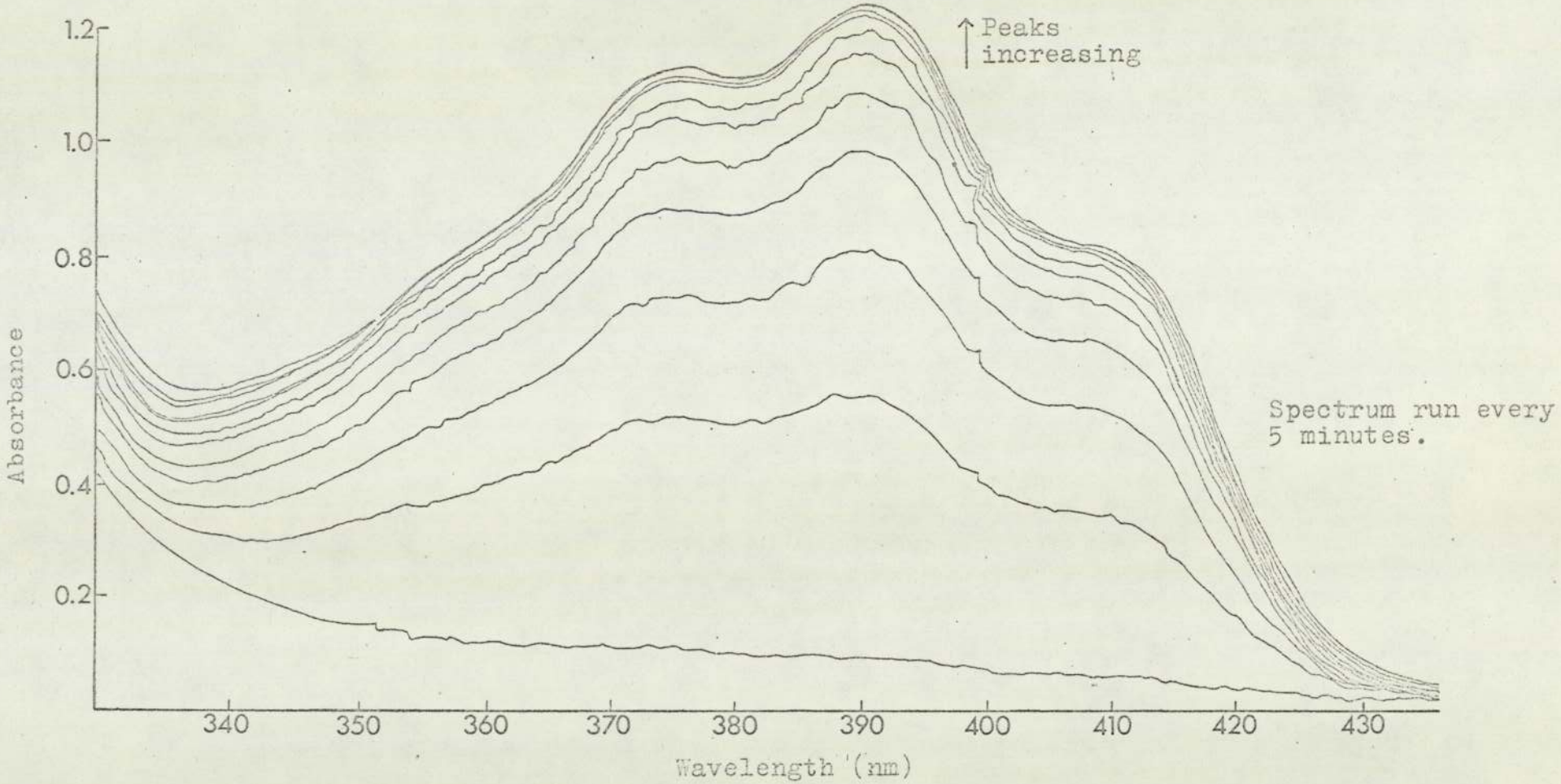


FIGURE 4.

Photolysis of 2,4 diamino-6-(2-azido-5-bromophenyl)-s-triazine in absolute ethanol.

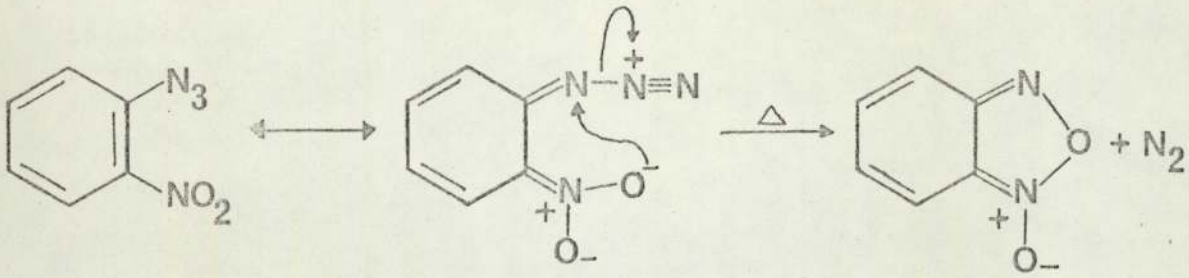


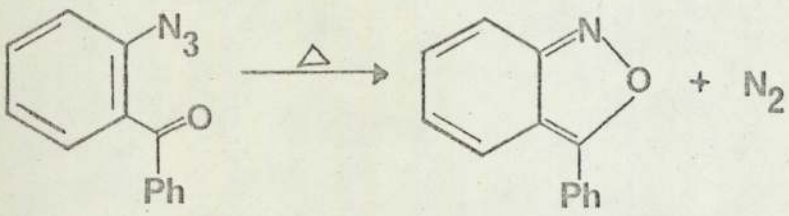
nitrogen (Scheme 14) not involving a nitrene. Examples of such a cyclisation are shown in Scheme 15.[157] Azides with a nucleophilic centre suitably placed within the molecule, i.e. ortho to the azide, readily lose nitrogen due to the low-activation energy required.[121] It is difficult to establish which mechanism is correct in this case, especially if a singlet nitrene were involved. If a triplet (i.e. diradical) species were involved this might be clarified by e.s.r. spectroscopy.

The synthesis of the monohydrochloride salts of these compounds was undertaken with a view to examine their photostability and ultimately their radiostability. The salt of the bromo compound (3.1b) could not be isolated as on recrystallisation it dissociated to the free base; this is probably due to the base-weakening effect of the bromo group. The hydrochloride salt from (3.1a) was, however, stable and could be recrystallised from water.

Photolysis of this salt was carried out in water in a 1 cm quartz cuvette. The course of the reaction was monitored by following changes in the electronic absorption spectrum (Figure 5.) It was found (t.l.c) that the initial reaction is dissociation of the salt to the free base which is then photolysed in a similar manner to the photolysis in alcohol. An isosbestic point was formed in the photolysis in water, but when over 65% of the azide had decomposed, the spectrum began to degrade and finally bore no resemblance to the triazinoindazole. In fact, the peaks between 410 and

SCHEME 15





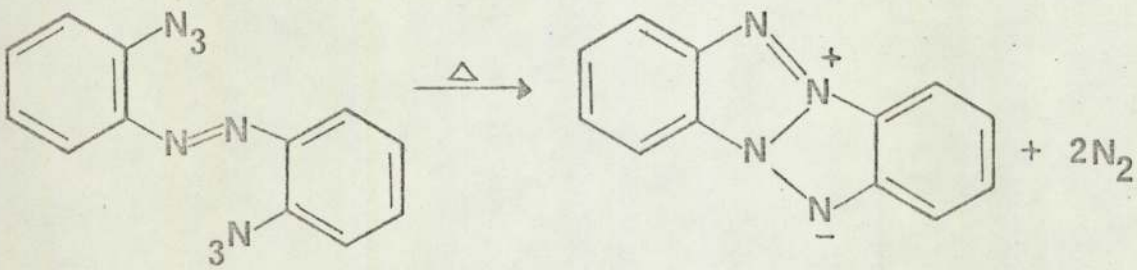
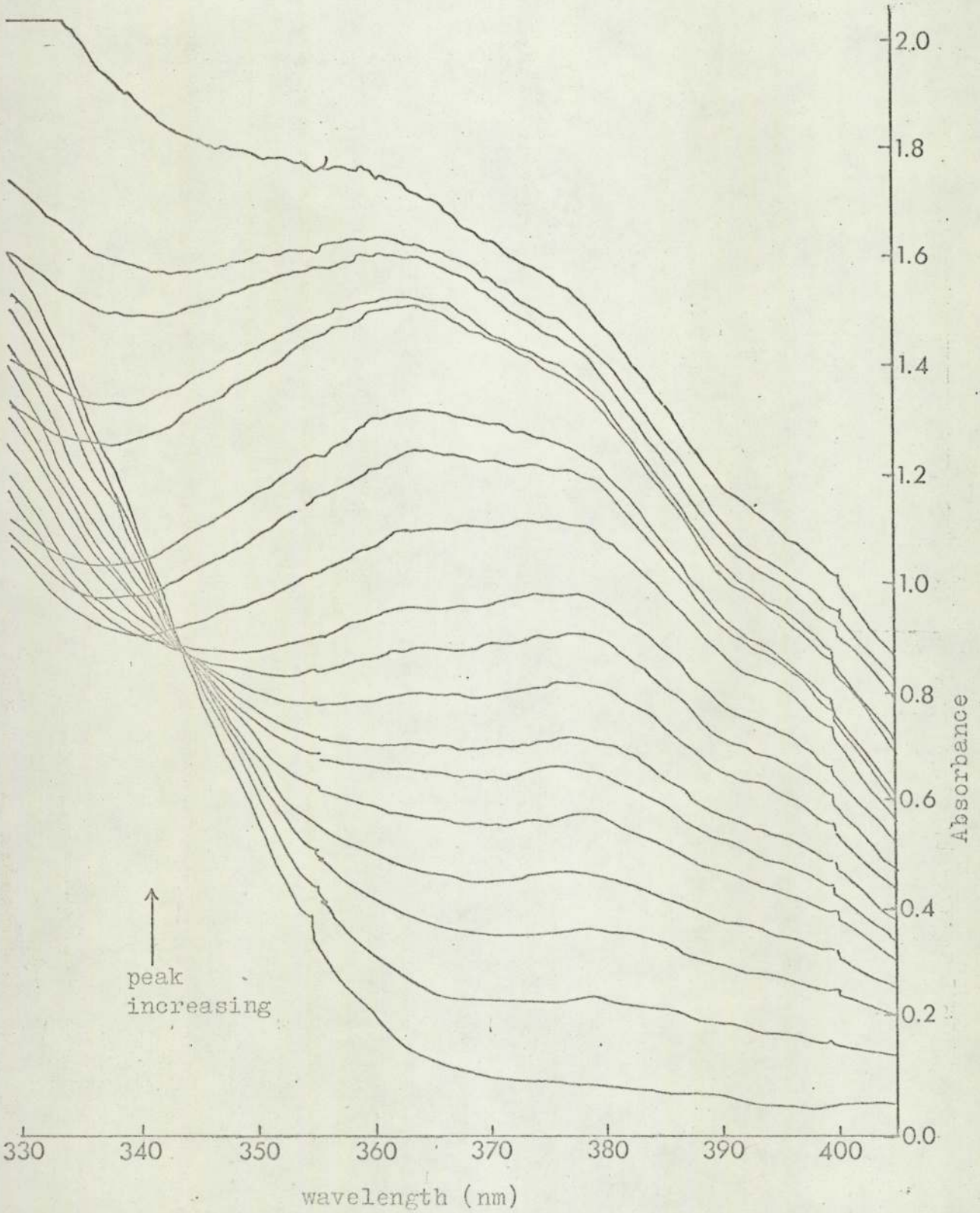
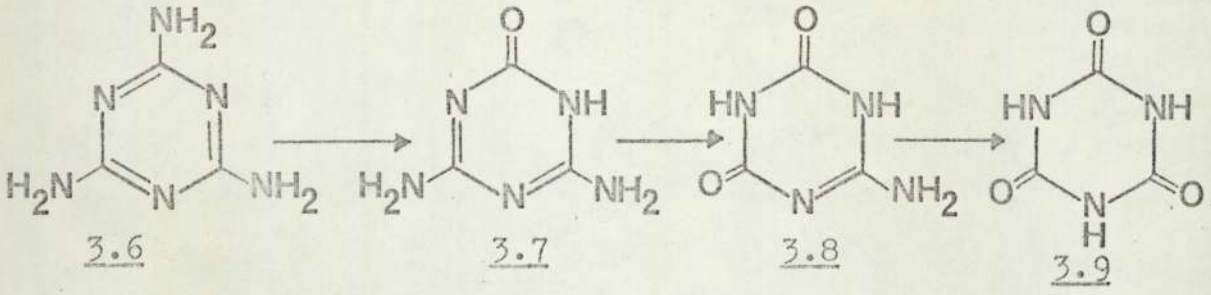


FIGURE 5.

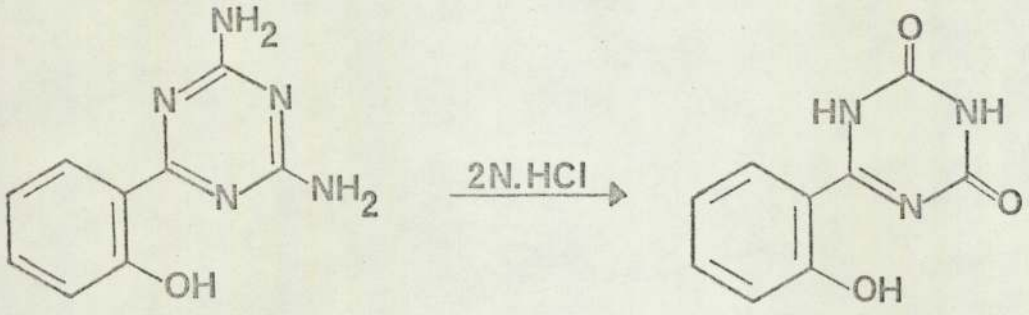
Photolysis of 2,4-Diamino-6-(2-azidophenyl)-s-triazine-hydrochloride in water.





3.6 3.7 3.8 3.9

SCHEME 16



3.10 3.11

SCHEME 17

350 nm. completely collapsed to form a broad peak at 360 nm. which then finally degraded completely. As this occurred t.l.c. revealed that at least three new compounds were formed other than the indazole.

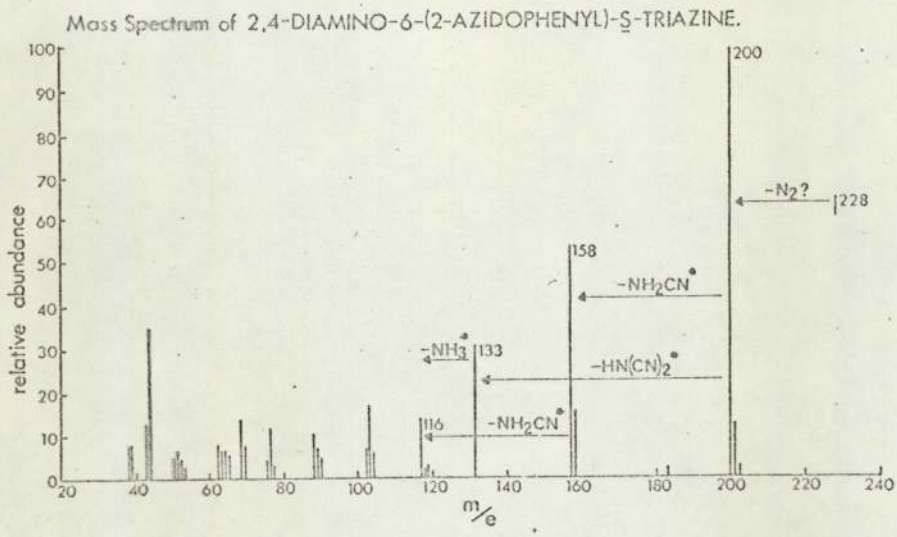
One possible explanation for this change involves hydrolytic replacement of the amino groups in the s-triazine ring. It is known that in aqueous alkaline or acidic solutions, the three s-triazine bases, melamine (3.6), ammeline (3.7) and ammelide (3.8) are hydrolysed to cyanuric acid (3.9) as shown in Scheme 16.[158,159] Also 2,4-diamino-6-(2-hydroxyphenyl)-s-triazine (3.10) is known to form the corresponding triazindione (3.11) in dilute hydrochloric acid (Scheme 17).[156] Conditions of the photolysis are favourable for this type of amine replacement.

Mass Spectra of 2,4 Diamino-6-(2-azidoaryl)-s-triazines.

The mass spectra of the s-triazines (3.1) are of some interest as very little has been reported on the mass spectra of azides.[160] The spectra were run on a A.E.I.M.S.9 instrument using a heated probe, at 70eV with a source temperature of 250°C. The base peak (m/e200) in the spectrum (Figure. 6) of triazine (3.1a) represents loss of nitrogen but it is debatable whether this is a nitrene fragment. No molecular ion at m/e228 was ever detected even though the spectra were run at reduced ionisation voltage (e.g. 15eV) and at the lowest possible source temperature.[161] The probe was not heated as this compound had a sufficiently high vapour pressure.

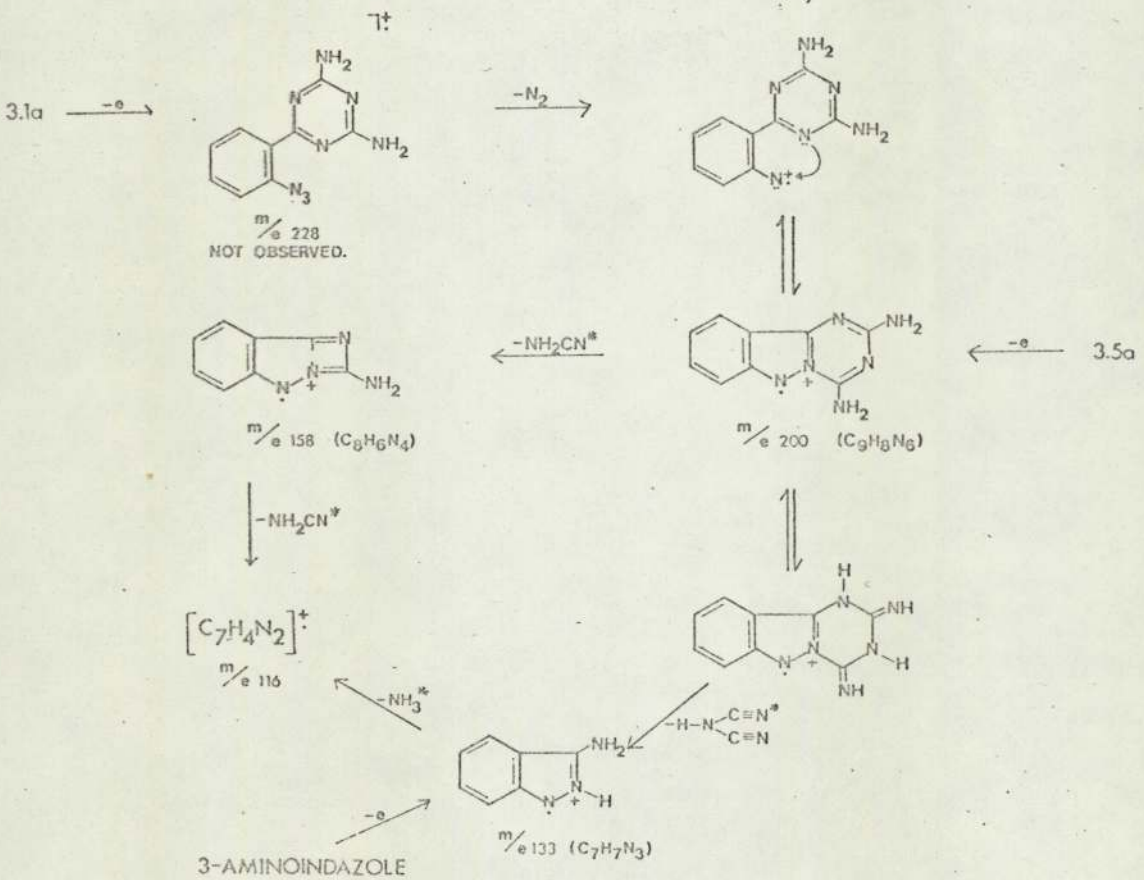
As the mass spectrum was in all respects identical

FIGURE 6.



SCHEME 18.

* Denotes transition supported by metastable ions.



to that of the corresponding triazinoindazole (3.5a) it is likely that the initial loss of nitrogen is exclusively a thermal process.[162] The breakdown pattern of this azide (3.1) is shown in Scheme 18, fragmentations marked with an asterisk are supported by metastable ion peaks. Other s-triazines behave similarly on electron impact.[163] The corresponding bromo-compound (3.1b) has comparable ions to those shown in Scheme 18.

The proposed structure of the ion at m/e133 was corroborated in that 3-aminoindazole also has an initial loss of ammonia. This loss is typical for compounds of this type.[164]

Radiolysis of 2,4-Diamino-6-(2-azidophenyl)-s-triazine and its Hydrochloride.

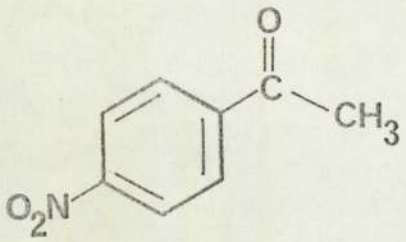
a. Radiation sensitizers - their role in radiation therapy.

It has been suggested that the hypoxic centres within a tumour mass are likely to be relatively resistant to sterilisation by ionising radiation.[165] Compounds which could be introduced into such hypoxic centres and which can mimic the radiation sensitizing effect of oxygen may therefore be of great value in the radiation therapy of cancer. Oxygen has been shown to radiosensitize mammalian cells by a close modifying mechanism, enhancing radiation-induced damage in the DNA of cells and is bound by radiation to biological molecules, e.g. enzymes.[166] A number of chemicals are known to sensitize mammalian cells to the effects of

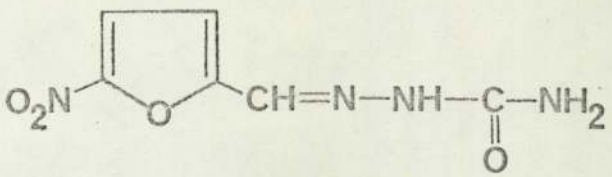
ionising radiation.[167]

One such compound is p-nitroacetophenone (3.12) which was selected as a potential radiosensitizer because of its strong electron affinity.[168] This compound is a good selective hypoxic radiosensitizer in mammalian cells. Adams[168] has postulated a mechanism of action for electron-affinic radio-sensitizers in which irreversible electron transfer from a target molecule to the sensitizer constitutes fixation of damage in the target and a potentially lethal event. Such a model fails to account for the observed binding of nitrofurazone (5-nitro-2-furaldehyde semicarbazone) (3.13)[166] to various macromolecules. A variation of this mechanism involves the sensitizer reacting with target radicals (generated by direct or indirect action of radiation) in such a way that irreversible binding of the sensitizer to the target results and constitutes a potentially lethal event. The target in the case of nitrofurazone and other nitrofurans is DNA.[166,169] Bridges[167] has reviewed the mechanisms whereby p-nitroacetophenone, nitrofurans and other derivatives exert their sensitizing effects.

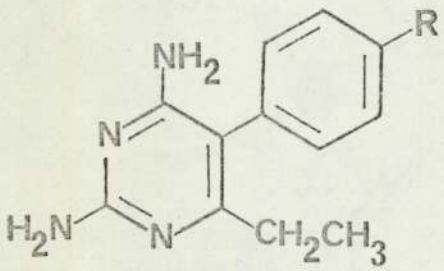
2,4-Diamino-s-triazines are reversible inhibitors of dihydrofolate reductase. The involvement of this enzyme with leukaemic cells is well known (Chapter 1). Originally the azide derivatives of the 2,4-diamino--s-triazines were synthesised to label the active-site of the enzyme (cf. Chapter 2) by photolysis of the enzyme-inhibitor complex. However, u.v. light does not



3.12



3.13



3.14

a : R = N_3

b : R = Cl (Pyrimethamine)

penetrate biological tissue and is therefore not of any value in generating a nitrene *in vivo*. If 2,4--diamino-6-(2-azidophenyl)-*s*-triazine has an affinity for dihydrofolate reductase and on radiolysis evolved a nitrene, this could act as a radiosensitizer: the only difference to the previously mentioned compounds would be that the target site would be an enzyme and not DNA.

b. *Solid phase Radiolysis of 2,4-Diamino-6-(2-azidophenyl)-s-triazine.*

2,4-Diamino-6-(2-azidophenyl)-*s*-triazine (3.1a) was repeatedly recrystallised to constant melting point (170^{o}). A measured amount of the free base was placed in pyrex glass evacuated ampoules (10cms long, external diameter 2 cms, internal diameter 1.5 cms.) which were then irradiated. It is essential to remove oxygen as this may interfere with any potential radiochemical reaction. The two sources used were an *X*-ray generator (Pantak L.C. 300) and a 5000ci ^{60}Co *γ*-source. When irradiating the sample it is important to place the centre of the sample on the central axis of the source. Having decided on this position, the apparatus must be designed such that the ampoule can be placed reproducibly in the same place for every experiment. This is vital because of the large changes in the dose rates with small changes in position. For example, the *γ*-source has a dose rate of 3.5 Mrads/hour (3.5×10^{6} rads) at the central axis whereas at a distance of 2.54 cms. the dose rate drops to 1.5 Mrads/hour (1.5×10^{6} rads).

Since the sample does not absorb all the emitted radiation, it was necessary to perform a dosimetric experiment. In this case the Fricke[150-152] dosimetry was done under identical conditions to the radiolysis. The Fricke dosimeter is based on the production of Fe^{3+} ion in aqueous ferrous sulphate solution on exposure to ionising radiation. A solution of ferrous sulphate (10^{-3}M), sodium chloride (10^{-3}M) in 0.8N-sulphuric acid was used.

Solutions were made up with water which had been distilled over potassium permanganate and then triple distilled. Permanganate is used to remove organic impurities which have a detrimental effect on the performance of the dosimeter. Solutions were given doses between 10,000 and 40,000 rads. The usual method of analysis is a spectrophotometric determination of the Fe^{3+} ion at 304 nm. using unirradiated solution as the blank. The absorbed dose received by the Fricke dosimeter in 0.8N-sulphuric acid can be calculated from the formula:-

$$Dm \text{ (in rads)} = 4.42 \times 10^5 \left[1 - 0.007\ (t-20)\right] A/G$$

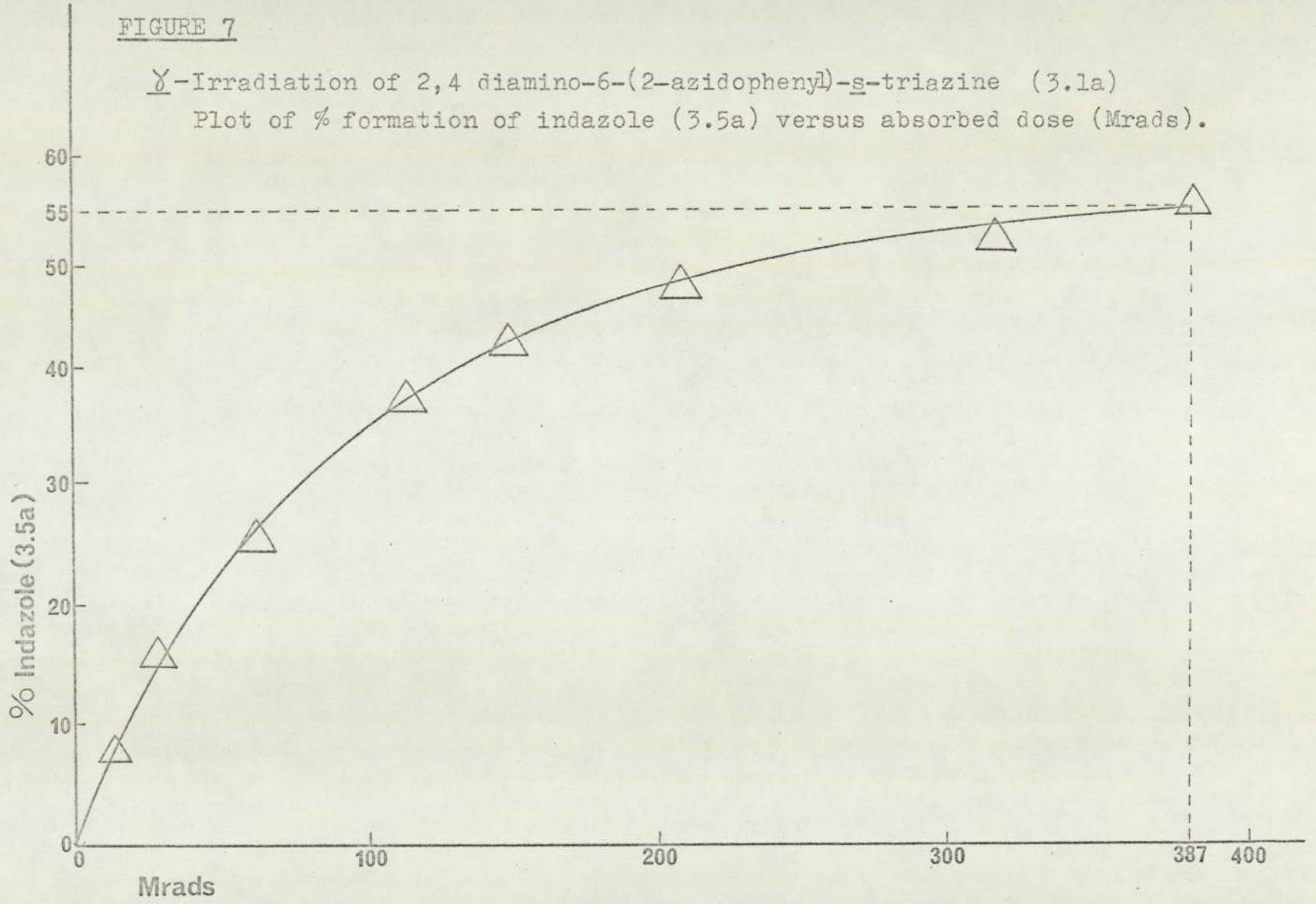
where A is the absorbance of the irradiated solutions at 304 nm, measured in 1 cm cells, G is the yield of the reaction for the radiation in use (known standards)[150-152] and t is the temperature in ^{o}C at which the absorbance is measured. The temperature term can be reduced to unity without any appreciable error resulting, assuming room temperature to be $20^{o} \pm 2^{o}C$. From the figures obtained from this equation the absorbed dose of the sample was calculated.[150-152]

The value of these doses suffer from three errors. Firstly, the direct application of the Fricke dosimeter depends on the assumption that the density of the solid and the ampoule is unity. It is possible to correct for these discrepancies, but since the error is small and the calculation cumbersome, this is not normally performed in preliminary experiments. Secondly, in calculating the dose rates using the γ-source an error results in the length of time the Fricke solution is exposed to the source. At the start of the radiolysis the source takes about seven seconds to rise, and at the end takes three seconds to full; i.e. there is a "dead time" of approximately 3 - 4 seconds. It is not possible to calculate the exact dead time due to the rise and fall times varying. Thirdly, over the period the experiments were performed the source itself was decaying. The half-life of the ^{60}Co source is 5.3 years; hence over the period this work was done there was about a 4% decay in output.[170]

The analysis of the radiated samples was carried out by extracting the sample into absolute ethanol. These extracts were then monitored by electronic absorption spectroscopy and t.l.c. From this it was observed that only one product had formed; this product was identified as the triazinoindazole (3.5a). A graph of the absorbed dose against percentage formation of triazinoindazole was then drawn (Figure 7.) The maximum conversion to the triazinoindazole was 55% (considerably lower than the photolytic process).

FIGURE 7

γ-Irradiation of 2,4 diamino-6-(2-azidophenyl)-s-triazine (3.1a)
Plot of % formation of indazole (3.5a) versus absorbed dose (Mrads).



The mechanism for the radiolytic process may well be similar to the photolytic and thermal process (see Schemes 13 and 14). In radiation chemistry the first step in any reaction is formation of a positive ion or an excited radical formed from the parent compound, (cf. mass spectra). In this case the azide required 1 Mrad (10^6 rads) for the formation of this primary species. X-rays had no effect on the azide at all (maximum practical dose 500 krads) (500 x 10^3 rads). Once this primary species forms the reaction then proceeds in a typical manner for most organic compounds on radiolysis.[150-152]

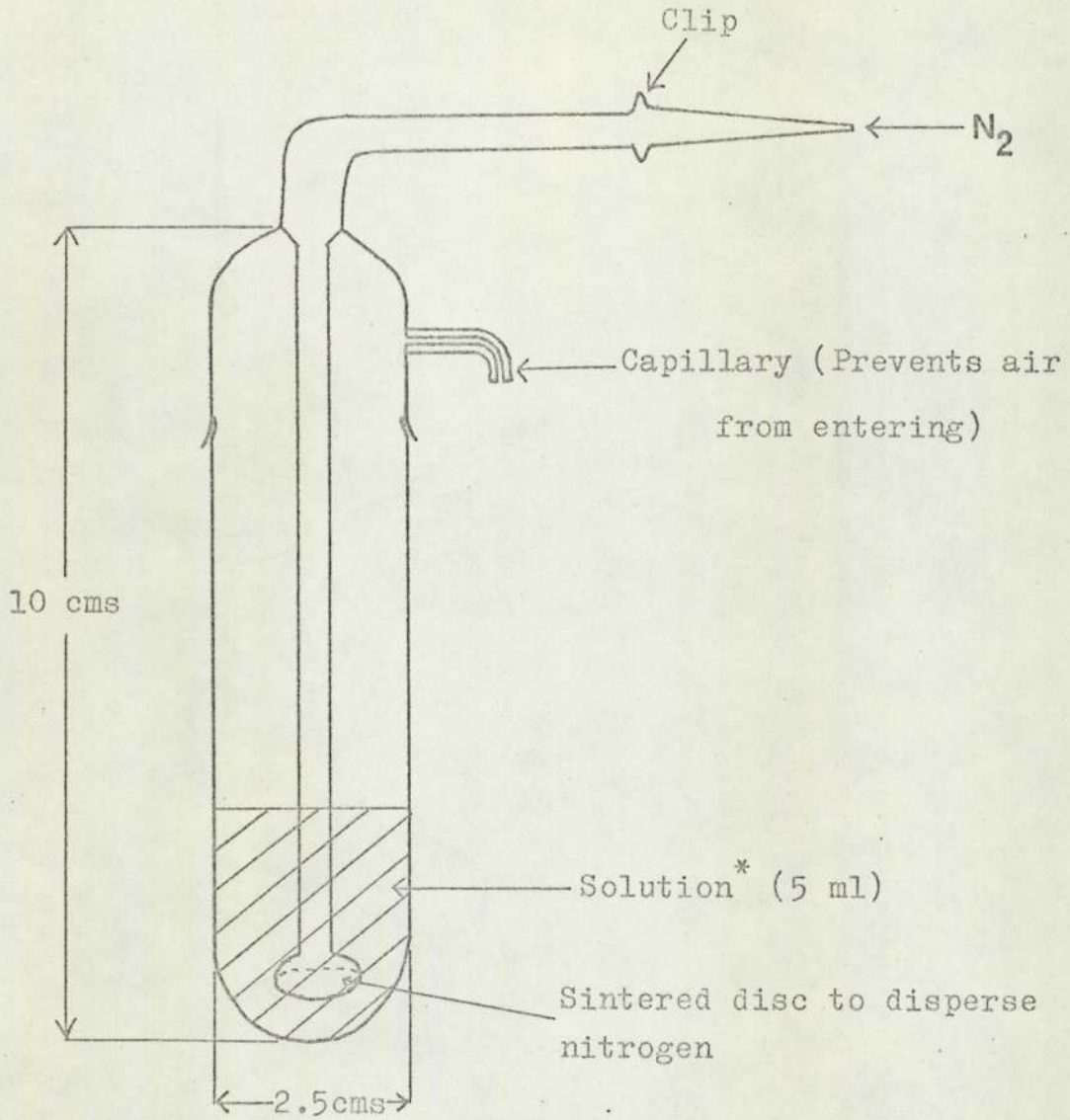
c. Radiolysis in the aqueous phase.

A 7.5 x 10^{-5}M solution of 2,4-diamino-6-(2-azidophenyl)-s-triazine hydrochloride in water was prepared. The water used was distilled from permanganate and then triple distilled. This strength of solution was used in order that the triazinoindazole would be produced in a concentration suitable for spectroscopic analysis without further dilution. Accordingly the progress of the radiolysis could be easily followed.

The special apparatus (Figure 8) had to fit the source compartment (25.4 cms^3) and be placed reproducibly for every experiment. Nitrogen was passed through the solution for thirty minutes before radiolysis and continued during the experiment to remove oxygen. Again a dosimetric experiment had to be done to calculate the absorbed dose; the Fricke dosimeter was used. In this work only γ-radiolysis was performed.

The parameters involved in the solution work are the

RADIATION CELL



* Solution is 2,4-diamino-6-(2-azidophenyl)-s-triazine monohydrochloride in water.

FIGURE 8

same as mentioned previously for the solid phase work.

Results from the radiolysis of the salt in water proved disappointing. The u.v. examination of the radiolysed solution showed that no triazinoindazole had formed. At least six products were detected (t.l.c.). It would seem that the water is forming the normal radiolytic reactive species in dilute solutions, i.e. hydrated electrons. These species could react with the s-triazine ring resulting in hydroxyl replacement of the amino groups or hydroxylation of the aryl group. The electronic absorption spectrum of the aqueous solutions was similar to the spectrum of the photolytic solution (Figure 5) once the isosbestic point was passed. From these results it is apparent that no nitrene intermediate was formed in the radiolysis of this azide in solution.

d. Conclusions

The solid phase radiolysis was carried out in order to discover if the reaction followed a similar course to the photolysis. The dose required to mimic the photolytic process was extremely high (200×10^6 rads). The nitrofuran radiosensitizers react at a dose level of the order of 50 krads (50×10^3 rads).

Thiols have the ability to protect biological macromolecules from radiation.[150] Oxygen on the other hand has a synergistic effect on radiation sensitivity.[166,169] If the tumour under interest could be saturated with oxygen and the healthy cells protected by thiols then compounds which normally decompose at high dose levels might well be useful clinically. However, even

allowing for this 2,4-diamino-6-(2-azidophenyl)-*s*-triazine (3.1a) under study here is not sufficiently susceptible to be of any potential value in radiation chemotherapy. It was hoped that had this azide decomposed to a nitrene *in situ* at potentially clinical useful doses then covalent attachment to dihydrofolate reductase could occur on irradiation. If the mechanism of the radiolytic decomposition involved a concerted loss of nitrogen (Scheme 14), then intra-molecular covalent attachment to this enzyme could occur if the azide was constrained within the active-site.

No conclusions can be drawn from the preliminary radiolyses of the hydrochloride salt of (3.1a) in the aqueous phase.

Developments of this work should be done on azides which cannot cyclize by *ortho*-assistance. For example the azide (3.14a) is an analogue of Pyrimethamine (3.14b) which is a reversible inhibitor of dihydrofolate reductase. The synthesis, and thermolytic, photolytic and radiolytic stability of (3.14a) would be an interesting problem to examine.

CHAPTER 4.

CHAPTER 4

Interaction of Acridines with Nucleic Acids.

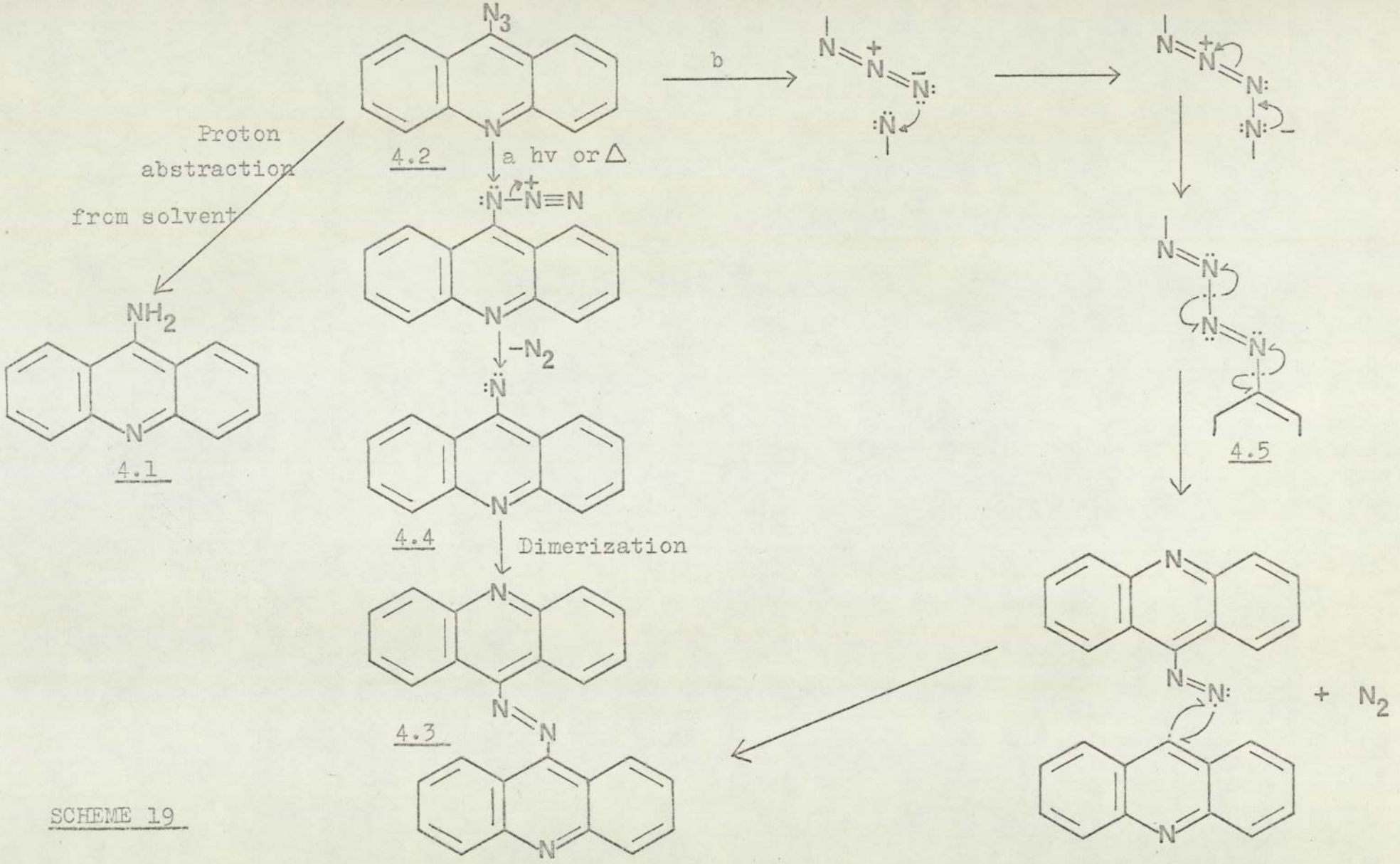
The aminoacridines have been known to exhibit antibacterial properties for a long time. Perhaps the most intensive investigation ever made of the relationship between the structure of a small molecule and its biological activity has been the study which was conducted by Albert[1,171] and his colleagues. These investigations have established that only those acridines which are substantially ionised at physiological pH (i.e.with pKa>7.8) are active as antibacterial agents. Structural features in the molecule which decrease the basicity or distort the planar character of the acridine nucleus have a dyschemotherapeutic effect. The antibacterial and mutagenic properties of aminoacridines and related compounds are thought to be a consequence of their ability to "intercalate" DNA, although the exact geometry of this association remains a subject of controversy.[172-179]

In view of this interaction with nucleic acids it was thought likely that some of the aminoacridines might be carcinogenic. All attempts to induce tumours with aminoacridines, so far have failed. However, Mellanby[180] has shown that Acriflavine prevented growth of transplantable tumours; and it is now known that Acriflavine does have anti-tumour activity.[171]

Photolysis, Thermolysis and Radiolysis of 9-Azidoacridine.

9-Aminoacridine (4.1) is amongst the most active of the antibacterial acridines[1,171] and the azido analogue (4.2) was synthesised in order to compare its biological properties.[181] The photolysis and thermolysis of this azide has been studied.[181] It was hoped that if 9-azidoacridine (4.2) complexed with nucleic acids, on subsequent activation a reactive intermediate could disorganise the macromolecule.

On photolysis in methanol or benzene the major product was 9,9'-azodiacridine (4.3). This product is also formed on thermolysis in o-dichlorobenzene or nitrobenzene or on pyrolysis at 150°C. Alternative mechanisms for azoacridine formation are possible. Smith[182] argued that reaction of an arylnitrene with an undecomposed molecule of azide is a statistically more probable process than direct dimerisation. The possible mechanisms for this reaction are outlined in Scheme 19 a and b. The simplest path to the azo compound would be cleavage of the azide to a singlet nitrene (4.4) followed by dimerisation (Scheme 19a). However, Scheme 19b is more likely. In this mechanism a tetrazadiene (4.5) is produced, which could form the azo compound by direct loss of nitrogen via a radical mechanism. Tetrazadienes are known to decompose thermally or photochemically by this homolytic process.[183] Thermolysis of 9-azidoacridine (4.2) in dekalin afforded 9-aminoacridine (4.1) probably through a stepwise radical abstraction of hydrogen from the


Proton
abstraction
from solvent
4.1
4.2
a hν or Δ
b
4.4
Dimerization
4.3
4.5
+ N2

SCHEME 19

solvent by a triplet nitrene. The abstraction of hydrogen from the solvent is a common side reaction in the decomposition of aryl azides.[182]

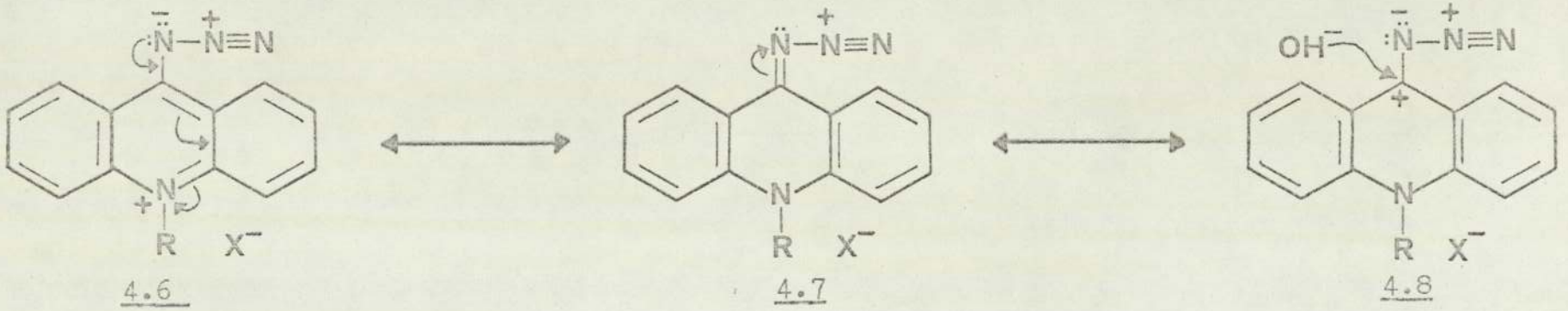
Solid phase radiolysis of 9-azidoacridine using a ^{60}Co γ-source also yielded the azo compound. The experiments were carried out in the same manner as those described in Chapter 3. Analysis of the radiolysed solid was carried out by extracting the solid into ethanol. A small amount of a purple solid did not dissolve (insufficient for spectroscopic study). Examination of the ethanol extract (t.l.c) showed that only two compounds were present, even at a dose of 200 Mrads(200×10^6 rads). These were unreacted 9-azidoacridine and the azo compound. Analysis of the yields of the azo compound was difficult due to the insolubility of the azo compound in most solvents. However, the percentage unchanged starting material was calculated and shown to be $\simeq$ 90%. A calibration graph of concentration of 9-azidoacridine and absorbance was drawn . Thus the use of 9-azidoacridine as a radiosensitizer is precluded due to the high dose required to generate a reactive intermediate.

The mass spectrum of 9-azidoacridine showed a molecular ion of low intensity, the only peaks of any significance occurred at m/e 192 (M-28) and 194 (M-26), the former corresponding to loss of nitrogen to give the nitrene. Peaks corresponding to M-26 have been previously observed in the mass spectra of azidopyrimidines,[184] and have been shown to arise

by thermal fragmentation of the azides to give nitrenes which by H abstraction give the amines in an overall process $(M-N_2) + 2H$. The spectra of this azide at low ionisation voltage (15eV) showed these two ions still present, suggesting that the loss of nitrogen is not a thermal process. Most other azides behave similarly losing N_2 from the molecular ion.[162] This is not thought to be due to thermal decomposition in the mass spectrometer source.[162]

Reactivity of 9-Azidoacridine and its Salts with Nucleophiles.

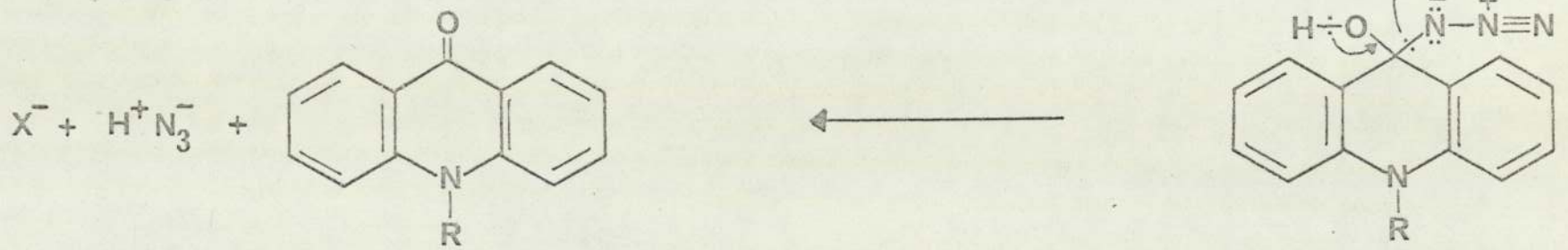
The radiolysis of the azide free base proved disappointing. Therefore the hydrochloride salt (4.6a) and the quaternary salt (4.6b) were synthesised.[181] The characteristic property of these salts is their susceptibility to nucleophilic attack *e.g.* hydrolysis to acridones (4.9 a & b). This may be explained in terms of the electron-donating character of the azido group and the electron withdrawing characteristics of the electropositive nitrogen group. Electron transfer from the azido group to the electropositive nitrogen can be represented 4.6 ↔ 4.7 ↔ 4.8 (Scheme 20). Mesomer (4.8) would be particularly susceptible to nucleophilic attack. Compound (4.6b) would be even more likely to undergo substitution than (4.6a) due to the more powerful electron-withdrawing effect of $\overset{+}{N}-CH_3$. This is indeed the case as can be seen from the relative half-lives of these salts in water



4.6 4.7 4.8

a: R=H, X= Cl

b: R=CH_3, X= CH_3SO_4



4.9

SCHEME 20

at 37°C (Table 6).

TABLE 6.

TABLE OF HALF LIVES OF THE ACRIDINE SALTS ON HYDROLYSIS IN WATER AT 37°C	
Compound	Half-life (hours)
9-Azidoacridine hydrochloride	10
9-azido-10-methyl acridinium methosulphate	8
9-Chloro-10-methyl acridinium methosulphate	1.5

The progress of hydrolysis of 9-azidoacridine hydrochloride in water at 37° was followed by electronic absorption spectroscopy (Figure 9). The spectra for all three salts (Table 6) are similar.

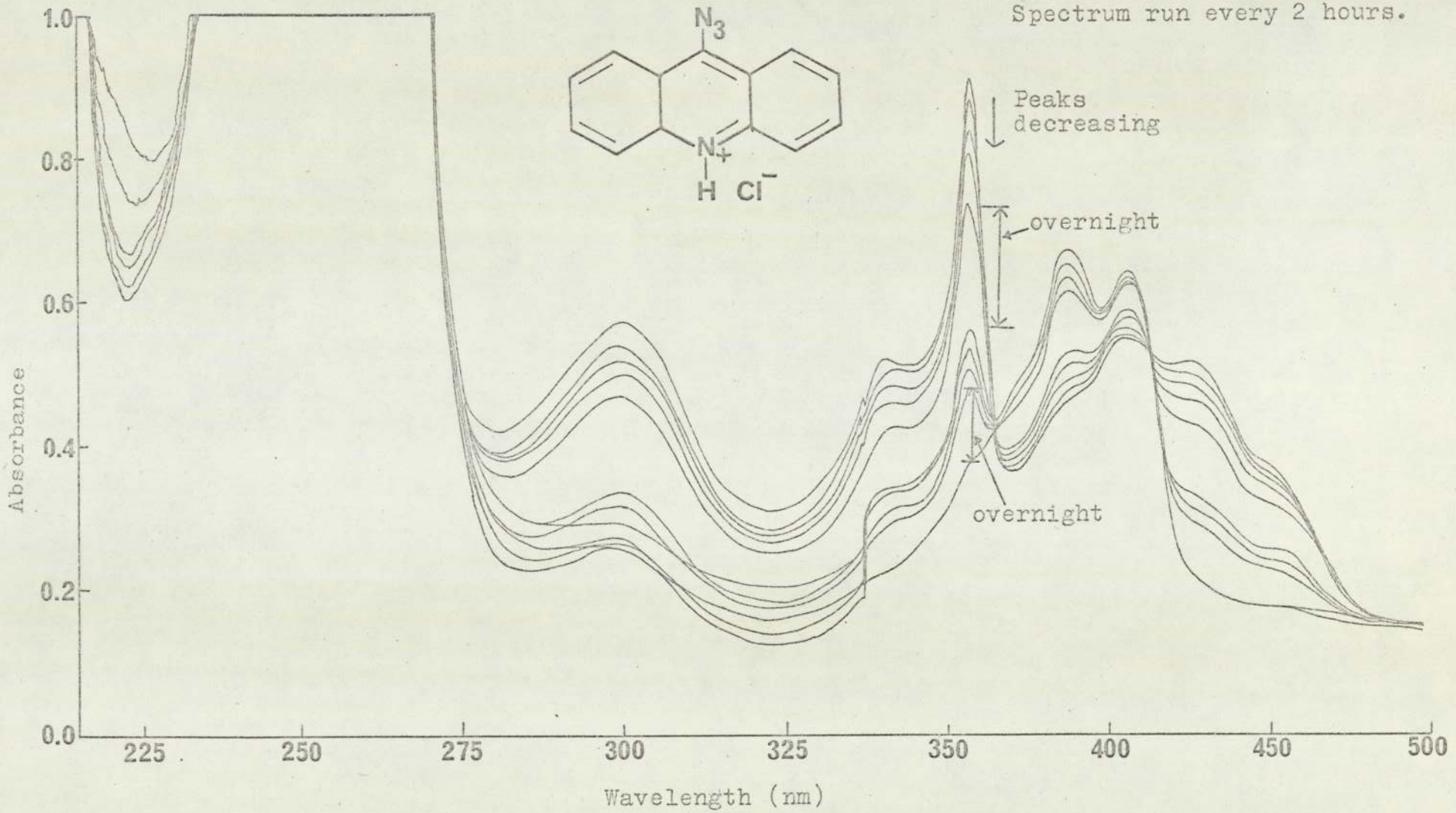
The kinetics of the hydrolysis of the quaternary salts showed that the azide salt follows zero order kinetics (i.e. rate of hydrolysis is independent of initial concentration) whereas the chloro salt is first order (i.e. rate of hydrolysis is dependent on the initial concentration).

Reaction of 9-azidoacridine hydrochlorides and the quaternary salt (4.6b) with amines has been described by Mair and Stevens.[181] In view of the susceptibility of (4.6b) to nucleophilic attack, it is likely that this compound could act as a hetarylating agent; possibly the salt could intercalate DNA and subsequently interact with nucleophilic

Hydrolysis of 9-azidoacridine-10-hydrochloride in water at 37°C.

FIGURE 9.

Spectrum run every 2 hours.



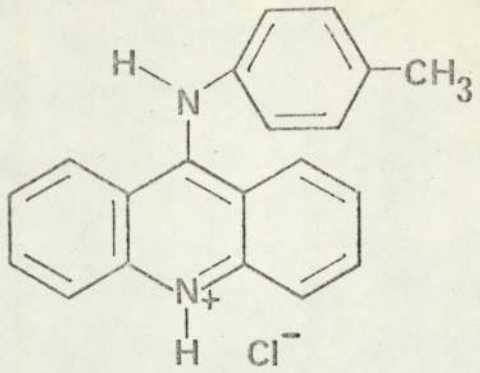
centres within the helix. During this work,[181] one compound, 9-(4-methylanilino) acridine hydrochloride (4.10) was found to have weak but significant tumour-inhibitory properties against lymphoid leukaemia (L-1210) Two recent reports,[185, 186] have confirmed activity in derivatives of 9-anilinoacridine.

It was therefore decided to synthesise more 9-anilinoacridines to examine structure-activity relationships in this series. These compounds may be prepared by condensation of arylamines with either 9-chloroacridine or 9-azidoacridine hydrochloride. As the former compound is more accessible, the chloro-acridine was used in all subsequent preparations.

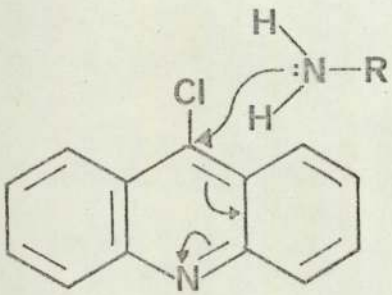
Synthesis of 9-Anilinoacridines.

The hydrochloride salts of 9-anilinoacridine were prepared by boiling 9-chloroacridine (4.11) with the appropriate arylamine in anhydrous methanol for 3 hours. The hydrochloride salts (4.12a-w) crystallised from solution on addition of ether. Yields in most cases were $\geq$ 95%. The free bases of some of the compounds were prepared by basifying the salt with aqueous sodium hydroxide.

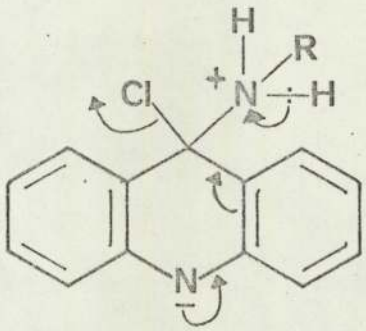
The reaction with 4-aminophenol was controversial. Russian workers suggested that the product formed was 9-(4-aminophenoxy)acridine (4.13). This acridine was prepared by reacting 9-chloroacridine with 4-nitro-phenol and reducing the resultant 9-(4-nitrophenoxy)-acridine.[188] The product differed from that obtained (4.12d) either from 9-chloroacridine and 4-aminophenol,

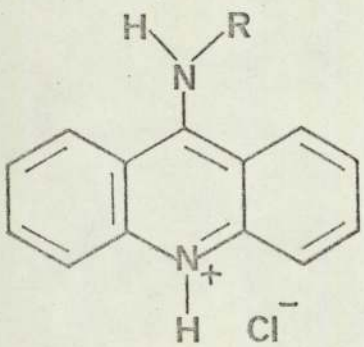


4.10

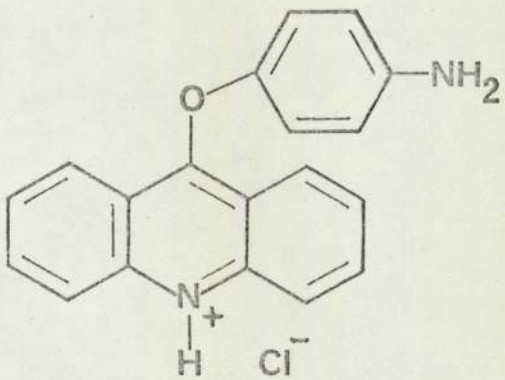


4.11





4.12



4.13

GROUP R.

a: Phenyl

b: 4-Aminophenyl

c: 2-Hydroxyphenyl

d: 4-Hydroxyphenyl

e: 4-Acetoxyphenyl

f: 2-Methoxyphenyl

g: 4-Methoxyphenyl

h: 4-Ethoxyphenyl

*i: 4-n-Propoxyphenyl

*j: 4-n-Butoxyphenyl

*k: 4-n-Pentoxyphenyl

*with Mr. T.C. Lam.

l: 4-Phenoxyphenyl

m: 2-Methylphenyl

n: 3-Methylphenyl

o: 4-Methylphenyl

p: 4-Ethylphenyl

q: 4-n-Butylphenyl

r: 2-Chlorophenyl

s: 4-Chlorophenyl

t: 4-Fluorophenyl

u: 4-Bromophenyl

v: 2-Pyridyl

w: 3-Pyridyl

or by hydrolysis of the acetate (4.12e).

Spectral Properties of 9-anilinoacridine hydrochlorides.

The 9-anilinoacridine hydrochlorides crystallised from ethanol-ether with variable amounts of water of crystallisation. Hydrates and hemi-hydrates were obtained in addition to unsolvated species. It was empirically observed that the i.r. spectra (KBr) of these three types fitted into a general pattern, and it proved possible to distinguish simply between them. For example, all the hydrates had a broad absorption at 3300-3400cm^{-1} (bonded O-H) together with a broad band at 2500-3100 cm^{-1} typical of a protonated amine. The hemi-hydrates all had two sharp absorptions at 3500 cm^{-1} (unbonded O-H and N-H) but the broad absorption at 3300-3400 cm^{-1} was absent. In the non-solvated derivatives, there were no absorptions in the 3500-3300 cm^{-1} range. The i.r. spectra of three typical examples are recorded in Figure 10.

The ^{1}H n.m.r. spectra of the 9-anilinoacridine hydrochlorides in DMSO-d_6 show that the acridine protons are relatively deshielded and occur at τ1.0-1.7 whereas the protons on the phenyl fragment occur upfield at τ2.0 - 2.7. The characteristic absorptions of the alkyl groups in many of the derivatives are recorded in the Experimental Section.

The mass spectra of all the anilinoacridine hydrochlorides showed molecular ions of high abundance The only major common feature was a peak at m/e 178 due to loss of the anilino fragment forming the

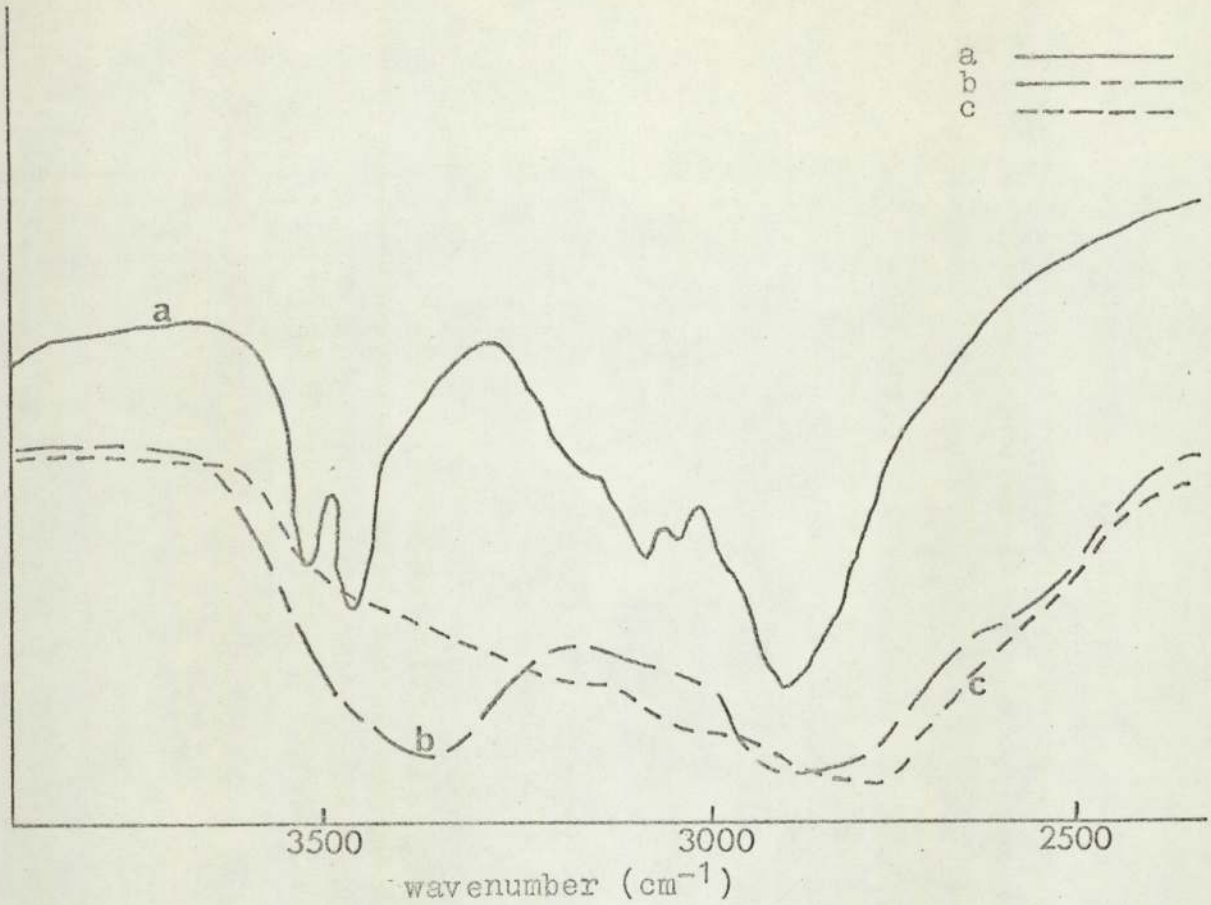


FIGURE 10

a. 9-(4-chloroanilino)acridine hydrochloride (4.12s)($0.5H_2O$)

b. 9-(4-ethoxyanilino)acridine hydrochloride (4.12h)($1.0H_2O$)

c. 9-(3-methylanilino)acridine hydrochloride (4.12n) (anhydrous)

acridinium ion. The mass spectral breakdown of this fragment has been well documented (see references 189 and 190).

The electronic absorption spectra of the anilinoacridine salts in water are reported in Table 7. The spectrum of 9-aminoacridine hydrochloride (4.1) shows fine structure and differs markedly from the 9-anilinoacridine salts. All the 9-anilinoacridine hydrochlorides have similar spectra and the substituent, whether ortho, meta or para exerts only a minor influence. The only notable differences are observed in the band at 240 nm which in the case of the ortho and meta substituted derivatives have a much lower extinction.

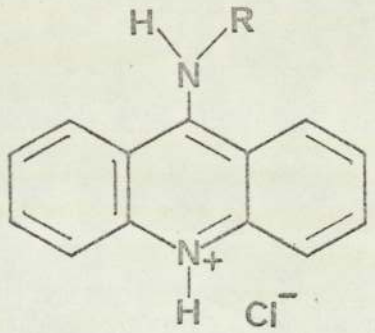
Comparison of the spectra of the non-protonated 9-anilinoacridines and their salts in very dilute solutions (where hydrogen bonding, micelle formation precipitation and decomposition are minimal) confirmed that the site of protonation is on the endocyclic N-atom. Protonation produces a profound bathochromic shift. This is in accord with the greater resonance permitted in the ion compared to that in the non-protonated species (Scheme 21).

If protonation had occurred at the exo-cyclic substituent, a hypsochromic shift would have occurred (cf. protonation of aniline).

Examples of the I.R. ^{1}H N.M.R. and U.V. spectra of a typical anilinoacridine hydrochloride are shown in Figure 11.

ELECTRONIC ABSORPTION SPECTRA OF SUBSTITUTED ANILINOACRIDINE HYDROCHLORIDES IN WATER.

TABLE 7.



4.12 a-w

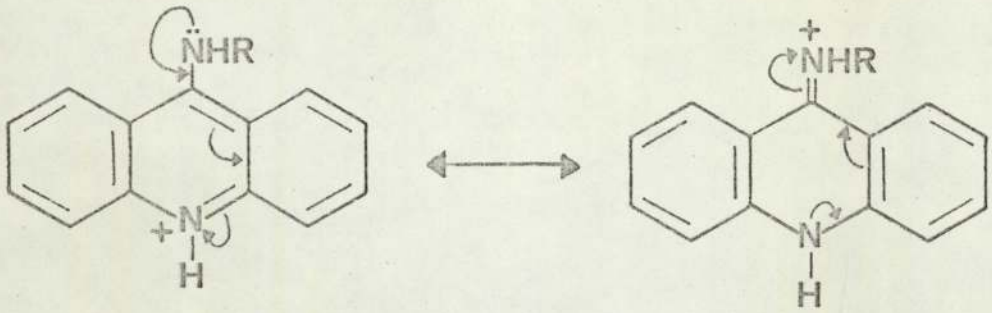
COMPOUND	λ MAX (n.m.) (LOG ϵ)										* Inflexion
4.12a	438	(4.0),	416	(3.96),	335	(3.53),	320*	(3.47),	267	(4.66),	239 (4.48)
b	440	(4.06),	418*	(3.96),	335	(3.43),			265	(4.64),	248 (4.74)
c	440*	(3.89),	418	(3.92),	335	(3.44),			265	(4.58),	244 (4.30)
d	440	(3.99),	418*	(3.96),	335	(3.49),			265	(4.64),	244 (4.55)
e	438	(3.93),	418	(3.92),	335	(3.45),			265	(4.59),	244 (4.37)
f	438	(3.66),	418	(3.65),	333	(3.35),			265	(4.41),	242 (4.09)
g	438	(4.01),	415	(3.98),	335	(3.44),	327*	(3.37)	266	(4.59),	240* (4.48)
h	440	(3.86),	418*	(3.83),	335	(3.43),			265	(4.46),	244 (4.41)

ELECTRONIC ABSORPTION SPECTRA OF SUBSTITUTED ANILINOACRIDINE HYDROCHLORIDES.IN WATER.

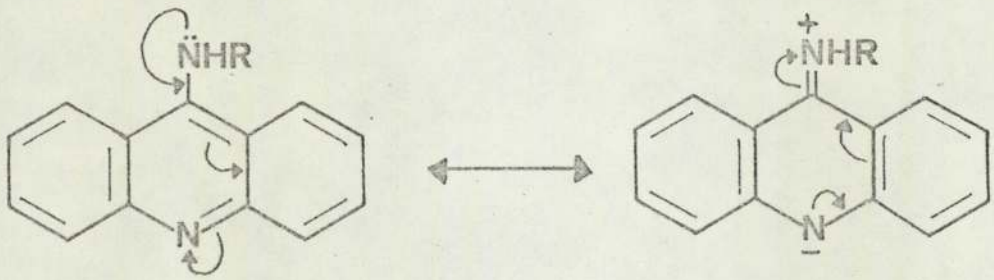
TABLE 7. Continued.

COMPOUND	λMAX (n.m.) (LOG ϵ)									
4.12i	437 (4.06),	413* (4.01),		334 (3.50),	318* (3.45)	264 (4.61),	244 (4.52)
j	436 (4.06),	413* (4.00),		333 (3.49),	317* (3.43)	265 (4.59),	244 (4.53)
k	434 (4.02),	412* (4.00),		331 (3.41)	316* (3.36)	265 (4.57),	244 (4.50)
l	440 (3.91),	420* (3.88),		335 (3.72)		265 (4.40),	244 (4.31)
m	440 (3.92),	420* (3.90),		335 (3.49),		265 (4.58),	238 (4.30)
n	438 (4.05),	418 (4.01),		335 (3.59),		265 (4.60),	238 (4.41)
o	438 (3.97),	418* (3.94),		335 (3.54),		266 (4.67),	242* (4.50)
p	438 (4.09),	418* (4.01),		335 (3.63),		265 (4.66),	241* (4.49)
q	438 (3.92),	418* (3.89),		335 (3.53),		265 (4.44),	242 (4.28)
r	440*(3.84),	418 (3.90),		335 (3.49),		265 (4.70)	
s	440 (3.95),	420* (3.92),		335 (3.59),		267 (4.61),	242 (4.39)
t	438 (4.03),	418* (4.02),		335 (3.62),		265 (4.68),	238 (4.43)
u	460 (3.78),	432 (3.90)	406 (3.84)	335 (3.53),		265 (4.68),	244* (4.43)
v	460 (3.87),	432 (3.90)		345 (3.71),		265 (4.64),	236* (4.40)
w	430 (3.94),			335 (3.69),		265 (4.74),	252 (4.72)

Protonated Species



Non-protonated Species



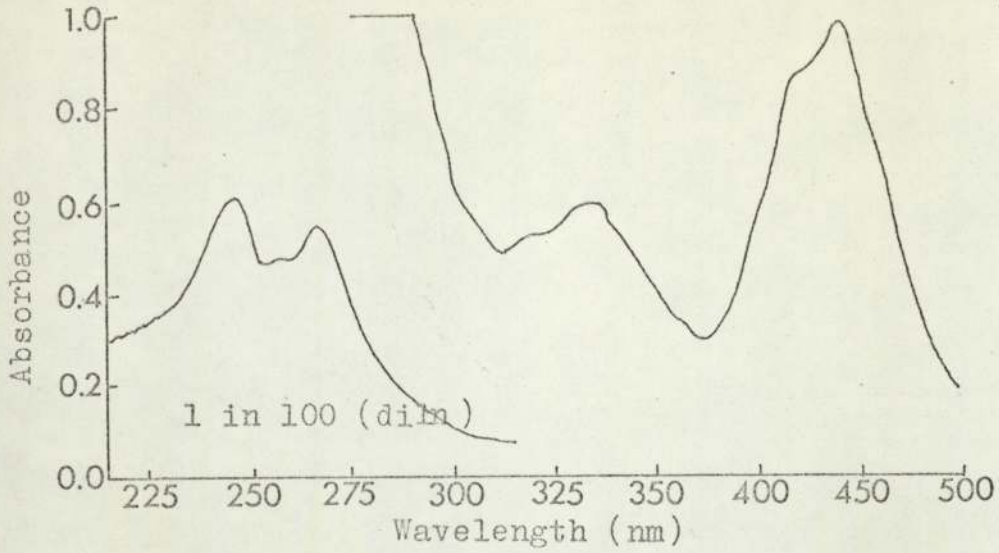
Resonance less favoured due to large separation of charge in the neutral molecule.

SCHEME 21.

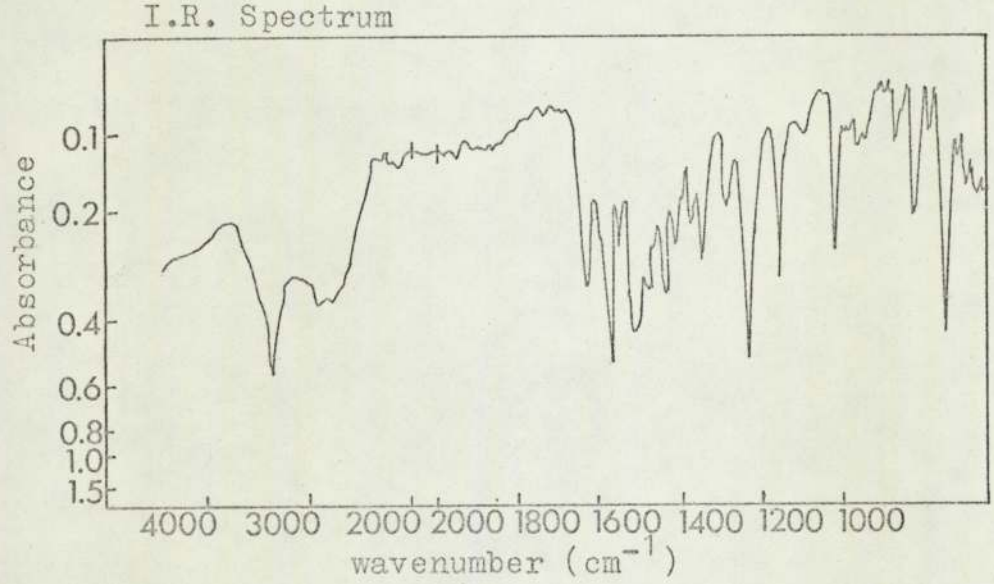
FIGURE 11

Spectra of 9-(4-methoxyanilino)acridine hydrochloride (4.12g)

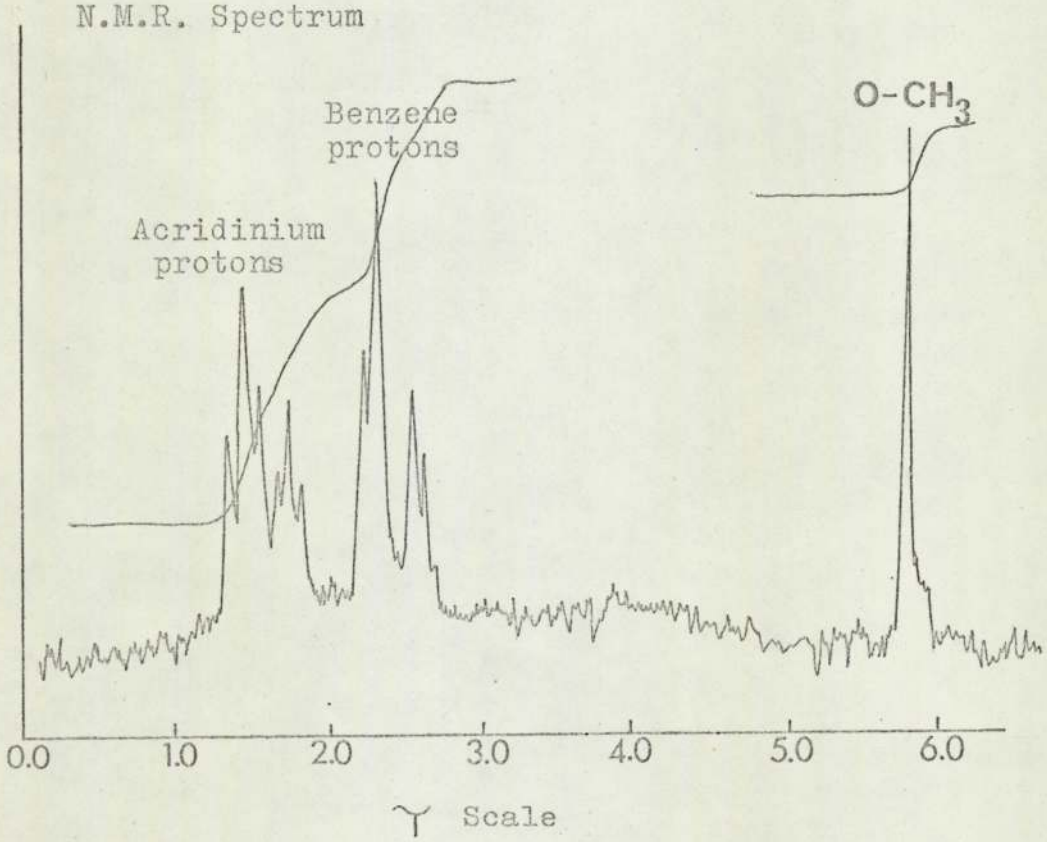
U.V. Spectrum



I.R. Spectrum



N.M.R. Spectrum



pKa Determination of the Anilinoacridines.

It is of considerable interest to know the basic strengths of acridine derivatives since their antibacterial activity closely parallels their basicity.[171] Two methods for determining the pKa of the anilinoacridines were considered - the potentiometric and the spectroscopic methods. Difficulties arose in using the first method, because of the low water solubility of the anilinoacridines. A suitable concentration in water for a potentiometric titration is about 1×10^{-2}M;[191] with special refinements it is possible to use 1×10^{-3}M solutions. Unfortunately, the maximum concentrations of the anilinoacridine hydrochlorides attainable in water was $\leq 5 \times 10^{-3}$M. Although the anilinoacridine hydrochlorides were more soluble in hot water substantial hydrolysis occurred (cf. Scheme 20) and acridone (4.9) was deposited. It is possible to use organic solvents in potentiometric titrations but it has been observed by Albert[171] that the basic strength of the acridines (in ethanol) is depressed irregularly ranging from 0.4 to 1.5 pKa units. If the pKa values are to be correlated with biological activity this error cannot be tolerated.

The spectroscopic method of Albert and Sergeant[191] had to be modified in view of the instability of the anilinoacridine hydrochlorides in base. It was observed that the spectra at alkaline pH were inconsistent. To minimise this effect the length of time between making the solution and recording the spectra was

standardised (2 minutes).

The method consisted of determining the relative proportions of ion to molecule in the acridine when it was dissolved in a series of buffers. This was measured at 265 nm (analytical wavelength) as this peak showed the greatest difference in absorbance values between ion and molecule in this series. The pKa values of the anilinoacridines were calculated by the modified Henderson-Hasselbach equation:[191]

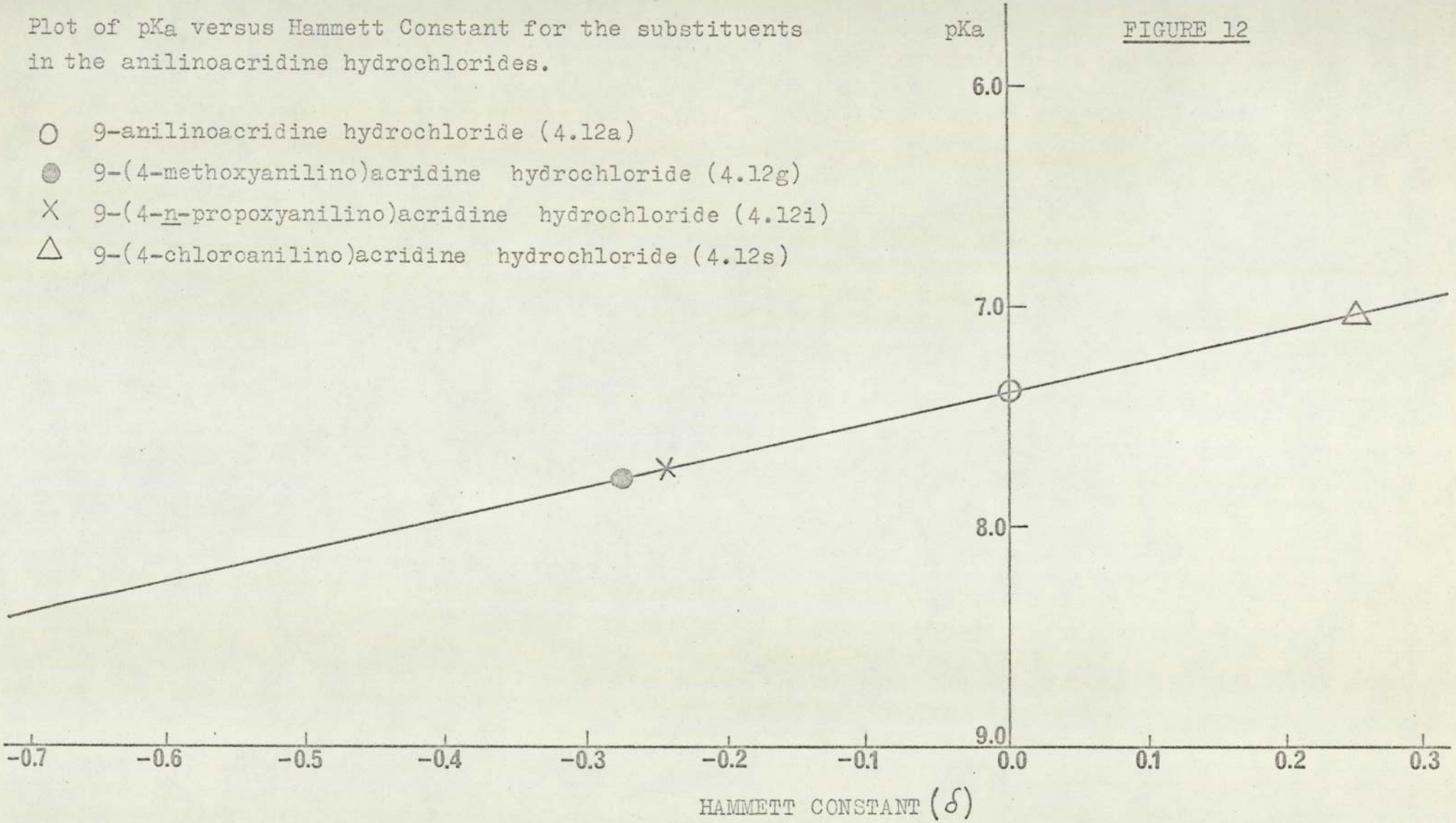
$$pKa = pH + \log \frac{d - dm}{d_1 - d}$$

where d_1 is the absorbance of the ion at the analytical wavelength, dm is the absorbance of the molecule, and d is the absorbance of the mixture of ion and molecule both at the same wavelength. The pKa of 9-anilinoacridine was found to be 7.41 ± 0.02 (Table 8). The pKa values of the other anilinoacridines were similarly calculated or predicted by using the method of Clark and Perrin.[192] These values are shown in Table 9.

This method of predicting pKa values is based on the known amount that any substituent influences the basicity of the parent compound in any chemical series. This is usually expressed as a Hammett constant. Thus by plotting these constants against the calculated pKa values it is possible to draw a calibration graph (Figure 12) from which the predicted pKa values can be obtained knowing the Hammett constant for the substituent group (R - see Table 8). This graph can only be applied to <u>meta</u> - and <u>para</u> - substituents.

Plot of pKa versus Hammett Constant for the substituents in the anilinoacridine hydrochlorides.

FIGURE 12



○ 9-anilinoacridine hydrochloride (4.12a)

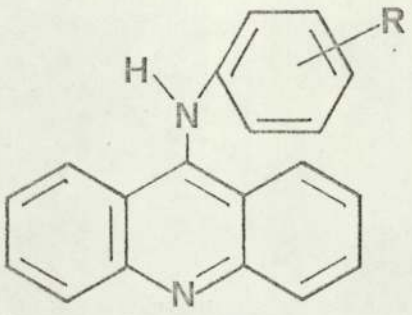
● 9-(4-methoxyanilino)acridine hydrochloride (4.12g)

× 9-(4-n-propoxyanilino)acridine hydrochloride (4.12i)

△ 9-(4-chlorcanilino)acridine hydrochloride (4.12s)

DETERMINATION OF THE pKa VALUE OF 9-ANILINOACRIDINE (R = H)

TABLE 8.



pH	pKa	MEAN pKA	DEVIATION FROM MEAN
7.09	7.39		-0.02
7.24	7.42	7.41	+0.01
8.00	7.40		-0.01
8.34	7.41		0

$\therefore$ pKa = 7.41 ± 0.02

TABLE OF pKa VALUES OF ANILINOACRIDINES. TABLE 9.

R =	CALCULATED pKa	PREDICTED pKa
H	7.41 (±0.02)	-
4 - OMe	7.78 (±0.08)	-
4 - OEt	-	7.74
4 - O-n-Pr	7.74 (±0.06)	-
4 - O-n-But	-	7.85
4 - O-n-Pent	-	7.88
3 - Me	-	7.50
4 - Me	-	7.65
4 - Et	-	7.60
4 - n-But	-	7.63

TABLE OF pKa VALUES OF ANILINOACRIDES. TABLE 9.
(Continued)

R =	CALCULATED pKa	PREDICTED pKa
4 - F	-	7.33
4 - Cl	7.04 ($\pm$0.08)	-
4 - Br	-	7.01
4 - OPh	-	7.85
4 - OAc	-	6.98
4 - NH_2	-	8.3
4 - OH	-	7.88
2 - Me	7.21 ($\pm$0.08)	7.26^{o}
2 - OMe	7.23 ($\pm$0.04)	7.41^{o}
2 - Cl	-	6.41^{o}
2 - OH	-	7.55^{o}

^{o} From modified Hammett Equation.

In order to predict the pKa values of the ortho - substituted anilinoacridine hydrochlorides it is necessary to use the modified Hammett equation:-

$$pKa = pKa^{o} - k (\Sigma \delta)$$

where pKa^{o} is the known pKa of the parent compound, k is the constant for the series under study and δ is the Hammett constant for the substituent group (R).

The influence of the substituent on the pKa values of these anilinoacridines is important since all the values are within ± 0.5 of a pKa unit of physiological pH. 9-Anilinoacridine (pKa = 7.41) is considerably weaker as a base than 9-aminoacridine (pKa 9.6). Albert[171] suggested that the large depression of basic strength ($\approx$ 2 units) found in 9-substituted aminoacridines was due to the steric effect of the substituent. The bulky aryl group must be twisted out of the plane of the rest of the molecule by the peri-hydrogen atoms of the acridine nucleus and this departure from planarity weakens the resonance possible in 9-aminoacridine. Substituents in the aryl group that are electron-donating (e.g.alkyl) are base-strengthening whereas electron-withdrawing groups (e.g.Cl)are base-weakening as expected. With regard to the para-substituents, electronic effects exert a major influence in altering the pKa value of the parent compound.

The influence of ortho-substituents on the basicity of 9-anilinoacridine is governed more by steric factors. It was observed that ortho-substituents

always depress the basicity regardless of the electronic character of the substituent. Probably the ortho-substituents twist the 9-anilino groups further from coplanarity with concomitant decrease in basic strength.

A relationship between the biological activity and the pKa values of these anilinoacridine derivatives was observed and will be discussed in Chapter 5.

CHAPTER 5.

CHAPTER 5.

Biological Results.

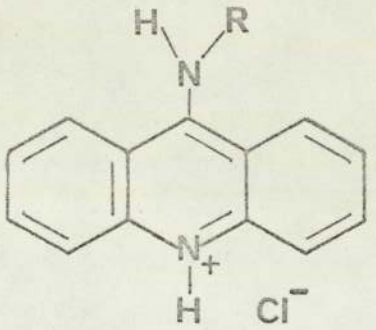
Anti-tumour activity of acridine derivatives.

Many of the acridines prepared in the foregoing Chapter were screened for anti-tumour activity by the Cancer Chemotheraphy National Service Centre, Bethesda U.S.A. The test systems used were lymphoid leukaemia L-1210, Lymphocytic leukaemia P 388 and a cell culture tube assay on Human Epidermoid Carcinoma of the Nasopharynx. The hosts in the leukaemic trials were BDF_1 and CDF_1 mice. The compounds were administered by intraperitoneal injection in saline, saline plus Tween 80 or Klucel (hydroxypropylcellulose). Mortality and weight changes were recorded throughout the trial. The test performed was an evaluation of the survival time in days. An increase in survival of test animals resulting in a T/C of greater than 125% was considered necessary for further screening. This latter definition of activity does not apply to the cell culture tube assays where activity is measured as a parameter of the ED_{50}. The results of this screening are summarised in Tables 10 - 14.

Structure-activity studies indicated the following:

1. Anilinoacridine hydrochlorides with -I substituents (e.g. halo compounds) are inactive whether substitution is in ortho-, meta- or para-positions.

BIOLOGICAL ACTIVITY OF 9-SUBSTITUTED ACRIDINES. TABLE 10.



4.12

Tumour:- Lymphoid leukaemia (L 1210) Host:- BDF^{1} or CDF_{1}^{*} Mice.

Vehicle:- Saline (S) or Saline + Tween 80 (T) † 2 Cures

DRUG R =	DOSE Mg/Kg	SURVIVORS				AVERAGE DAYS OF SURVIVAL Treated	Control	% T/C
Phenyl	400 (S)	3	"	"	6	10.3	9.2	-
	200	6	"	"	6	7.3	9.2	-
	100	6	"	"	6	10.6	9.2	115
4-Aminophenyl	400 (T)	0	"	"	6	0.0	9.0	-

BIOLOGICAL ACTIVITY OF 9-SUBSTITUTED ACRIDINES.

TABLE 10. (Continued)

DRUG R =	DOSE Mg/Kg	SURVIVORS				AVERAGE DAYS OF SURVIVAL Treated	Control	% T/C
4-Aminophenyl	200	0	"	"	6	0.0	9.0	-
	100	2	"	"	6	11.0	9.0	-
"	25.0(S)	6	"	"	6	10.8	9.4	114
	12.5	6	"	"	6	10.5	9.4	111
	6.25	6	"	"	6	9.5	9.4	101
4-Acetoxyphenyl	400 (T)	0	"	"	3	0.0	8.6	-
	200	2	"	"	3	7.0	8.6	-
	100	3	"	"	3	15.3	8.6	117
"	200	3	"	"	3	7.0	10.3	-
	100	3	"	"	3	18.7	10.3	181
	50.0	3	"	"	3	15.0	10.3	145
	25.0	3	"	"	3	11.0	10.3	106
"	225	5	"	"	10	6.4	10.3	-

TABLE 10. (Continued)

DRUG R =	DOSE Mg/Kg	SURVIVORS		out of		AVERAGE DAYS OF SURVIVAL Treated	Control	% T/C
4-Acetoxyphenyl	150	1	"	"	10	11.0	10.3	-
	100	10	"	"	10	15.7	10.3	152†
	66.0	10	"	"	10	11.3	10.3	109
	44.0	10	"	"	10	12.3	10.3	119
* "	225	1	"	"	10	9.0	9.8	-
	150	10	"	"	10	11.4	9.8	116
	100	9	"	"	10	12.1	9.8	123
	66.0	10	"	"	10	11.7	9.8	119
	44.0	10	"	"	10	11.4	9.8	116
4-Methoxyphenyl	400 (S)	2	"	"	6	10.0	9.2	-
	200	3	"	"	6	10.7	9.2	-
	100	5	"	"	6	12.0	9.2	130

TABLE 10. (Continued)

DRUG R =	DOSE Mg/Kg	SURVIVORS	AVERAGE DAYS OF SURVIVAL Treated	Control	% T/C
4-Methoxyphenyl	150	5 " " 6	14.4	9.4	153
	100	6 " " 6	12.6	9.4	134
	66.0	6 " " 6	12.3	9.4	130
	44.0	6 " " 6	12.3	9.4	130
4-Ethoxyphenyl	400 (T)	0 " " 6	0.0	9.0	-
	200	4 " " 6	8.6	9.0	88
	100	6 " " 6	11.8	9.0	131
4-Phenoxyphenyl	400 (T)	3 " " 3	9.0	8.6	104
	200	3 " " 3	8.7	8.6	101
	100	3 " " 3	8.7	8.6	101
2-Methylphenyl	400 (T)	0 " " 6	0.0	9.2	-
	200	1 " " 6	9.0	9.2	-
	100	5 " " 6	7.8	9.2	-

TABLE 10. (Continued)

DRUG R =	DOSE Mg/Kg	"	"	SURVIVORS out of	"	"	AVERAGE DAYS OF SURVIVAL Treated	Control	% T/C
2-Methylphenyl	50.0	6	"	"	6		8.2	9.4	87
	25.0	6	"	"	6		9.8	9.4	104
	12.5	6	"	"	6		9.3	9.4	98
3-Methylphenyl	400 (T)	0	"	"	6		0.0	8.4	-
	200	2	"	"	6		9.0	8.4	-
	100	5	"	"	6		8.4	8.4	100
"	100	5	"	"	6		9.4	9.0	104
	50.0	6	"	"	6		9.0	9.0	100
	25.0	6	"	"	6		8.8	9.0	97
4-Methylphenyl	300	2	"	"	6		15.0	10.5	-
	150	6	"	"	6		13.5	10.5	128
	75.0	6	"	"	6		10.6	10.5	101

TABLE 10. (Continued)

DRUG R =	DOSE Mg/Kg	"	"	SURVIVORS out of	"	"	AVERAGE DAYS OF SURVIVAL Treated	Control	% T/C
4-Methylphenyl	150	6	"	"	6		14.1	8.8	160
	75.5	6	"	"	6		12.1	8.8	137
	44.0	6	"	"	6		10.6	8.8	120
"	225 (T)	6	"	"	6		11.8	10.0	118
	150	6	"	"	6		8.8	10.0	88
	75.0	6	"	"	6		12.3	10.0	123
	65.0	6	"	"	6		10.0	10.0	100
"	150	4	"	"	6		6.6	9.1	-
	75.0	6	"	"	6		9.3	9.1	102
	37.5	6	"	"	6		9.2	9.1	101
4-Ethylphenyl	400 (T)	1	"	"	6		11.0	9.0	-
	200	6	"	"	6		7.5	9.0	-
	100	5	"	"	6		11.8	9.0	131

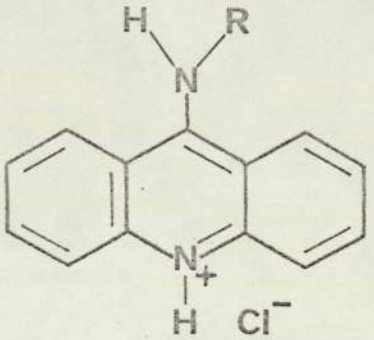
TABLE 10. (Continued)

DRUG R =	DOSE Mg/Kg	"	SURVIVORS " out of	"	"	AVERAGE DAYS OF SURVIVAL Treated	Control	% T/C
4-n-Butylphenyl	400 (T)	2	"	"	3	9.5	9.0	105
	200	3	"	"	3	10.3	9.0	114
	100	3	"	"	3	9.7	9.0	107
2-Chlorophenyl	400 (S)	6	"	"	6	8.7	9.0	96
	200	6	"	"	6	9.2	9.0	102
	100	6	"	"	6	9.0	9.0	100
4-Chlorophenyl	400 (S)	6	"	"	6	6.8	11.3	-
	200	6	"	"	6	12.5	11.3	110
	100	6	"	"	6	11.0	11.3	97
4-Fluorophenyl	400 (T)	0	"	"	6	0.0	9.0	-
	200	2	"	"	6	9.0	9.0	-
	100	6	"	"	6	8.7	9.0	96
	100	5	"	"	6	9.4	8.1	116

TABLE 10. (Continued)

DRUG R =	DOSE Mg/Kg	"	SURVIVORS " out of "	"	AVERAGE DAYS OF SURVIVAL Treated	Control	% T/C
4-Fluorophenyl	50.0	5	"	6	9.6	8.1	118
	25.0	5	"	6	7.5	8.1	92
4-Bromophenyl	400 (T)	0	"	6	0.0	9.2	-
	200	6	"	6	9.5	9.2	103
	100	6	"	6	9.5	9.2	97
3-Pyridyl	400 (S)	6	"	6	10.3	11.3	91
	200	6	"	6	10.0	11.3	88
	100	6	"	6	10.0	11.3	88
	400	4	"	6	10.0	10.6	94
	200	6	"	6	10.5	10.6	99
	100	6	"	6	10.3	10.6	97

TABLE 11.



Tumour:- Lymphocytic leukaemia (P 388)

Host:- BDF_1 Mice

Vehicle (1) Saline + Tween 80

(2) Klucel (hydroxypropyl-cellulose)

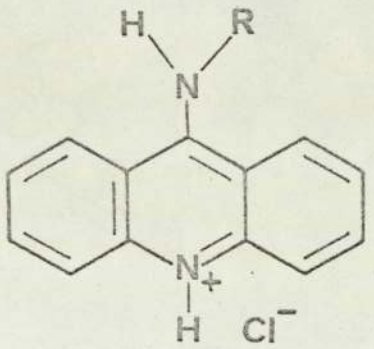
DRUG R =	DRUG Mg/Kg	SURVIVORS " " out of " "	AVERAGE DAYS OF SURVIVAL Treated	Control	% T/C
4-Methylphenyl	400(2)	6 " " 6	12.5	11.0	113
	200	6 " " 6	13.0	11.0	118
	100	6 " " 6	12.0	11.0	109
4-Bromophenyl	200(1)	4 " " 6	7.0	10.5	-
	100	6 " " 6	10.5	10.5	100
	50.0	6 " " 6	10.0	10.5	95

TABLE 11. Continued.

DRUG R =	DRUG Mg/Kg	SURVIVORS " " out of " "	AVERAGE DAYS OF SURVIVAL Treated	Control	% T/C
2-Chlorophenyl	400(1)	6 " " 6	11.5	11.0	104
	200	6 " " 6	14.0	11.0	127
	100	6 " " 6	11.0	11.0	100

CELL CULTURE TUBE ASSAY

TABLE 12



4.12

TUMOUR:- Human Epidermoid Carcinoma of the Nasopharynx

DRUG R =	SLOPE	ED_{50}	CONCLUSION
Phenyl	-0.81	1.8 x 10 (1)	Inactive
4-Aminophenyl	-0.0	1.0 x 10 (0)	Weak activity
"	-1.09	2.7 x 10 (-1)	Moderate activity
4-Methoxyphenyl	-0.43	1.0 x 10 (0)	Weak activity
"	-1.08	2.6 x 10 (-1)	Moderate activity
4-Ethoxyphenyl	-0.0	1.0 x 10 (0)	Weak activity
"	-0.97	2.3 x 10 (-1)	Moderate activity

TABLE 12. (Continued)

DRUG R =	SLOPE	ED_{50}	CONCLUSION
2-Methylphenyl	-0.71	1.4 x 10 (1)	Inactive
3-Methylphenyl	-0.53	8.7 x 10 (0)	Inactive
4-Ethylphenyl	-0.57	8.9 x 10 (-1)	Moderate activity
"	-0.0	1.7 x 10 (0)	Inactive
4-Chlorophenyl	-0.78	1.6 x 10 (1)	Inactive
4-Fluorophenyl	-0.7	1.3 x 10 (1)	Inactive
4-Bromophenyl	-0.84	1.9 x 10 (0)	Inactive
3-Pyridyl	-0.63	4.2 x 10 (1)	Inactive

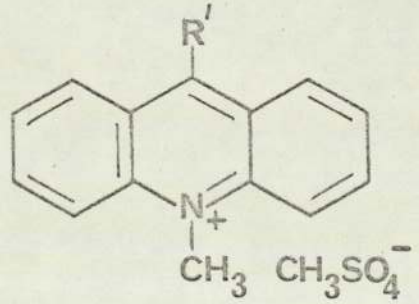
ED_{50} :- Dose that inhibits 50% of control growth expressed in micrograms per ml.

Slope:- Change of response for each one log change of dose.

TABLE 13

HOST: MOUSE CDF_1

TUMOUR:- lymphoid leukaemia L1210



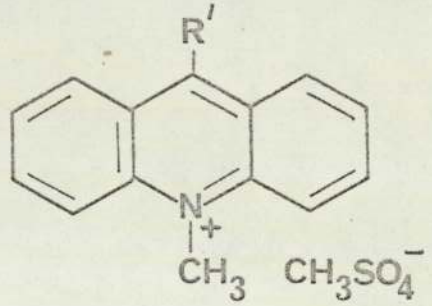
Vehicle: Saline + Tween 80

DRUG R' =	DOSE mg/Kg/	SURVIVORS " " OUT OF " "	WEIGHT CHANGE	AVERAGE DAYS OF SURVIVAL TREATED	CONTROL	% T/C
Chloro	400	0 " 6	0.9	0.0	8.4	-
	200	5 " 6	0.9	9.2	8.4	109
	100	6 " 2	0.9	8.8	8.4	104
Azido	400	0 " 6	0.9	0.0	8.4	-
	200	0 " 6	0.9	0.0	8.4	-
	100	0 " 6	0.9	0.0	8.4	-

TABLE 14

CELL CULTURE TUBE ASSAY

Tumour: Human Epidermoid Carcinoma of the Nasopharynx



DRUG R' =	SLOPE	ED_{50}	CONCLUSION
Chloro	-0.84	1.9 x 10 (1)	Inactive
Azido	-1.01	2.4 x 10 (0)	Weak activity
	-0.66	1.2 x 10 (0)	Moderate activity

2. Anilinoacridine hydrochlorides with +I substituents (e.g. alkyl derivatives) show marginal activity only when the substituent is in the para - position.

3. Anilinoacridine hydrochlorides with +M substituents (e.g. alkoxy, amino) show pronounced tumour-inhibitory activity especially when the substituent is in the para-position.

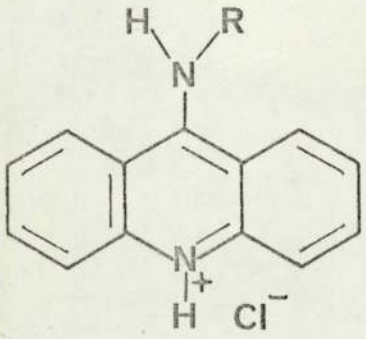
These conclusions are in accord with earlier work on similar derivatives by Cain.[186] There is a clear correlation with pKa and the biological activity of these anilinoacridine hydrochlorides. Those derivatives with pKa greater than 7.5 are active, whereas those below are inactive (Table 15).

However, it must be born in mind that other factors besides basic strength may well come into play. The possible mechanism for the anti-tumour activity of the anilinoacridine hydrochlorides is open to speculation: whether the acridines react within the cell or not has not been determined. However, by analogy with anti-bacterial acridines it is probable that the nucleic acids are the vulnerable sites.

It is possible that the hydrophilic-lipophilic balance is another limiting factor in determining anti-tumour activity.[172,185,186,193] Thus those anilino-acridines with pKa below 7.78 (minimum value for anti-bacterial acridines) could have the advantage by providing adequate neutral species for optimum cellular penetration by passive diffusion to elicit the

TABLE 15

RELATIONSHIP BETWEEN pKa AND BIOLOGICAL ACTIVITY.



4.12

DRUG R=	pKa	% IONISATION AT PHYSIOLOGICAL PH(7.3)	BIOLOGICAL ACTIVITY
Phenyl	7.41	56.3	-
4-Aminophenyl	8.30	90.9	±
4-Acetoxyphenyl	6.98	32.4	++
2-Methoxyphenyl	7.23	46.0	-
4-Methoxyphenyl	7.78	75.0	+
4-Ethoxyphenyl	7.74	73.4	±
4-Phenoxyphenyl	7.85	78.0	-
2-Methylphenyl	7.21	44.9	-
3-Methylphenyl	7.50	61.3	-
4-Methylphenyl	7.65	69.1	±
4-Ethylphenyl	7.60	66.6	±
4-n-Butylphenyl	7.63	68.1	-
2-Chlorophenyl	6.41	11.41	-
4-Chlorophenyl	7.04	35.5	-
4-Fluorophenyl	7.33	51.7	-
4-Bromophenyl	7.01	33.1	-

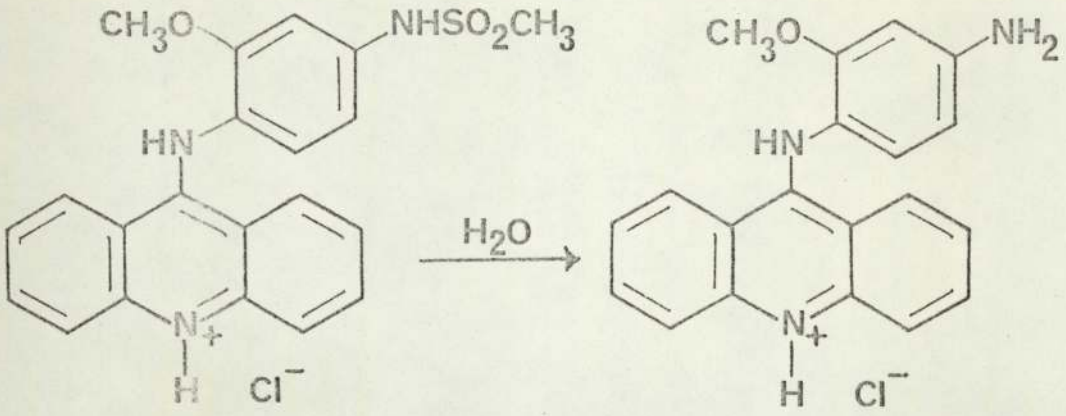
++ = Strongly active (> 175%)
+ = Active (>150%)
± = Weakly active (> 125%)
- = Inactive (< 125%)

biological response. However, once inside the cell these compounds will not intercalate DNA as readily as their more strongly ionised counterparts. The final biological result may be ascribed to the integrated physico-chemical properties of the total molecule.

Another possibility is that the ortho-substituted acridines cannot complex with the nucleic acids due to purely steric factors: equally some of the bulky para-substituted acridines, e.g. 9-(4-phenoxy-anilino) acridine hydrochloride (4.12 1)(pKa 7,85) may also be incapable of intercalating DNA.

One of the anilinoacridines, 9-(4-acetoxy-anilino) acridine hydrochloride (4.12e) showed the greatest activity of all these derivatives; in fact two cures against lymphoid leukaemia (L 1210) were obtained. The predicted pKa of this compound is 6.98 (32.4% ionised at physiological pH). This acetoxy derivative could have desirable transport characteristics and be bio-transformed to the active moiety within susceptible cells. This active moiety may well be the corresponding phenol (4.12d) (predicted pKa 7.88 — % ionised at physiological pH = 79.2) which could form by in vivo hydrolysis of the acetate. This phenol is known to have significant anti-tumour properties.[186]

Another anilinoacridine hydrochloride recently prepared [194] also shows significant anti-tumour activity with a predicted pKa value of approximately 6.5. This compound, 9-(2-methoxy-4-methylsulphonamido-anilino)-acridine hydrochloride (5.1) has marked activity against



5.1 5.2

Predicted pKa ≈ 6.5

% ionisation at physiological pH = 13.6%

Predicted pKa = 8.3

% ionisation at physiological pH = 90.90%

SCHEME 22

leukaemia L 1210 and contains a substituent sulphonamide group. The sulphonamide group could possibly undergo hydrolysis *in vivo* to form the aminomethoxyanilinoacridine (5.2) (Scheme 22).

The anti-tumour properties of the methosulphates (Tables 13 and 14) were also examined. These compounds were highly toxic but inactive. The lack of activity of these acridines may be due to the fact that these salts are permanent cations and hence cannot penetrate the cell wall. They are also rapidly de-activated by hydrolysis.

Anti-cholinergic activity of the acridinium methosulphates.

9-Azido-10-methylacridinium methosulphate (4.6 b) and its 9-chloro analogue (4.14) have been shown to produce toxic effects in mice; it was suggested that one possible cause of death might have been a result of inhibition of cholinesterase enzymes. As a result of the quaternary nature of these two compounds it was suspected that there might be an interaction between the organic molecules and the anionic site on the cholinesterase enzymes. The nature of the enzyme-substrate complex with the natural ligand *i.e.* acetylcholine is thought to involve an ionic bond between the quaternised portion of acetylcholine and the anionic site at the active-site of the enzymes. This is followed by covalent acylation of a reactive serine in the esteratic portion of the active site.[5]

The effects of these compounds were determined

on both acetylcholinesterase (specific or true cholinesterase), and cholinesterase (pseudo- or non-specific enzyme) [195] by Mr. D. Bradshaw, University of Aston. The inhibition produced by these two compounds was compared on different enzyme preparations using different substrates, by incubating the enzymes with a number of different concentrations of the inhibitor and estimating the I_{50} values (Table 16).

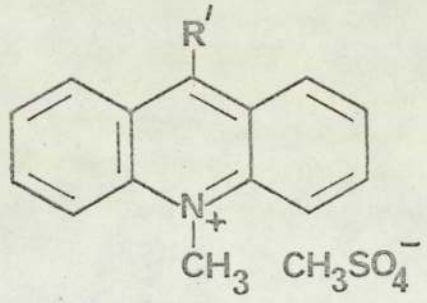
With all the enzyme preparations and with all substrates used the azido compound was more potent than the chloro analogue. Both compounds inhibited pseudo-cholinesterase to a greater extent than acetylcholin-esterase. N-Methylacridone, the product of hydrolysis (See Chapter 4) was also tested for activity against cholinesterase and was found to inhibit the enzyme by 40% at a concentration of 10^{-3}M. This is of interest as this compound lacks the quaternary nitrogen atom.

However, the results show that the azido compound (4.6 b) inhibited cholinesterase by 68% at a concentration of 10^{-5}M, and the chloro analogue by 21% at the same concentration. These compounds were compared to a known inhibitor of cholinesterase (Physostigmine) which under comparable conditions inhibited the enzyme by 98% at a concentration of 10^{-5}M.

The reduced activity of the acridinium salts against acetylcholinesterase may well be due to this enzymes requirement of two sites of attachment to the active-site.

The overall toxicity of the two compounds to

TABLE OF I_{50} VALUES AS MOLAR CONCENTRATIONS FOR THE INHIBITION OF THE CHOLINESTERASES BY THE METHYLACRIDINIUM SALTS.



$R' = N_3$ (4.6b)
$R' = Cl$ (4.14)

ENZYME	SOURCE OF ENZYME	(4.6b)	(4.14)	SUBSTRATE
Acetylcholinesterase	Bovine erythrocytes	2.9×10^{-5}	5.5×10^{-3}	Acetylcholine
Cholinesterase	Horse serum	1.9×10^{-6}	3.3×10^{-4}	"
Mixed Cholinesterases	Rat brain homogenate	4.2×10^{-6}	1.2×10^{-3}	"
" "	" " "	3.6×10^{-6}	1.0×10^{-3}	Acetyl-β-methylcholine
" "	" " "	1.5×10^{-6}	4.4×10^{-4}	Butyrylcholine

I_{50} - Dose required for 50% inhibition of the enzyme.

mice correlates well with their anticholinergic activity, the azide compound is the most toxic and the strongest anticholinesterase.

Both compounds have potential use as insecticides or rodenticides.

CHAPTER 6.

CHAPTER 6.

Arylation of Nucleophilic Sites within Nucleotides.

9-Azido-10-methylacridinium methosulphate (4.6b) is an hetarylating agent (Chapter 4). The analogous chloro compound (4.14), although not tested with amines, should react similarly. The high toxicity of these compounds, observed in biological testing, was thought possibly to involve anti-cholinergic activity (Chapter 5). However, an alternative toxic action might well involve the nucleic acids. Arylation of nucleophilic sites within the nucleic acids could explain the extreme toxicity of these compounds (cf. alkylation Chapter 1). Identification of these sites might give an insight into the mechanism of action of these acridinium salts.

As yet no work has been done on arylation of DNA using 2,4-dinitrohalobenzenes or picryl halides. However, the arylation of nucleotides has been studied using 2,4-dinitrofluorobenzene. Two groups of workers[196-197] have shown that the major product of these reactions is the formation of 2,4-dinitrophenyl esters at the 5 -OH group, with minor reactions at the 2' - and 3'- positions with the ribonucleotides. This result was confirmed by other workers.[198] 2,4-Dinitrochlorobenzene was shown not to react with the sugar moieties under comparable conditions.[97]

In the present work the arylation of adenine with 2,4-dinitrochlorobenzene (6.1) and 9-chloroacridine was examined. In view of the number of reactive sites within the adenine molecule, preliminary experiments

using the isomeric aminopyridines as model compounds were carried out. Previous arylation studies of adenine derivatives[199,200] using 2,4-dinitrofluorobenzene under basic conditions showed that the exocyclic nitrogen of the adenine molecule was the most susceptible site.

Ring-opening of Zincke Salts.

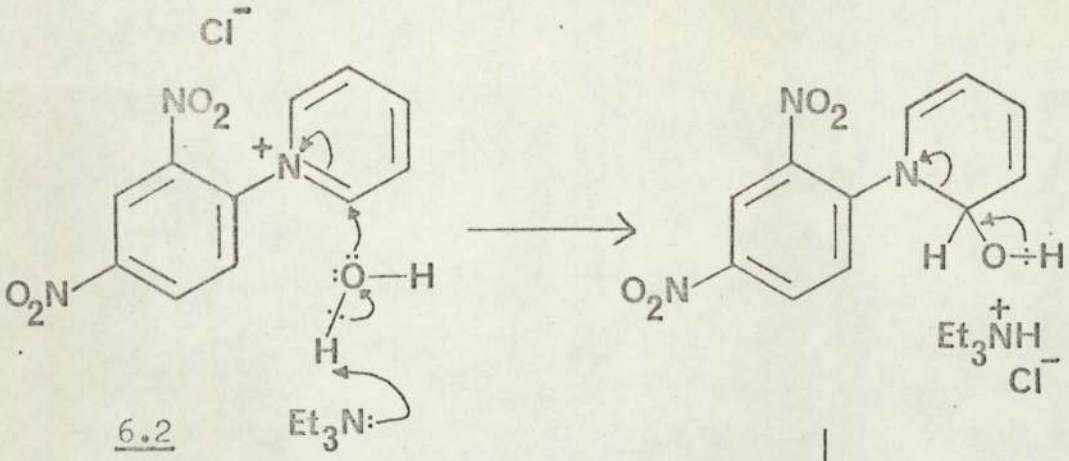
Pyridine reacts with 2,4-dinitrochlorobenzene under relatively mild conditions to form 2,4-dinitrophenylpyridinium chloride (6.2).[201] This "Zincke" salt is very sensitive to nucleophilic ring-opening. Treatment with triethylamine in aqueous ethanol leads to 5-(2,4-dinitroanilino)-2,4-pentadienal (6.3)(Scheme 23)[202]. The configuration of (6.3) was shown tentatively to be all-trans by ^{1}H n.m.r. spectral analysis (60MHz)[202] This was confirmed in the present work by a 100MHz spectrum (Figure 13). The coupling constants are recorded in Table 17 from the expanded spectrum and are typical for trans-polyenes.

Other nucleophiles that have been used in this ring-opening reaction (Scheme 23) include hydroxylamine[202] hydrazine[202] and aniline.[203] In these cases the Schiff bases of the pentadienal were isolated.

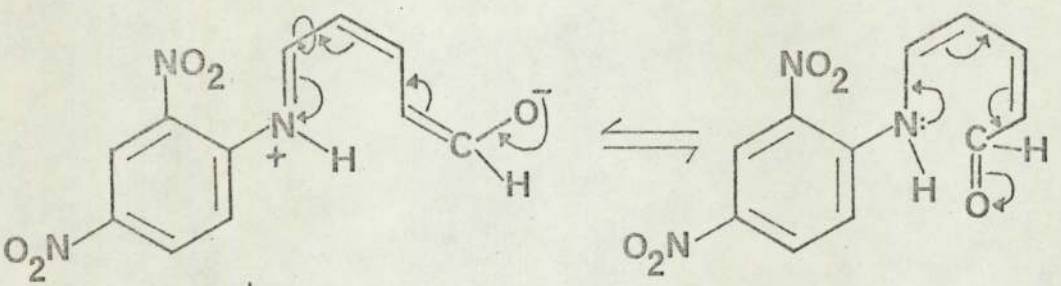
Reaction of 9-Chloroacridine with Aminopyridines.

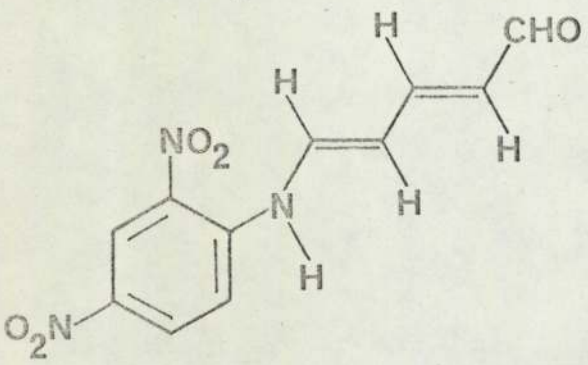
Reaction of aminopyridines with 9-chloroacridine resulted in formation of 9-(pyridylamino) acridine hydrochlorides (4.12 v & w). The products obtained were identical to those obtained by reaction of these aminopyridines with 9-azidoacridine hydrochloride.[181,204]

Proof for the structure of these acridines rested on their electronic absorption spectra, which were similar to the anilinoacridines shown in Table 7.



6.2

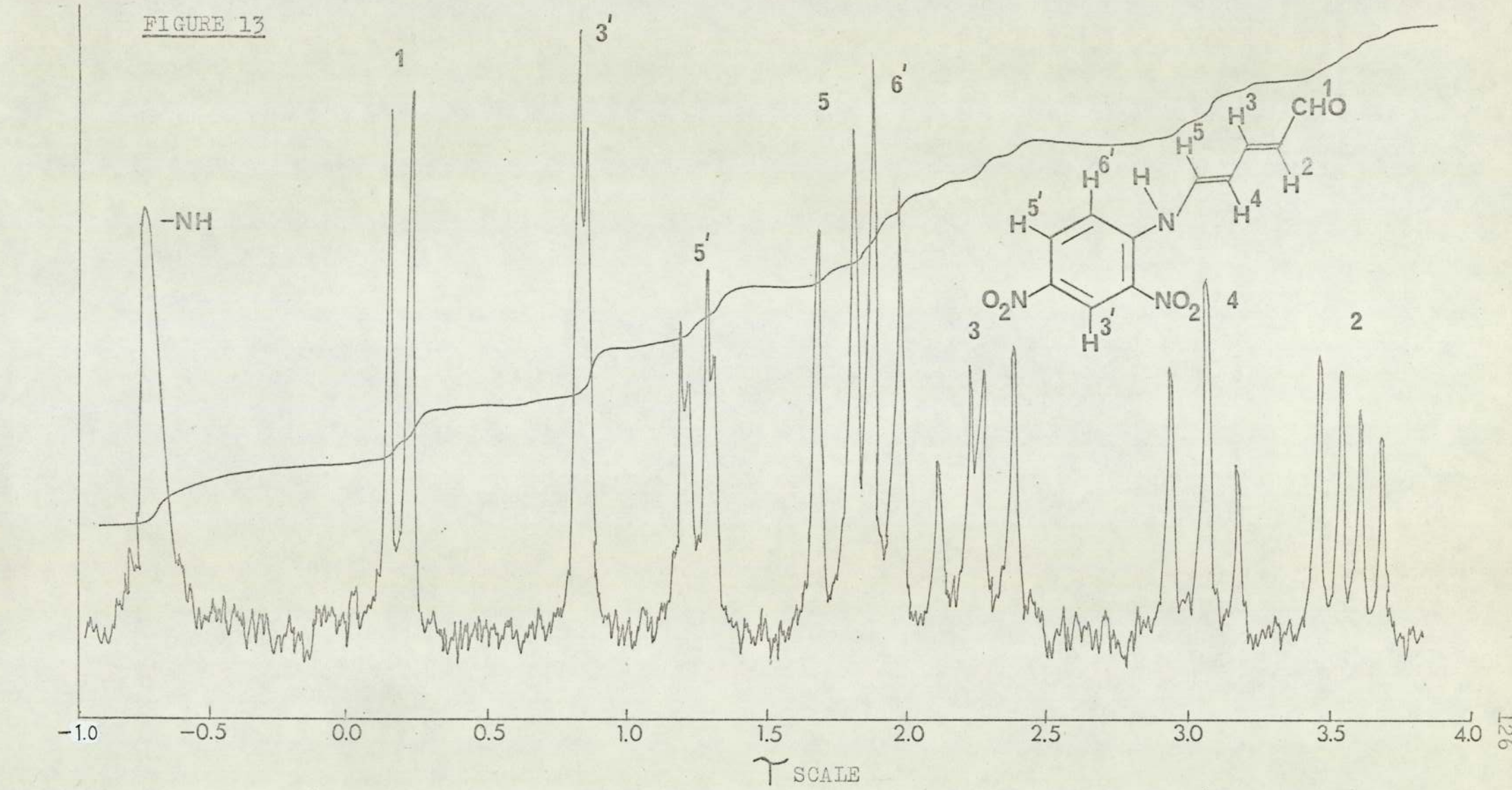




6.3

SCHEME 23

FIGURE 13



1H NMR of 5-(2,4-dinitroanilino)-2,4-pentadienal (6.3) Sweep width 500 MHz

TABLE 17

Table of Coupling Constants (J) for 5-(2,4-dinitro-anilino)-2,4-pentadienal (6.3) (Figure 13.)

COUPLING PROTONS	COUPLING CONSTANTS (J) Hz
1,2	7.8
2,3	15.3
3,4	12.9
4,5	12.9
3',5'	2.8
5',6'	9.8

The 2-pyridylamino derivative (4.12v) analysed as a monohydrate and its i.r. spectrum showed the characteristic broad water band at 3400-3500 cm^{-1} (See Figure 10).

The reason why the exocyclic nitrogen is always attacked in this reaction may be due to some steric interaction between the pyridine ring and the acridine nucleus.

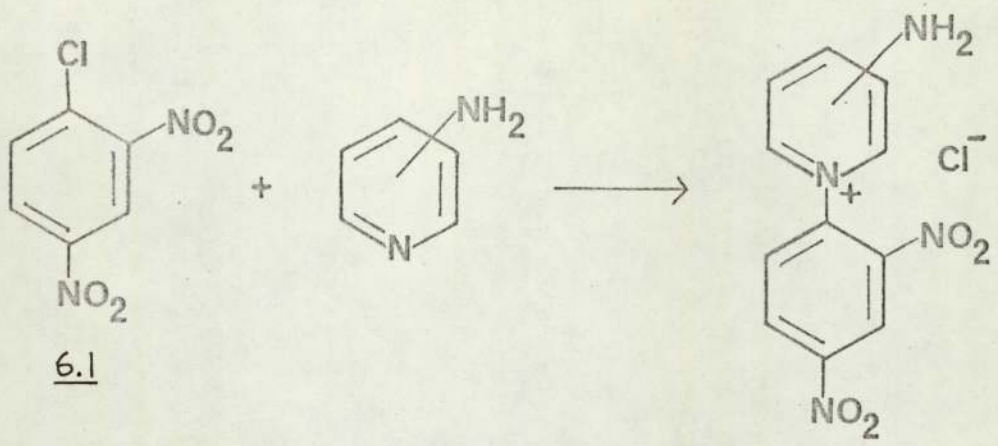
Reaction of 2,4-Dinitrochlorobenzene with 4- and 3-Aminopyridine.

These aminopyridines react with 2,4-dinitrochlorobenzene under relatively mild conditions to form 3- and 4- amino-1-(2,4-dinitrophenyl)pyridinium salts (6.4) (Scheme 24).[205] These quaternary Zincke salts were shown to contain ionic chloride, but were not titrable with alkali carbonates (i.e. they are not hydrochloride salts).

Reaction of the aminopyridine quaternary salts with triethylamine in alcohols leads to a completely different product from that obtained from the parent Zincke salt (6.2). In this case the attacking nucleophile is the alkoxide ion generated by abstraction of the alcoholic proton by triethylamine (Scheme 25). 2,4-Dinitrophenyl-ethers (6.5; R = Me or Et) are formed. Spectroscopic evidence for the structure of these ethers is recorded in the Experimental Section. No reaction occurred in the absence of the base catalyst.

Reaction of 2,4-Dinitrochlorobenzene with 2-Aminopyridine.

Reaction of 2-aminopyridine with 2,4-dinitrochlorobenzene failed to furnish a clean product. Many



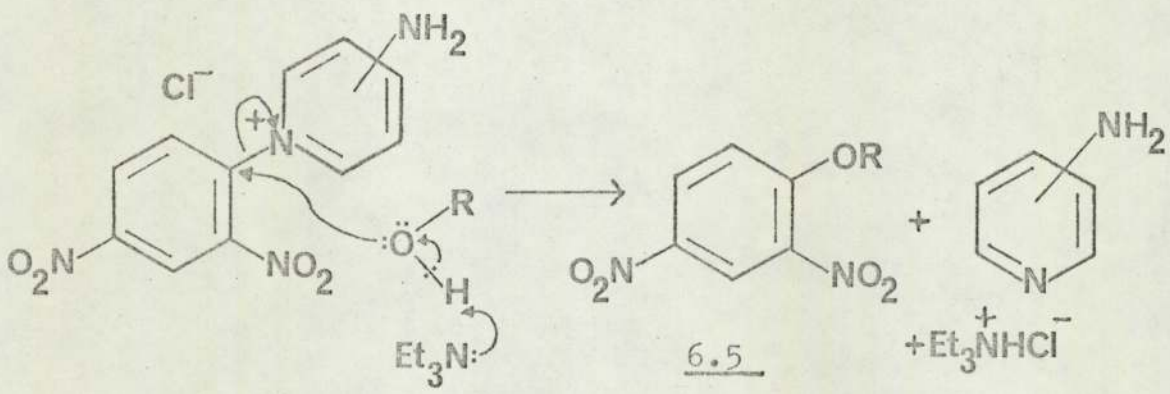
6.1

6.4

SCHEME 24

a = m-NH_2

b = p-NH_2



6.5

R = Me or Et.

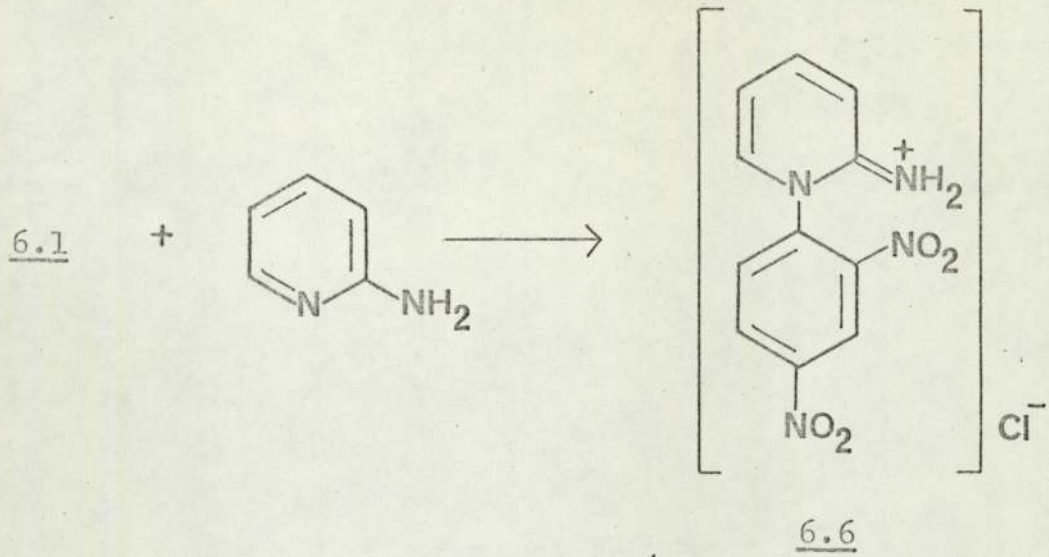
SCHEME 25

attempts were made to isolate the 2-amino Zincke salt by employing a range of solvents but all resulted in the formation of a black oil. Treatment of this black oil with base resulted in rapid formation of 2-(2,4-dinitroanilino)pyridine (6.7) which was characterised by its electronic absorption spectrum which was nearly identical to that of 1-anilino-2,4-dinitrobenzene. This derivative (6.7) is also formed by fusion of 2-amino-pyridine and 2,4-dinitrochlorobenzene at 100° [206] or by refluxing these solids in toluene or ethanol. Probably the initial reaction is formation of the imino hydro-chloride (6.6) which then rearranges to the pyridylamine (6.7) (Scheme 26) (Dimroth rearrangement). The failure to form a stable quaternary salt is probably due to steric factors.[205] The suggestion that a Dimroth rearrangement might be involved finds support in the isolation of a Dimroth intermediate (6.8) from the related reaction between picryl chloride and 2-amino-pyrazine.[207]

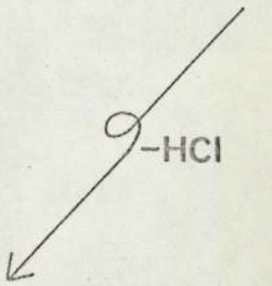
Attempted Displacement of the Pyridine Fragment from Zincke Salts.

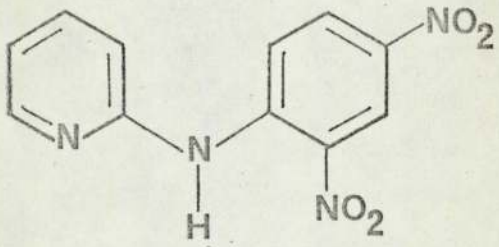
The nucleophilic replacement of the aminopyridines by the stronger alkoxide nucleophile (Scheme 25) led to the idea that the 2-iminopyridinium salt (6.6) might be synthesised by replacement of 3-aminopyridine or pyridine in the corresponding quaternary salts by 2-aminopyridine.

Reaction of the 3-aminoquaternary salt (6.4a) with 2-aminopyridine in ethanol resulted in formation of 1-ethoxy-2,4-dinitrobenzene (6.5; R = Et)(cf. Scheme 25)



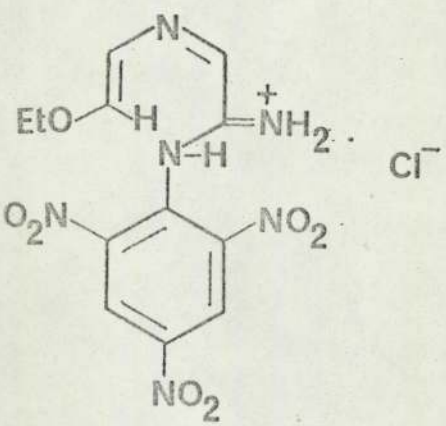
6.6





6.7

SCHEME 26



6.8

Presumably, 2-aminopyridine is acting in a similar manner to triethylamine.

Fusion of (6.4a) with 2-aminopyridine at 120° for 2 hours led to a very violent reaction taking place; a black oil formed. This oil was extracted into benzene. When the benzene solution was concentrated a yellow precipitate formed: this was the pyridylamine (6.7). The filtrate was shown to contain (t.l.c.) more of the pyridylamine, 2,4-dinitroaniline, 2,4-dinitrophenol and 2- and 3-aminopyridines. Chromatographic fractionation of the benzene extract on an alumina column allowed the isolation of these components which were characterised by spectroscopic analysis.

Surprisingly, reaction with the analogous 4-amino-quaternary salt (6.4b) resulted in a similar reaction, the only difference being that formation of the pyridylamine (6.7) was reduced.

In both these reactions it is likely that 2-aminopyridine is acting both as a reactant (giving eventually the pyridylamine) and also as a base, catalysing ring-opening (cf. triethylamine). In the latter case a pentadienal intermediate could be involved which might undergo hydrolysis to 2,4-dinitroaniline. It is likely that 2,4-dinitrophenol is formed by a reaction mechanism analogous to Scheme 25 but involving traces of water in the reactants.

However, the major product is the pyridylamine (6.7). It is likely that the mechanism for formation of this product involves formation of the 2-iminopyridinium

salt (6.6) as an intermediate which then undergoes a Dimroth rearrangement (Scheme 26).

A final attempt to synthesise the 2-iminopyridinium salt (6.6) was made by reacting the parent Zincke salt (6.2) with 2-aminopyridine in boiling ethanol. 2,4-Dinitroaniline was the only isolated product, formed probably by hydrolysis of an intermediate pentadienal (6.3). 3-Aminopyridine reacted similarly.

Treatment of the parent Zincke salt (6.2) with 4-aminopyridine in cold ethanol resulted in a quantative formation of the pentadienal (6.3).

Attempted Arylation of Adenine.

a. With 2,4-dinitrochlorobenzene.

Examination of the arylation of adenine proved difficult because of the insolubility of adenine in most organic solvents. Attempts to arylate adenine as a suspension in ethanol, acetonitrile and acetone all resulted in a quantitive return of unchanged adenine. Equally, refluxing in water or 50% ethanol resulted in a quantitive recovery of adenine. With 2,4-dinitrochlorobenzene in hot dimethylacetamide, adenine gave a deep red solution from which a low yield of an orange solid precipitated. All attempts to purify this high-melting solid failed. The only facts known on this compound are:

1. Nitro absorptions are present (1530 and 1335 cm^{-1}) in the i.r. spectrum.
2. ^1H n.m.r. spectrum in DMSO- d_6 shows two dinitrophenyl fragments.

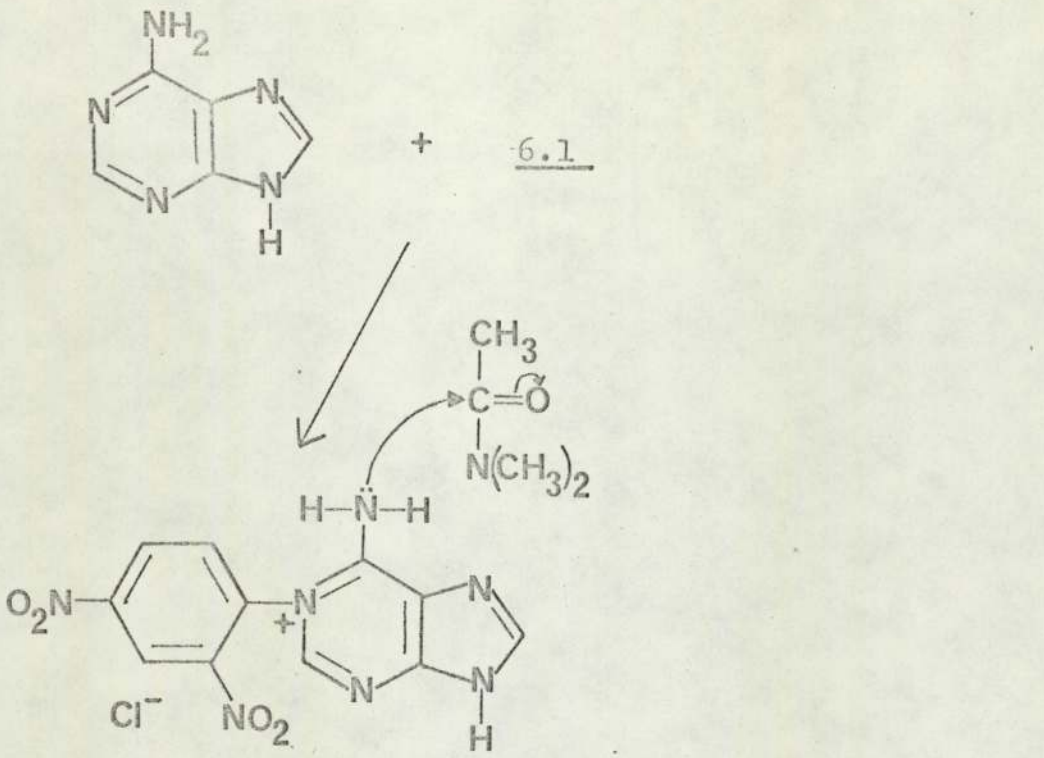
Since a mass spectrum of this solid could not be

obtained (poor volatility), a convincing structure could not be assigned to it. It is possibly a bis-2,4-dinitrophenyl derivative of adenine.

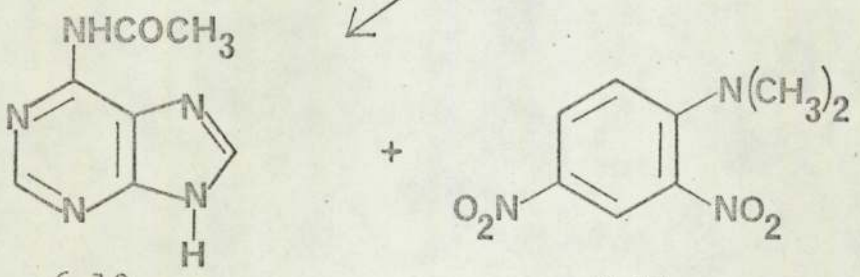
Fractional preciptitation from the mother liquors afforded samples of the following compounds: 6-acetamidopurine (6.10); N,N-dimethyl-2,4-dinitroaniline (6.11); and dimethylamine hydrochloride, which were characterised by spectroscopic comparisons (i.r.) with authentic samples.

Since both adenine and 2,4-dinitrochlorobenzene are stable in hot dimethylacetamide it is likely that the initial reaction is arylation of N(1) of adenine, this site being the most basic centre [46] (Scheme 27). Once formed this salt could react with dimethylacetamide. The disposition of substituents in the tetrahedral intermediate (6.9) postulated in this mechanism is such that the acylated nitrogen is ortho to the arylated heteroatom. In this situation it would be expected that an intramolecular reaction would be greatly facilitated. (Scheme 27). On the other hand an intermolecular reaction must be involved in the reaction of the 4-aminoquaternary salt (6.4b) with dimethylacetamide to form N,N-dimethyl-2,4-dinitroaniline (6.11) and 4-aminopyridine as the major products (Scheme 28).

The observation that the adenine arylation and decomposition (Scheme 27) is complete in 1 hour, whereas the 4-aminoquaternary salt (6.4b) decomposition requires 24 hours for completion adds further support to different mechanisms being involved.



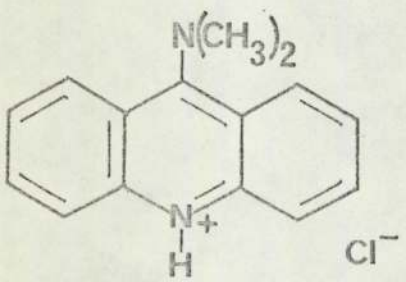
6.9



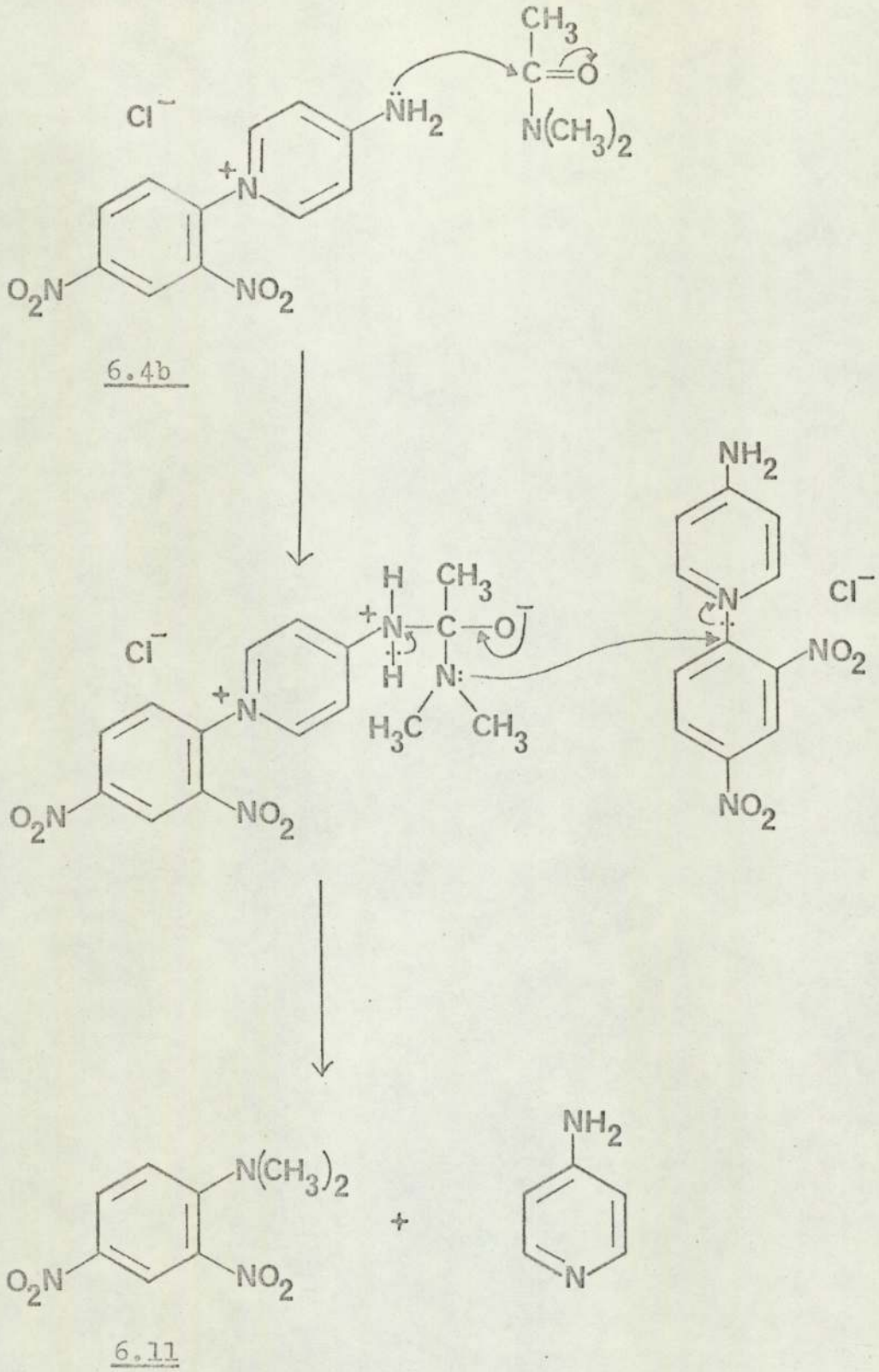
6.10

6.11

SCHEME 27.



6.12



6.4b

6.11

SCHEME 28

The failure to isolate a stable arylated derivative of adenine in this work is puzzling. The governing factor may be pH since earlier workers[199,200] arylated adenine at slightly alkaline pH (suitable for a Dimroth rearrangement) whereas in this work the pH was 5 - 6.

b. With 9-Chloroacridine.

A similar reaction was carried out using 9-chloroacridine. In this case no initial precipitation occurred. On cooling a precipitate of 6-acetamidopurine (6.10) was formed. Removal of the acetamidopurine and treatment of the filtrate with ether resulted in the formation of a brown oil. T.l.c. examination of this oil showed it to contain two components. One, with a blue fluoresence under u.v. light, was shown to be acridone (also isolated from the reaction); another, with a green fluoresence was possibly 9-dimethylaminoacridine hydrochloride (6.12) which has been reported to have a green fluorescence.[171] The formation of this product (6.12) is not surprising assuming an analogous mechanism to that outlined in Scheme 27.

CHAPTER 7.

CHAPTER 7.

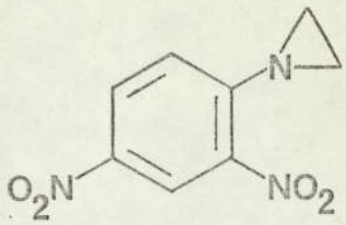
Search for Ortho-Interactions in Tumour-inhibitory 2,4-Dinitrophenylaziridines.

2,4-Dinitrophenylaziridine (7.1a)[208] is one of a number of ethyleneimines with cytotoxic activity.[64] The 5-carboxamide derivative (7.1b) discovered by Ross and his colleagues at the Chester Beatty Institute[209] (and designated CB1954) shows remarkable toxicity towards the transplantable Walker 256 tumour, but is less active or inactive against other tumours.[68,210-212]

Recent evidence points to CB1954 acting *in vivo* as an alkylating agent,[69, 213] and since its biochemical effects in ascites tumour cells appear identical to those of the mustard Melphalan[214] it has been speculatively suggested that it could be metabolised in susceptible cells to the difunctional agent (7.2).[215]

Other forms of covalent reactivity are possible. Bio-nucleophilic substitution of the activated nucleus could lead to displacement of cytotoxic ethyleneimine in cells; alternatively, substitution of a nitro group or even nucleophilic addition to form a Meisenheimer σ-complex could, if they occurred subsequent to alkylation of a biologically-significant macromolecule by the aziridine fragment, imply cross-linking capability for CB1954.

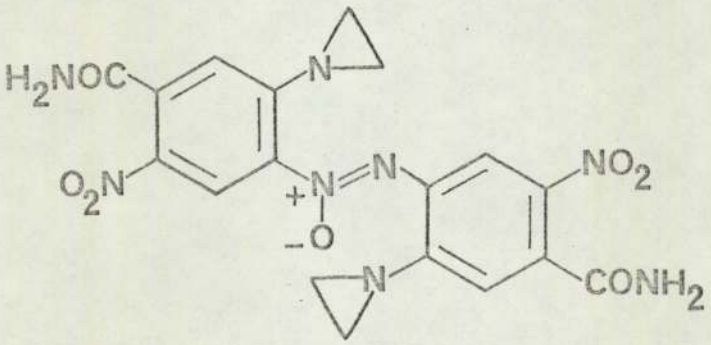
Another possible mechanism for the activity of CB1954 is that it may act as an alkylating anti-metabolite in purine biosynthesis.[69,210,215] In support of this view, these workers showed that 4-amino-



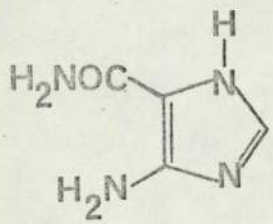
7.1

a: R=H

b: R=CONH$_2$

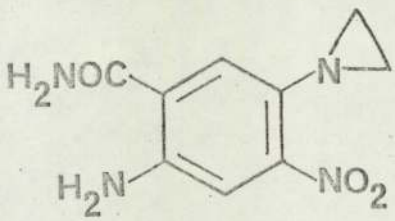


7.2



7.3

7.1b —metabolism→



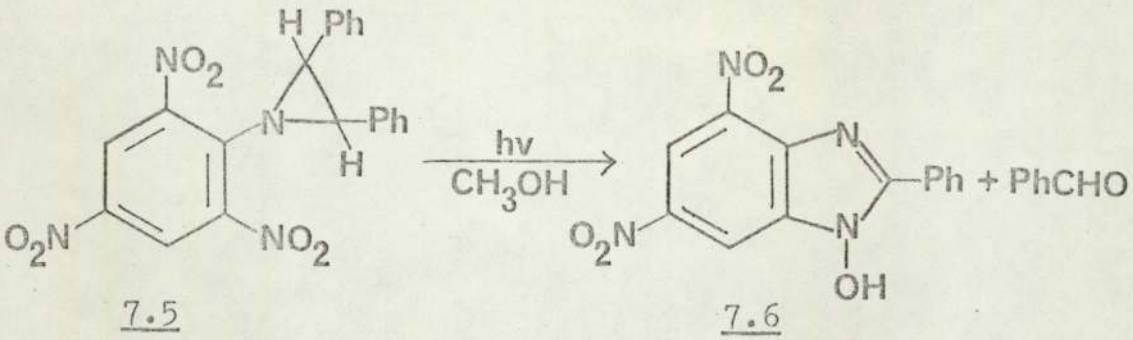
7.4

-imidazole-5-carboxamide (7.3) can completely protect the tumour from the cytotoxic effects of CB1954. The implication is that reduction of the nitro group ortho to the amido group occurs in vivo and the anthranilamide (7.4) formed binds to a cellular receptor from which it can be displaced by other o-aminoamides.[69]

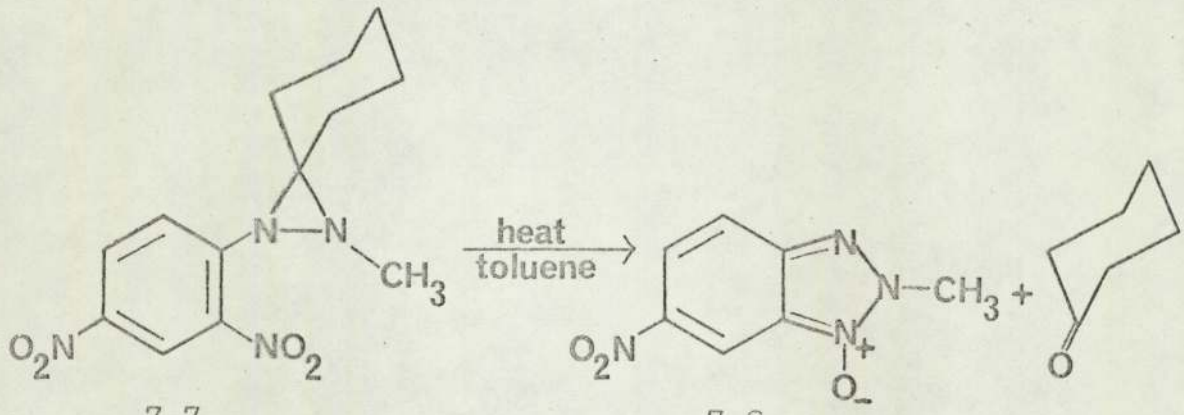
However, a more intriguing possibility is that the dinitrophenylaziridines could be bio-activated by an ortho-nitro interaction.[216,217] There are several reasons to encourage this line of investigation:

1. The o-nitro substituent in 7.1a and b is essential for activity: it cannot be replaced by other electron-attracting groups.[210] The p-nitro group is not essential for activity.
2. Precedents for this type of reaction have been reported (Scheme 29). For example, the photochemical transformation of N-(2,4,6-trinitrophenyl)--2,3-diphenylaziridine (7.5) to the hydroxybenzimidazole (7.6) has been described.[218] More recently it has been reported that the diazirine (7.7) is smoothly transformed to the benzotriazole N-oxide (7.8) in high yield in toluene.[219] The chemistry of the closely related o-nitro-t-anilines (7.9) has been extensively reviewed.[220] These compounds readily form benzimidazole derivatives (7.10).
3. Perhaps significantly 2-substituted benzimidazoles (7.11) and bisbenzimidazoles (7.12) exhibit anti-viral activity which presumably involves interaction at the nucleic acid level.[221-224]

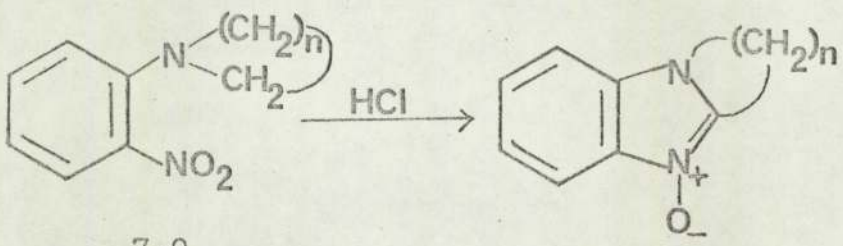
SCHEME 29



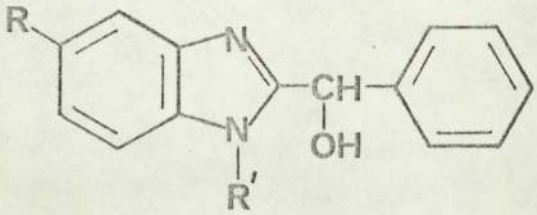
7.5 7.6



7.7 7.8



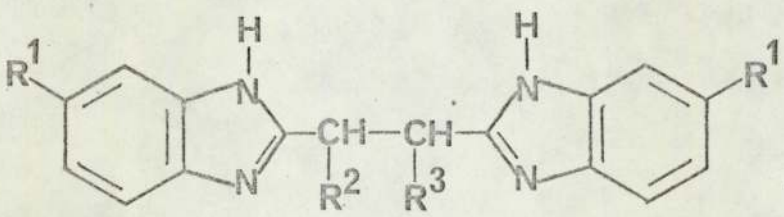
7.9 7.10



7.11

a: R = H, R' = Alkyl

b: R = Cl, R' = H



7.12

R^1 = Alkoxy: R^2 and R^3 = H, OH or OAc

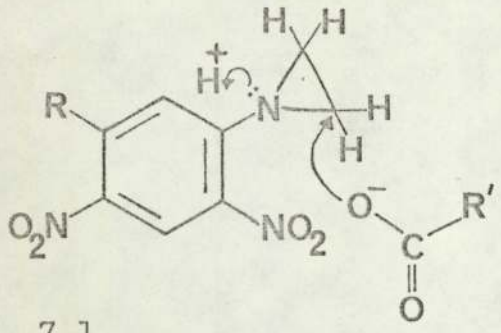
Of the two possibilities for substitutent interactions, the aziridino-nitro or carboxamido-nitro, the former appears more likely.

Reactions with Acids.

Previous work [225] has shown that the aziridines (7.1a and b) with a range of organic acids in boiling toluene afford the esters (7.13) in high yield (Scheme 30). The spectroscopic characteristics of these esters have already been documented.[225] The product from aziridine (7.1a) and toluene-p-sulphonic acid had spectroscopic properties in accord with structure (7.14a).[226] Hydrolysis of the acetate (7.13, R'=CH_3) in boiling 2 N-hydrochloric acid gave the expected 2,4-dinitro-1-(2-hydroxyethylamino) benzene (7.14b).

In the non-polar solvent toluene, ring-opening by the acid anion may involve the non-protonated weakly basic aziridine. The -M nitro substituents would stabilise the negative charge developing on the aziridino N atom in the SN2 transition state. Similar aziridines with electron-attracting N-substituents have been described as activated aziridines.[64]

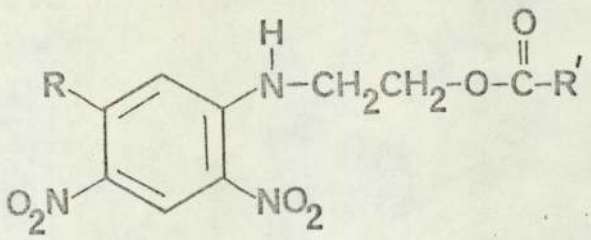
When the aziridine (7.1a) was heated with carboxylic acids alone, the same series of esters (7.13) formed.[226] However, with the strongly acidic chloroacetic acids a by-product was isolated. In 98% formic acid this product, formed in an exothermic reaction, accounted for 65% of the yield, the remainder being the formate (7.13,R'=H). This by-product, which was only soluble in dimethylformamide and dimethylsulphoxide was tentatively



7.1

a: R=H

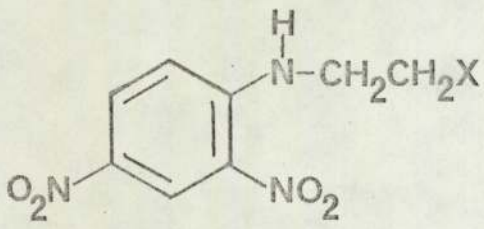
b: R=CONH$_2$



7.13

R′= alkyl or H

SCHEME 30



7.14

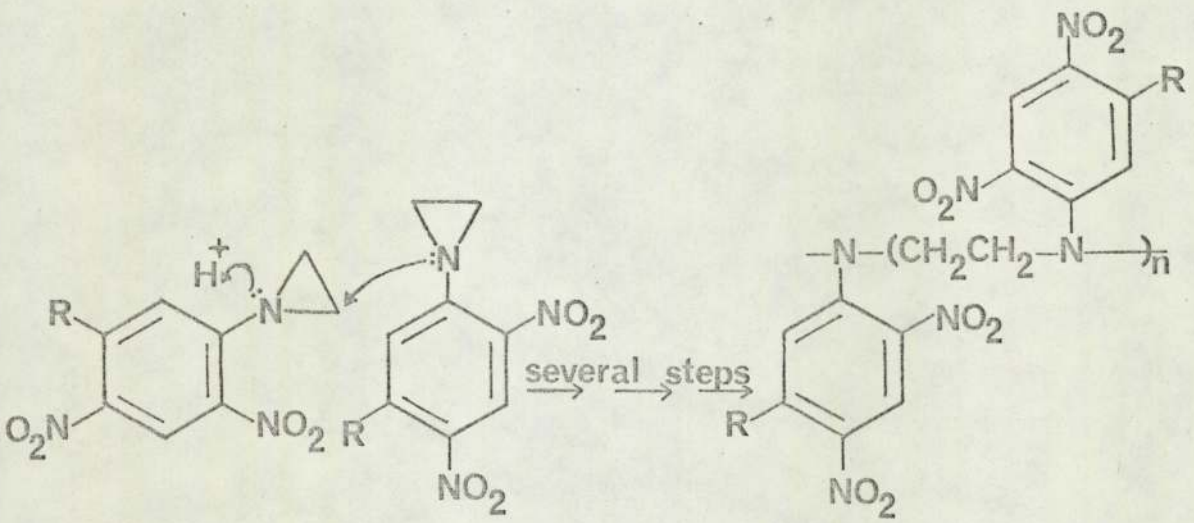
a: X= O-Tosyl

b: X=OH

c: X=Cl

d: X=I

e: X=Br



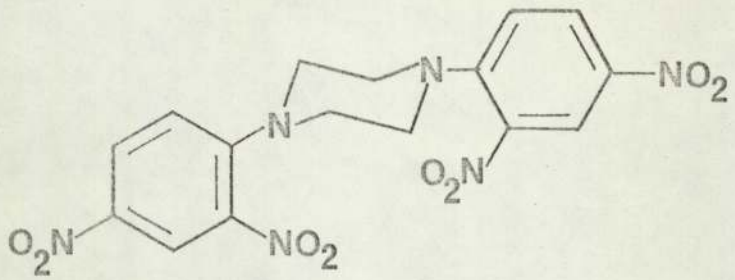
7.1 7.15

SCHEME 31

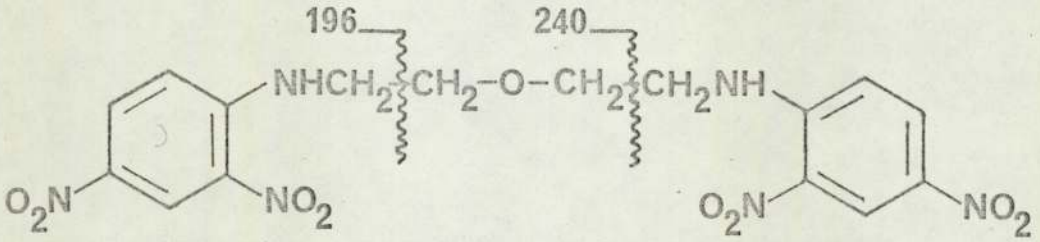
assigned the polymer structure (7.15; R=H) (Scheme 31) because its i.r. spectrum showed no NH absorptions and it differed from the piperazine dimer (7.16). However, branched or crown polymers are equally possible. CB1954 reacts similarly with 98% formic acid [225] to yield polymer (7.15; R= $CONH_2$).

It is significant that only the strong acids, trichloroacetic (pKa 0.66) dichloroacetic (1.25), chloroacetic (2.84) and formic acid (3.75) yield polymers and these must partially protonate the aziridine allowing unprotonated aziridine to compete with the nucleophilic acid anions (Scheme 31). The balance between polymer and ester is determined by such factors as the pKa of the aziridine substrate and the reacting acids, the nucleophilicity of the acid anions and the polarity of the solvent. This sensitivity was illustrated by the behaviour of 2,4-dinitroaziridinobenzene towards mineral acids. In 2N-hydrochloric acid the product was exclusively the 2-chloroethylamine (7.14c), whereas in 2N-sulphuric acid the product was the polymer (7.15; R=H). The differences can be attributed to the relative nucleophilic strengths of the chloride ion (strong) and the hydrogen sulphate ion (weak). Interestingly, N-(2,4-dinitrophenyl) pyrrolidine and its larger ring homologues cyclise to benzimidazole N-oxides in hydrochloric acid.[227] CB1954 (7.1b) has been reported to form the appropriate 2-chloroethylamine with dry hydrogen chloride in acetone.[210]

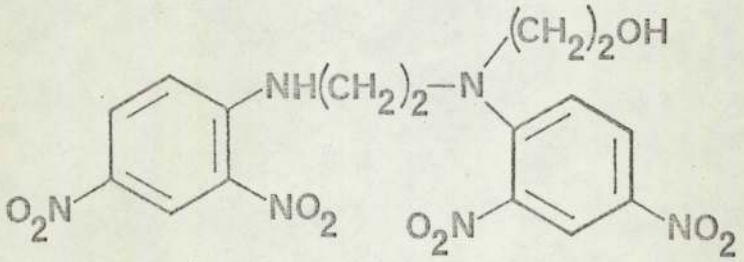
In an attempt to suppress the competing ring-opening reactions and encourage an ortho-nitro inter-



7.16



7.17



7.18

action, the aziridine (7.1a) was boiled in anhydrous xylene containing excess vacuum-dried acidic alumina as the acid catalyst. The yellow product (76%) was tentatively assigned structure (7.17) on the following evidence. The product analysed correctly for $C_{16}H_{16}N_6O_9$, and, although the mass spectrum did not show a molecular ion at m/e 436 corresponding to this formula, this was not unexpected (see later). Instead the spectrum showed a base peak at m/e 196, indicative of a ring-opened derivative and a major ion at m/e 240, both formed by cleavage of the C-C bond adjacent to heteroatoms. The alternative structure (7.18) was discarded because no ion at m/e 31 (characteristic of primary alcohols) was observed in the mass spectrum.

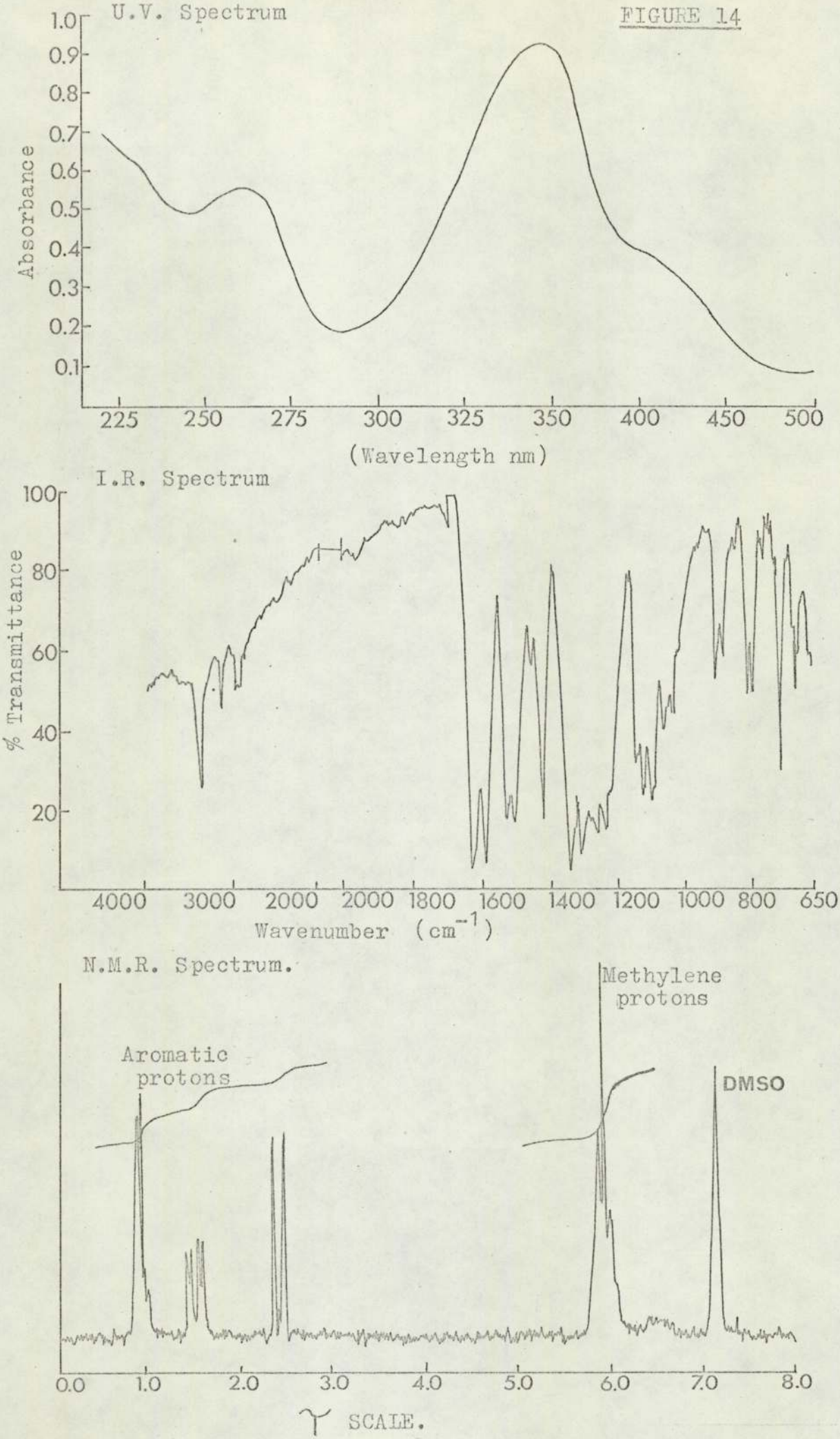
The i.r. spectrum (Figure 14) showed ν NH at 3390 cm^{-1} and νC-O at 1140 cm^{-1}, and the electronic absorption spectrum (Figure 14) showed a λ-max at 345 nm (typical for ring-opened derivatives).[225] The H^1 n.m.r. spectrum (Figure 14) showed the methylene protons as an overlapping multiplet indicating that the flanking substituents are of comparable electronegativity. The spectrum integrated correctly for structure (7.17).

Appropriate control experiments established that the 2-hydroxyethylamine (7.14b) was not an intermediate in the formation of ether (7.17) since the hydroxyethylamine neither reacted alone nor did it react with aziridine (7.1a) in the presence of alumina.

Surprisingly, the same ether was obtained from the aziridine and basic alumina in xylene or acetone and

FIGURE 14

Spectra of 2,2'-Bis-(2,4-dinitroanilino)diethyl ether (7.17)



it is possible that the ether is formed directly from two molecules of aziridine and water tenaciously absorbed to the catalytic surface.

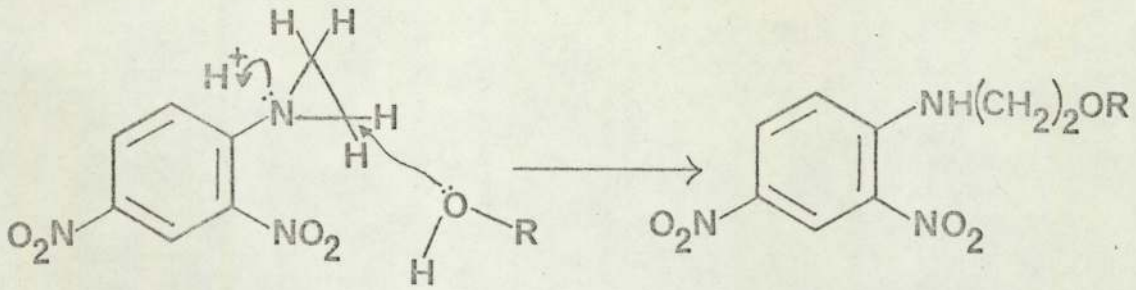
Reactions with Alcohols.

The aziridine (7.1a) was recovered unchanged after prolonged boiling in methanol or ethanol, but with traces of sulphuric acid,[225] or acidic alumina,[226] the ring-opened ethers (7.19) were formed (Scheme 32) (*cf.* the behaviour of the activated aziridine, 1-ethoxycarbonyl-aziridine).[228] With hydrochloric acid both aziridines (7.1a and b) in methanol or ethanol afforded the 2-chloroethylamines following intervention of the chloride ion in the ring-opening step.

Alcohols by themselves are presumably too weakly nucleophilic to bring about ring-opening of the aziridine ring but with catalytic amounts of mineral acids or acidic alumina, ring-opening occurs involving the labile ethyleneimmonium ion.

When toluene-*p*-sulphonic acid was employed as the acid catalyst in the reaction of the aziridine and alcohols, mixtures of the ethers (7.19) and (7.14a) were isolated. The strongly nucleophilic toluene-*p*-sulphonate ion evidently competes with the alcohol in the ring-opening step.

Reaction of the aziridine (7.1a) with methyl or ethyl iodide in methanol also afforded a high yield of the methyl ether (7.19a); with the alkyl iodides in ethanol a mixture of the ether (7.19b) and 2-iodoethyl-amine (7.14d) was isolated, the yield of the ether increasing at the expense of the 2-iodoethylamine



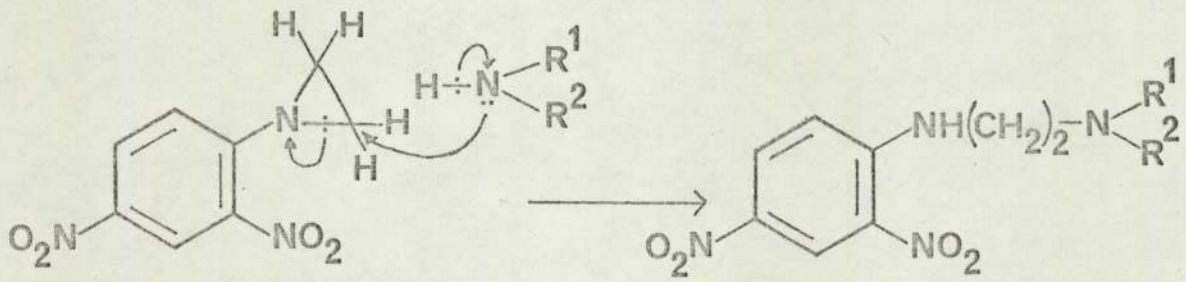
7.1a

7.19

a: R=Me

b: R=Et

SCHEME 32.



7.1a

7.20

SCHEME 33.

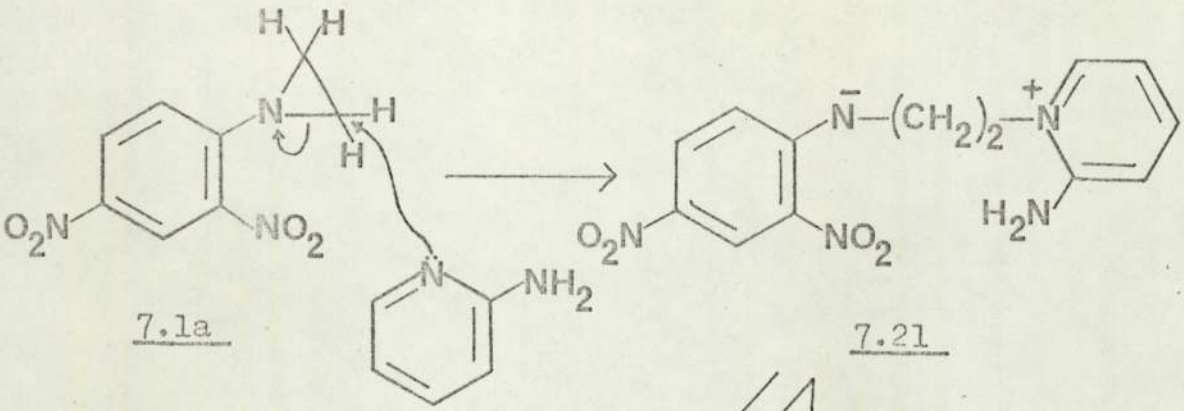
derivative as reaction time was prolonged. In no cases were "exchange" ethers detected (i.e. the methyl ether from the reaction of the aziridine with methyl iodide in ethanol or the ethyl ether from reaction with ethyl iodide in methanol). There is little doubt that the 2-iodoethylamine formed as intermediate in these reactions, arises from the hydroiodic acid generated in situ from the alkyl iodides and alcohols, since the aziridine (7.1a) is stable in both alkyl iodides alone, and in the presence of potassium carbonate the reaction is suppressed.

Reaction of the aziridine (7.1a) with excess benzyl bromide in refluxing xylene afforded the 2-bromoethylamine (7.14c) in substantial yield probably from hydrobromic acid liberated in the decomposition of benzyl bromide.

Similar ring-opening reactions have been reported for other aziridines. 1-Methylaziridine on reaction with halogenated nitrobenzenes forms a ring-opened tertiary amine.[229] Other activated aziridines have been recently reported to ring-open in the presence of thiols.[230,231]

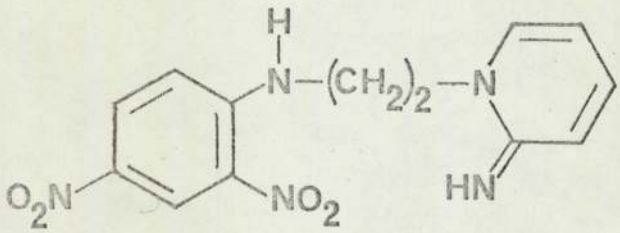
Reactions with Bases.

Bases have been frequently used to promote cyclisation reactions of ortho-nitroarenes.[216,217] Aziridine (7.1a) reacted with various amines at 100° to afford the ethylenediamines (7.20) (Scheme 33);[225] no catalyst was required. Indeed, when ammonium or toluene-p-sulphonates were employed as the base components no reaction occurred. In pyridine, aziridine (7.1a) reacted

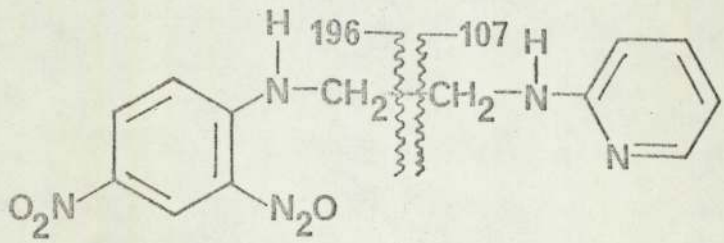


7.1a

7.21



7.22



7.23

SCHEME 34

exothermically to yield a black solid, which on repeated crystallisation from dimethylformamide-ethanol gave a product identical (i.r.) to polymer (7.15; R=H). A cleaner product was obtained by employing xylene or toluene as solvent. Aziridine (7.1a) and 3- or 4-aminopyridines, with or without toluene as solvent, similarly afforded polymer (7.15; R = H).

2-Aminopyridine and aziridine (7.1a) gave two isomeric products in variable yield in addition to the polymer (Scheme 34). The high-melting isomer was identified as the diamine (7.23) because its electronic absorption spectrum ($\underline{\lambda}$-max 350 nm; log ε 4.19) was similar to that of the anilino analogue ($\underline{\lambda}$-max 349 nm; log ε 4.15) and its mass spectrum showed appropriate ions at m/e 196 and 107. The unstable low-melting isomer, which was not obtained pure, was probably the imine (7.22) as opposed to the betaine (7.21), since it rearranged to the high-melting isomer in aqueous sodium hydroxide. Similar Dimroth rearrangements are a feature of the chemistry of 1-alkyl-2(1H)-iminopyridines.[232]

Triethylamine catalysed ring-opening of aziridine (7.1a) to the methyl ether (7.19a) in methanol although the process was less efficient than acid catalysis. No displacement of the aziridine occurred although substitution of the basic fragment is the major pathway in the reactions of related 1-(2,4-dinitrophenyl) pyridinium salts (cf. Scheme 25 Chapter 6). The aziridine (7.1a) does however yield sodium 2,4-dinitrophenoxide when boiled in aqueous 2N-sodium hydroxide.

Photolysis of the Aziridine (7.1a) in Methanol.

Photolysis of the aziridine (7.1a) in methanol led to a puzzling result. No change in the electronic absorption spectrum of the solution was detected during 48 hours illumination by an unfiltered 100 W medium pressure arc in an Hanovia photochemical reactor using a quartz filter. When the lamp was extinguished a progressive bathochromic shift from 327 to 350 nm was observed and after 48 hours the methyl ether (7.19a) was isolated in 25% yield. However, no reaction occurred when control solutions of the aziridine in methanol were kept in the dark for the same period. Equally, exposure to ambient light without prior irradiation in the u.v. region also did not afford the ether. A possible explanation could be that ring-opening occurs when sufficient formic acid has been generated in the oxidative u.v. photolysis of the methanol solvent. Acid-catalysed ring-opening could then yield the methyl ether (7.19a). Support for this hypothesis came from an independent experiment in which the methyl ether (7.19a) was formed in 65% yield in methanol containing 0.1% formic acid. The lack of ring-opening under illumination is as yet not understood.

The possibility that a reversible reaction (7.1a $\rightleftharpoons$ 7.19a) might be involved with irradiation favouring the cyclic species (7.1a) was discounted, since the ether (7.19a) was indepently shown to be photo-stable both in methanol alone, or methanol containing 0.1% formic acid. Although nucleophilic ring-opening of the aziridine by

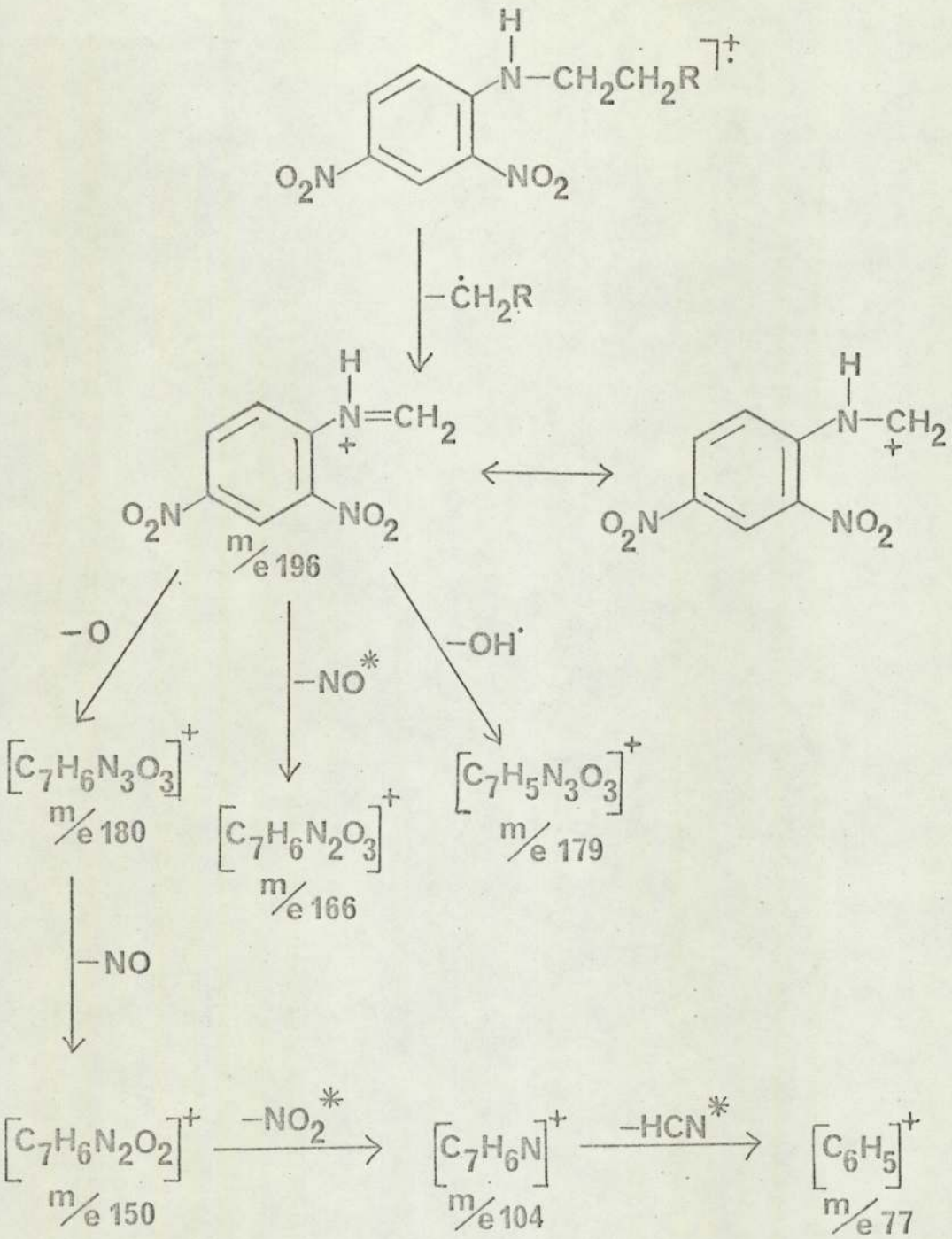
methanol is undoubtedly involved, the precise details of the mechanism remain obscure.

Spectral Properties of Aziridines and their Ring-Opened Derivatives.

The i.r. spectrum of the aziridine (7.1a) is similar to that of N-(2,4-dinitrophenyl) piperidine and shows nitro absorptions at 1338 and 1515 cm^{-1}. The ring-opened derivatives all have sharp NH absorptions in the region 3320-3390 cm^{-1} with other absorptions characteristic of the appropriate substituent. The polymers (7.15) have broad featureless absorptions.

The mass spectrum of the aziridine (7.1a) showed an abundant molecular ion, but no M-17 or M-18 ions were evident indicating no ion-impact promoted o-nitro interactions were occuring.[233,234] The mass spectrum of the aziridine (7.1a) was notable for the large number of abundant fragment ions at low m/e values - the identification and fragmentation routes of these ions has as yet not been determined. The ring-opened derivatives show abundant ions at m/e 196 but the molecular ions are of low abundance or absent. The ion at m/e 196 is the base peak except in cases where alternative fragmentations afford more favourable stabilised ions (i.e. 7.23; m/e 107). Such is this dominating fragmentation process of the C-C bond adjacent to the amino group that the mass spectra of the ring-opened derivatives are nearly identical in the region below m/e 196: the major fragmentations are outlined in Scheme 35. The constitutions of the ions in Scheme 35 have been corroborated by high resolution mass measurements.

SCHEME 35



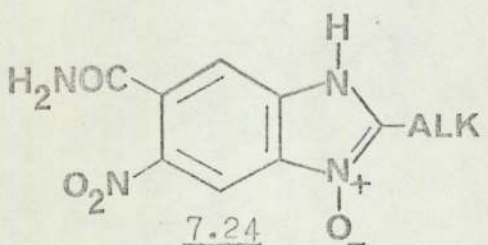
* Denotes metastable ion

The H^1 n.m.r. spectrum of aziridine (7.1a) shows an AMX pattern for the aromatic protons with chemical shifts and coupling constants similar to those of other 2,4-dinitrophenyl derivatives. Physical processes, which could lead to non-equivalence of the methylene protons - i.e. restricted rotation about the aziridine-aryl bond or slow inversion of the tertiary nitrogen - were sufficiently rapid on the n.m.r. time scale to result in the signal for these protons appearing as a sharp singlet (7.43 τ) at magnet temperature and -30°, with only slight broadening at -70°C. The methylene protons of other activated aziridines are similarly uninfluenced at temperatures above -70°. [235]

Conclusion

No chemical evidence for ortho-nitro interactions was discovered, although it is possible that such reactions could be mediated by enzymes within susceptible cells. The work described in this chapter lends further support to the conclusions of the Chester Beatty workers regarding the alkylating ability of aziridines (7.1). It would be of interest, however, to prepare 2-alkyl-5-carboxamido-6-nitrobenzimidazole-1-oxides (7.24) by alternative routes and to examine their biological properties.

Most of the ring-opened derivatives prepared in this and other work[225] were screened for tumour-inhibitory activity against lymphoid leukaemia (L1210) in mice. None of the derivatives were active.



7.24

PART III

EXPERIMENTAL

CHAPTER 8.

PART 3.

CHAPTER 8.

Experimental

Notes - Unless otherwise stated.

1. All melting points (m.p.) are uncorrected.

2. "Ethanol" refers to 95% ethanol.

3. I.r. spectra were recorded on a Perkin-Elmer 257, a Perkin-Elmer 157 or a Pye Unicam S.P. 200 (as KBr discs).

4. U.v. spectra were recorded on a Perkin-Elmer 402, Pye Unicam S.P. 8000 or a Beckman Acta V.

5. 60 MHz ^{1}H n.m.r. spectra were recorded on a Perkin-Elmer R 14 (normal probe temperature 33.4°C) and 100 MHz on a Varian H-A100D) (normal probe temperature 36°C).

6. Mass spectra were recorded on an A.E.I. - M.S.9 spectrophotometer.

7. Photolyses were carried out with an unfiltered 100W medium-pressure arc in an Hanovia photochemical reactor using a quartz filter.

8. X-radiolyses were performed using a Pantak L.C. 300 ^{60}Co source.

9. γ-radiolyses were performed using a 5000 ci ^{60}Co source.

10. The pKa values of the anilinoacridines were calculated according to a modified spectroscopic

method of Albert and Serjeant.[191] The only modification was that the length of time between making up the alkaline solutions and recording the absorbance was kept constant (2 minutes).

The buffers used were Walpoles acetate buffer,[236] Phosphate buffer[237] and Borate Buffer,[237] at 0.01M strength.

The instrument used was Pye Unicam S.P. 500 (single beam instrument). The analytical wavelength was confirmed by the S.P. 500 to be 265 nm. for every acridine.

Synthesis of 2,4-Diamino-6-(2-azidoaryl)-s-triazines.

2,4-Diamino-6-(2-aminophenyl)-s-triazine.

2-Aminobenzonitrile (16g), cyanoguanidine (11.2g) and potassium hydroxide (2.4g) were boiled (4 hours) in 2-ethoxyethanol (60ml). The cooled mixture precipitated as a gum on being poured into iced water (1 litre) but rapidly solidified to give an off-white precipitate of the triazine (60%), which crystallised from water as needles, m.p. 179 - 181° (Lit.,[238] 167-168°).

2,4-Diamino-6-(2-azidophenyl)-s-triazine (3.1a)

2,4-Diamino-6-(2-aminophenyl)-s-triazine (5g) was mixed with 2N-hydrochloric acid (150 ml) at 0°C and diazotised by gradual addition of a solution of sodium nitrite (2g) in water (10 ml). Sodium azide (2.5g) was added slowly with stirring to the ice-cold solution of diazonium chloride. The mixture was stirred for a further 1.5 hours at 0°C. The precipitate was collected, suspended in water, and neutralised with sodium acetate to yield 2,4-diamino-6-(2-azidophenyl)-s-triazine (90%) which recrystallised from aqueous ethanol as colourless plates of the hydrate which decomposes at 170° to a solid which finally melts at 310-313° (Lit.,[156] m.p. 170° eff).

ν max 2126 and 2080 cm^{-1} (N_3)

2,4-Diamino-6-(2-azidophenyl)-s-triazine monohydrochloride.

2,4-Diamino-6-(2-azidophenyl)-s-triazine (4.0g) was stirred with 4N-hydrochloric acid (100 ml) at room temperature for 2 hours. A precipitate of the hydrochloride (82%) formed and was collected and

recrystallised from water, as light-brown needles, m.p. 204-205° (eff).

Found: C,40.7;H,3.4;N,42.6.

$C_9H_9N_8Cl$ requires C,40.9;H,3.4;N,42.5%.

2-Amino-5-bromobenzonitrile.

2-Aminobenzonitrile (21g) in acetic acid (250 ml) at 15° was treated with bromine (29.4g) at a rate sufficient to keep the temperature from rising. The product, 2-amino-5-bromobenzonitrile, in the form of a hydrobromide was collected, basified with concentrated aqueous ammonia and the 2-amino-5-bromobenzonitrile (80%) crystallised from carbon tetrachloride as buff flakes, m.p. 93-94° (Lit.[239] 96-97°)

2,4-Diamino-6-(2-amino-5-bromophenyl)-s-triazine.

2-Amino-5-bromobenzonitrile (2.85g), cyanoguanidine (1.12g) and potassium hydroxide (0.12g) were boiled in 2-ethoxyethanol (10g) for 4 hours. The cooled mixture was poured into excess ice-water and the precipitate collected, washed to neutral pH, and dried. Recrystallisation from ethanol yielded golden platelets of 2,4-diamino-6-(2-amino-5-bromophenyl)-s-triazine (60%), m.p. 282-283° (Lit.[156] 281-282°).

2,4-Diamino-6-(2-azido-5-bromophenyl)-s-triazine.

2,4-Diamino-6-(2-amino-5-bromophenyl)-s-triazine (5.6g) was suspended in 2N-hydrochloric acid (120 ml) at 0°C and diazotised by addition of a solution of sodium nitrite (1.6g) in water (7 ml). Sodium azide (2g) was slowly added to the stirred ice-cold diazonium chloride solution and the resultant mixture was stirred at 0°C

for 2 hours. The yellow precipitate was collected, suspended in water and basified with 2N-ammonium hydroxide to afford 2,4-diamino-6-(2-azido-5-bromophenyl)-s-triazine (85%) which recrystallised from aqueous dimethylformanide as cream needles, m.p. indefinite above 180° (Lit.,[156] 160°) with effervescence and resolidification. The residue melted at 305-310°.

ν max 2135 and 2095 cm^{-1} (N_3)

2,4-Diamino-s-triazino [1,2-b] indazoles (3.5)

1. 2,4-Diamino-6-(2-azidophenyl)-s-triazine hydrochloride (0.01g) was thermolysed in a sealed evacuated pyrex glass ampoule at 200° for 30 minutes. 2,4-Diamino-s-triazino [1,2-b] indazole (90%) was collected on vacuum sublimation (300°/5mm) as a yellow amorphous solid m.p. 313° (Lit.,[41] 313°).

2. 2,4-Diamino-6-(2-azidophenyl)-s-triazine hydrochloride (0.0048g) was dissolved in water (25 ml). This solution was photolysed at room temperature for one hour in a 1 cm quartz cuvette. Electronic absorption spectroscopy showed that the final product was 2,4-diamino-s-triazino [1,2-b] indazole (80%). Similar treatment of the free base (3.1a) also afforded the indazole.[156]

3. 2,4-Diamino-6-(2-azidophenyl)-s-triazine (0.005g) was placed in a sealed evacuated ampoule. γ-radiolysis resulted in conversion to 2,4-diamino-s-triazino-[1,2-b] indazole (55%) at a dose of 387 Mrads (387 x 10^6 rads).

4. γ-radiolysis of 2,4-diamino-6-(2-azidophenyl)-s-triazine hydrochloride (0.002g) in water (10 ml)

resulted in formation of at least six products. These have not been identified. No indazole was present.

5. A solution of 2,4-diamino-6-(2-azido-5-bromophenyl)-s-triazine (0.005g) in absolute ethanol was photolysed for 1 hour. The final product was 9-bromo-2,4-diamino-s-triazino-[1,2-b] indazole (100%). Examined by electron absorption spectroscopy.

Fricke Dosimetry.[150]

A solution of ferrous sulphate (10^{-3}M), sodium chloride (10^{-3}M) in 0.8N sulphuric acid was prepared. The water used to dilute the concentrated acid was distilled over potassium permanganate and then triply distilled. The Fricke solution (5ml) was placed in (a) the sealed ampoule and (b) the radiation cell. (Figure 8.)

Each sample was irradiated for eight different timed intervals. The times chosen are such that the normal range of Fricke doses are covered, i.e. 10,000 - 40,000 rads.

The times for the X-ray sources were 30 mins, 25 mins, 20 mins, 15 mins, 10 mins, 3 mins, and 1 min, and for the γ-source, 60 secs, 50 secs, 45 secs, 40 secs, 30 secs, 25 secs, 20 secs and 10 secs. These times are calculated on the known approximate dosimetry of these sources.

At the end of each exposure the absorbance of the irradiated solution was measured at 304 nm and 20°C using unirradiated solution as reference. From these absorbance values the absorbed dose can be calculated from the Fricke equation:

$$Dm \text{ (in rads)} = 4.42 \times 10^5 \left[1-0.007(t-20)\right] A/G$$

(See Chapter 3, page69)

From this equation the dose rate at 1.25 cm was calculated. The average of the eight samples was used as the dose rate for both sources. The solids under study (Chapter 3) were irradiated at various times in exactly the same position as the Fricke solutions. Knowing these times the approximate dose absorbed by the solids was calculated.

Synthesis of Substituted 9-Anilinoacridine Hydrochlorides (4.12).

General Method.

9-Chloroacridine[240] (2.14g) and the appropriate aniline derivative (1 mol.equiv.) were refluxed in anhydrous methanol (100 ml) for 3 hours. The solution was allowed to cool to 25° and was then filtered. On addition of excess ether a precipitate of the anilinoacridine hydrochloride formed which was collected and dried in a vacuum dessicator. The yields in all cases were 90-100%. The compounds were all recrystallised from ethanol-ether.

4.12

(a) 9-Anilinoacridine hydrochloride, m.p.295-296° (Lit.,[241] m.p. 296°), as yellow rosettes.

(b) 9-(4-Aminoanilino)acridine hydrochloride, m.p. 315-317° (Lit.,[186] 315-317°), as light brown flakes.

(c) 9-(2-Hydroxyanilino)acridine hydrochloride, m.p. 342° dec (Lit.,[186] 342° dec), as yellow-brown flakes.

(d) 9-(4-Hydroxyanilino)acridine hydrochloride, m.p. 350-352° (Lit.[186] 351°), as buff flakes.

(e) 9-(4-Acetoxyanilino)acridine hydrochloride, m.p. 276-278°, as bright-orange flakes.

Found: C,68.3; H,4.9; N,7.7

$C_{21}H_{17}N_2O_2Cl$ requires C,69.1; H,4.7; N,7.7%

ν max 1750 cm^{-1} (C = O)

τ 7.36 (s; - $C\underline{H}_3$).

(f) 9-(2-Methoxyanilino)acridine hydrochloride, m.p. 287-288°, as orange-yellow flakes.

Found: C,71.1; H,5.0; N,8.3.

$C_{20}H_{17}N_2OCl$ requires C,71.1; H,5.0; N,8.3%

νmax 1250 cm^{-1} (O-C ether)

τ 6.16 (s; -$OC\underline{H}_3$)

(g) 9-(4-Methoxyanilino)acridine hydrochloride, m.p. 272-274° (Lit.[242] 274°), as bright orange flakes.

τ 6.2 (S; $OC\underline{H}_3$)

(h) 9-(4-Ethoxyanilino)acridine hydrochloride, m.p. 252-254 (Lit.[242] 255°), as medium brown flakes.

τ 5.5 (q; -$C\underline{H}_2$-) and 8.36 (t; - $C\underline{H}_3$)

(i) 9-(4-n-Propoxyanilino)acridine hydrochloride, m.p. 274-275°, as yellow-orange flakes.

Found: C,72.6; H,5.9; N,7.7

$C_{22}H_{21}N_2$ ClO requires C,72.4; H,5.8; N,7.7%

τ 6.12 (t; -$OC\underline{H}_2$-CH_2-), 8.33 (sex; -$C\underline{H}_2CH_3$) and 9.08 (t; -CH_2-$C\underline{H}_3$)

(j) 9-(4-n-Butoxyanilino)acridine hydrochloride, m.p. 260° (decomp), as orange flakes.

Found: C,73.1; H,6.2; N,7.5.

$C_{23}H_{23}N_2ClO$ requires C,72.9; H,6.1; N,7.4%

(k) 9-(4-n-Pentoxyanilino)acridine hydrochloride, m.p. 259-260 (decomp), as orange flakes.

Found: C,71.8; H,6.4; N,7.2

$C_{24}H_{25}N_2ClO\frac{1}{2}H_2O$ requires C,71.7; H,6.5; N,7.0%

(l) 9-(4-Phenoxyanilino)acridine hydrochloride, m.p. 250-251°, as orange flakes.

Found: C,73.5; H,5.0; N,6.9

$C_{25}H_{19}N_2OCl$ requires C,74.0; H,4.9; N,7.0%.

ν_{max} 1270 cm^{-1} (o-Ph).

(m) 9-(o-Toluidino)acridine hydrochloride, m.p. 282-283°, as a green-yellow crystalline solid.

Found: C,71.4; H,5.4; N,8.4.

$C_{20}H_{17}N_2ClH_2O$ requires C,71.0; H,5.6; N,8.4%

7.32 (s; $-C\underline{H}_3$)

(n) 9-(m-Toluidino)acridine hydrochloride, m.p. 265-266°, as yellow flakes.

Found: C,74.0; H,5.6; N,8.5.

$C_{20}H_{17}N_2Cl$ requires C,75.0; H,5.4; N,8.7%.

τ 7.28 (s; $-C\underline{H}_3$)

(o) 9-(p-Toluidino)acridine hydrochloride, m.p. 256-257°, as shiny yellow flakes.

Found: C,70.9; H,5.4; N,8.3.

$C_{20}H_{17}N_2Cl.H_2O$ requires C,71.0; H,5.6; N,8.4%.

τ 7.32 (s; $-C\underline{H}_3$)

(p) 9-(4-Ethylanilino)acridine hydrochloride,
m.p. 266-268°, as a green crystalline solid.

Found: C,74.0; H,5.6; N,7.9.
$C_{21}H_{19}N_2Cl.\frac{1}{2}H_2O$ requires C,73.7; H,5.7; N,8.1%.

τ 6.8 (q; $-C\underline{H}_2-$) and 8.12 (t; $-C\underline{H}_3$)

(q) 9-(4-n-Butylanilino)acridine hydrochloride,
m.p. 247-248°, as orange microneedles.

Found: C,72.7; H,6.4; N,7.5.
$C_{23}H_{23}N_2Cl.H_2O$ requires C,72.5; H,6.5; N,7.4%.

τ 6.82 (t; $-C\underline{H}_2(CH_2)_{\overline{2}}$), 7.7 - 7.82 (m; $-CH_2-(C\underline{H}_2)_{\overline{2}}$)
and 8.6 (t; - $C\underline{H}_3$)

(r) 9-(4-Chloroanilino)acridine hydrochloride,
m.p. 238-239° (Lit.,[243] 268°), as a yellow crystalline solid.

(t) 9-(4-Fluoroanilino)acridine hydrochloride,
m.p. 220-222°, as green plates.

Found: C,68.4; H,4.3; N,8.6.
$C_{19}H_{14}N_2F.\frac{1}{2}H_2O$ requires C,68.2; H,4.5; N,8.4%.

(u) 9-(4-Bromoanilino)acridine hydrochloride,
m.p. 230-232° (Lit.,[243] 230-231°), as light brown flakes.

(v) 9-(2-Aminopyridyl)acridine hydrochloride,
m.p. 260° (Lit.,[181] 260°), as orange-yellow rosettes.

(w) 9-(3-Aminopyridyl)acridine hydrochloride, m.p.
288-290° (Lit.,[181] 288-290°) as orange-red rosettes.

Hydrolysis of 9-(4-Acetoxyanilino)acridine hydrochloride

9-(4-Acetoxyanilino)acridine hydrochloride
(o.5g) was dissolved in warm water (100 ml). On

addition of excess 2N-ammonium hydroxide solution a brown-white solid precipitated. This solid was shown to be 9-(4-hydroxyanilino)acridine; the i.r. spectrum was identical to the free base obtained from structure 4.12d.

Synthesis of 9-Azidoacridine Hydrochloride (4.6a).

9-Azidoacridine[244] (0.5g) was dispersed in dry ether and was dissolved by addition of absolute ethanol. Dry hydrogen chloride gas was passed (10 min) through the solution. The hydrochloride precipitated out as yellow microneedles (80%). Crystallisation from ethanol ether afforded pale-yellow rosettes, m.p. 270° (decomp) [(Lit.,[204] 270° (decomp)].

Synthesis of 9-Azido-10-methylacridinium methosulphate (4.6b).

9-Azidoacridine (2.5g) in anhydrous benzene (100 ml) was allowed to stand for 4 days with freshly distilled dimethylsulphate (3.0 ml) (in the dark). The methosulphate (1.0g) was deposited as a brown crystalline solid, m.p. 120° (decomp) (Lit.,[204] 120° decomp).

Synthesis of 9-Chloro-10-methylacridinium methosulphate (4.14).

9-Chloroacridine (2.2g) in anhydrous benzene (50 ml) was allowed to stand for 14 days with freshly distilled dimethylsulphate (3.0 ml). The methosulphate was deposited as yellow-green needles (1.43g) and was dried in a vacuum desiccator. It had m.p. 128° (eff).

Found: C,52.8; H,4.0; N,4.0.

$C_{15}H_{14}NClSO_4$ requires C,53.0; H,4.1; N,4.1%.

Kinetic Experiments for the Acridinium Salts.

The kinetic experiments were performed according to the spectroscopic method of Laidler.[245] A plot of absorbance against time was drawn; analytical wavelength 355 nm. In the case of the azido salt (4.6b) this was an ordinary plot ie. zero order, whereas with the chloro salt (4.14) it was a log plot ie. 1st order. In these experiments the rate of formation of the final product (N-methylacridone) was determined.

Radiolysis of 9-Azidoacridine.

9-Azidoacridine (0.005g) was placed in a sealed evacuated ampoule. On γ-radiolysis the yellow solid became purple. The radiolysed product was extracted into ethanol and the resultant solution examined by electronic absorption spectroscopy at 355 n.m. From a calibration graph it was observed that at a dose of 200 Mrads (200×10^6 rads) about 90% of the azide was undecomposed.

Synthesis of Quaternised Pyridines.

2,4-Dinitrophenylpyridinium chloride (62)

Pyridine (4g) and 2,4-dinitrochlorobenzene (10.4g) were refluxed in acetone (20 ml) for 15 hours. The solid which formed was recrystallised from acetone to give white needles of the quaternary compound, m.p. 198-200° (Lit.,[246] 190-191°) (Yield 99%).

4-Amino-1-(2,4-dinitrophenyl)pyridinium chloride (64b)

4-Aminopyridine (1.88g) and 2,4-dinitrochlorobenzene (4.04g) were dissolved in a minimum amount of acetone. The mixture was stirred for 12 hours. A pink amorphous solid formed which, on recrystallisation

from ethanol, afforded bright red plates of the quaternary salt, m.p. 262-263° (Lit.[205] 263°) (yield 99%).

3-Amino-1-(2,4-dinitrophenyl)pyridinium chloride (6.4a)

3-Aminopyridine (1.88g) and 2,4-dinitrochlorobenzene (4.04g) were dissolved in a minimum of acetone. The solution was stirred for 2 hours, and a bright yellow precipitate of the quaternary salt was isolated. Recrystallisation from ethanol afforded bright yellow needles, m.p. 246-248° (Lit.[205] 246°) of the pyridinium chloride.

Synthesis of 2-(2,4-Dinitrophenylamino)pyridine (6.7)

(1). 2,4-Dinitrochlorobenzene (8g) was added gradually to 2-aminopyridine (3.6g) dissolved in toluene (50 ml) and refluxed with stirring for 1 hour. The solution was filtered and concentrated to 20 ml. On cooling the aminopyridine separated as brown flakes, m.p. 154-155° (Lit.[206] 156-157°) (yield 90%).

(2) 2,4-Dinitrochlorobenzene (8g) and 2-aminopyridine (3.6g) were refluxed in ethanol (250 ml) for 12 hours. The solution was concentrated to 100 ml and left overnight at room temperature. A brown crystalline solid (8.2g) was collected, and recrystallised from ethanol as brown shiny flakes, m.p. 148-150°. It was identical (i.r.) to the aforementioned sample.

(3) 2,4-Dinitrochlorobenzene (8.0g) and 2-aminopyridine (3.6g) were dissolved in a minimum of (a) acetone, (b) dichloromethane, (c) acetonitrile and (d) ethanol. Solutions a - c were kept at room temperature

for 24 hours. On removing the solvent under vacuum a black oil remained. Similarly, after 10 minutes refluxing in ethanol, followed by removal of the solvent, the same black oil formed. On refluxing (2 hours) this oil in 2N- sodium hydroxide, quantitive amounts of the aminopyridine formed.

Reactions of the Quaternised Pyridines.

(1) To an ice cold solution of 2,4-dinitrophenyl pyridinium chloride (2.8g) in ethanol (100 ml) was added dropwise a solution of triethylamine (1.0g) with stirring. The reaction mixture was stirred at room temperature overnight, during which time the colourless mixture gradually changed to red, giving an orange precipitate. Recrystallisation from acetone afforded red crystals (45%) of 5-(2,4-dinitroanilino)-2,4-pentadienal (6.3), m.p. 174-175° (Lit.,[202] 175°).

(2) 4-Amino-1-(2,4-dinitrophenyl)pyridinium chloride (0.5g) was dissolved in ethanol (40.ml). To this solution triethylamine (3 ml) was added. On refluxing for 6 hours, the red solution became light brown. On cooling to room temperature, followed by treatment with excess water, a yellow precipitate of 1-ethoxy-2,4-dinitro-benzene (6.5) was formed. Recrystallisation from benzene-petrol (60-80°) afforded golden flakes of the ether, m.p. 82-83° (Lit.,[247] 84°) (yield 0.25g).

τ 5.6 (q; $-OC\underline{H}_2-$) and 8.4 (t; $-C\underline{H}_3$)

(3) 3-Amino-1-(2,4-dinitrophenyl)pyridinium chloride (0.5g) was dissolved in ethanol (40 ml). To this solution triethylamine (4 ml) was added and the

solution was refluxed for 24 hours. This solution was evaporated to dryness. The oil formed was triturated with water and the organic products were extracted into benzene 2 x 50 ml). Chromatographic fractionation on an acid alumina column resulted in isolation of a yellow solid from the first eluate. Crystallisation from benzene-petrol (60-80°) afforded 1-ethoxy-2,4-dinitrobenzene (0.1g) Similar treatment of 3-amino-1-(2,4-dinitrophenyl)pyridinium chloride, using methanol as solvent afforded 1-methoxy-2,4-dinitrobenzene, m.p. 88-89°,(Lit.[248] 88-89°)as fine cream needles (yield 0.3g).

τ 5.9 (S; $-OCH_3$)

Equally, when 2-aminopyridine was used as the base catalyst under identical experimental conditions again the ethers formed in almost identical yields.

(4) 3-Amino-1-(2,4-dinitrophenyl)pyridinium chloride (1.5g) and 2-aminopyridine (0.5g) were fused together at 120° (as an intimate mixture) for 2 hours. An extremely violent reaction took place with evolvement of copious yellow fumes. The residual black oil was extracted into benzene (5 x 50 ml). This extract was concentrated to 10ml and the yellow precipitate collected was shown to be 2-(2,4 dinitrophenylamino)pyridine (yield 0.4g). The i.r. spectrum was identical to an authentic sample. The filtrate was then passed through an acidic alumina column. The first band was evaporated to dryness and more of the pyridine (0.1g) was isolated. The second band was concentrated with petrol (60-80°) to afford a yellow precipitate. This solid was shown to be 2,4-dinitroaniline (0.4g) (i.r. spectrum identical to authentic material). The remaining fraction from the column was examined by t.l.c. and shown to consist of 2,4-dinitrophenol and unreacted quaternary salt.

The non-benzene soluble portion of the oil was extracted into ethanol. T.l.c. examination showed this extract to contain unreacted quaternary salt, 3- and 2-aminopyridine and the pyridylamine.

(5) 4-Amino-1-(2,4-dinitrophenyl)-pyridinium chloride (1.5g) and 2-aminopyridine (0.5g) were fused together as an intimate mixture at 200° for 2 hours. A black oil resulted and was treated as in the preceding experiment. The solids isolated were the pyridylamine (0.4g), 2,4-dinitroaniline (0.2g) and 2,4-dinitrophenol (0.1g). T.l.c. of the ethanol extract showed the presence of the unreacted quaternary salt, 4-aminopyridine, the pyridylamine, and 2,4-dinitroaniline.

(6) 2,4-Dinitrophenylpyridinium chloride (1.4g) and 4-aminopyridine (0.45g) were dissolved in ethanol (20 ml). After shaking for ten minutes at room temperature, a flocculent red precipitate was formed. On recrystallisation from ethanol red rosettes of 5-(2,4-dinitroanilino)-2,4,-pentadienol resulted (yield 1.1g).

(7) 2,4-Dinitrophenylpyridinium chloride (1.4g) and 3-aminopyridine (0.45g) were refluxed for 1.5 hours in ethanol (20 ml). On cooling to room temperature a yellow preciptiate formed which was shown (i.r.) to be 2,4-dinitroaniline (yield 1.4g).

(8) Similar treatment of 2,4-dinitrophenylpyridinium chloride with 2-aminopyridine also yielded 2,4-dinitroaniline (1.5g).

Attempted Arylation of Adenine.

1. Preliminary experiments.

Synthesis of 1-dimethylamino-2,4-dinitrobenzene (6.11)

2,4-Dinitrochlorobenzene (2.02g) and dimethylamine

(26% $^{W}/_{V}$ water) (5.0 ml) were refluxed in ethanol (50 ml) with triethylamine (1.1g) for 3 hours. On cooling yellow needles of the dimethylamine formed. Recrystallisation from ethanol afforded shiny yellow needles, m.p. 80 - 81° (Lit.[249] 86-87°) (yield 99%).

Synthesis of 6-acetamidopurine (6.10)

Adenine (1.35g) was refluxed in acetic anhydride (8.0 ml) for 3 hours. On cooling a white solid formed, which was filtered, washed with ether and dried. This solid was purified by vacuum sublimation (260°/5 min) to give white crystals of 6-acetamidopurine, m.p. 260° (sublimes) (Lit.[250] 260° sublimes). The i.r. spectrum was identical to a published spectrum[250] of authentic material.

2. Reaction of adenine with 2,4-dinitrochlorobenzene.

Adenine (1.35g) and 2,4-dinitrochlorbenzene (2.2g) were refluxed in purified dimethylacetamine (50 ml) for 2 hours. Whilst hot, an orange precipitate was formed. All attempts to obtain a pure sample of this solid failed, and its structure is unknown.

The filtrate was left for 2-3 days below 10°C. An off-white crystalline preciptate formed. Vacuum sublimation of this solid afforded 6-acetamidopurine (0.7g) m.p. 260° (sublimes). The i.r. spectrum of this solid was identical to authentic material.

The remaining solution was then treated with ether. A brown oil was deposited which ultimately solidified to form a hygroscopic solid (0.5g). The i.r. spectrum of this solid was identical to authentic dimethylamine hydrochloride.

On removal of the ether, followed by dilution with water, a yellow precipitate (1.4g) was obtained. Recrystallisation from ethanol afforded shiny yellow plates of 1-dimethylamino-2,4-dinitrobenzene. (I.r. spectroscopy and mixed m.p.).

T.l.c. examination of the remaining aqueous dimethylacetamide showed the presence of 2,4-dinitrochlorobenzene and unreacted adenine.

3. Reaction of adenine with 9-chloroacridine.

Adenine (1.35g) and 9-chloroacridine (2.1g) were refluxed in redistilled dimethylacetamide (50 ml) for one hour. After 24 hours at 10°C a green-white precipitate formed (1.5g). This solid was shown to be 6-acetamidopurine (i.r. spectroscopy and mixed m.p.). Treatment of the filtrate with ether afforded a brown oil, which was extracted into ethanol. T.l.c. examination of this extract showed the presence of only two spots - acridine, and a compound with a green fluorescence. This latter spot is possibly 9-dimethylaminoacridine hydrochloride.[171]

No reactions occurred between adenine and 9-chloroacridine or 2,4-dinitrochlorobenzene in 50% ethanol or acetonitrile and adenine was quantitively recovered.

4. Stability of 4-Amino-1-(2,4-dinitrophenyl)pyridinium Chloride in Dimethylacetamide.

4-Amino-1-(2,4-dinitrophenylpyridinium chloride (1.0g) was refluxed in dimethylacetamide (20 ml) for 24 hours. The solution was diluted with water and then sequentially extracted into benzene and ethanol. The

benzene extract was concentrated to dryness. The off-white solid formed was crystallised from ethanol and was shown to be 2,4-dinitrophenol.

The ethanol extract was reduced to 10 ml and a bright yellow precipitate was formed. This solid was shown to be 1-dimethylamino-2,4-dinitrobenzene (0.3g) (i.r. identical to authentic material). T.l.c. of the ethanol solution indicated the presence of 4-aminopyridine and unreacted quaternary salt.

Chemical Properties of 2,4-Dinitrophenylaziridine.

2,4-Dinitrophenylaziridine was prepared from 2,4-dinitrochlorobenzene and ethyleneimine according to the literature procedure,[210] it melted at 99-100°. (Lit.,[210] m.p. 100°).

Reactions with Acids.

(a) Organic acids.

2-(2,4-Dinitroanilino)ethanol toluene-p-sulphonic acid ester (7.14a).

A solution of 2,4-dinitrophenylaziridine (2.09g) and toluene-p-sulphonic acid (1.1 mol.equiv.) in toluene (20 ml) was boiled for three hours. The evaporated solution afforded the yellow sulphonate (75%) which crystallised from toluene-light petroleum (60 - 80°) as yellow needles, m.p. 162-163° (Lit.,[225] m.p. 152°).

Similarly prepared was 2-(2,4-dinitroanilino)-ethanol formate (7.13, R=H) in 90% yield, m.p. 133-234°.

Found: C,42.5; H,3.6; N,16.6.

$C_9H_9N_3O_6$ requires C,42.4; H,3.5; N,16.5%.

2-(2,4-Dinitroanilino)ethanol acetate (7.13, R=CH_3)

2,4-Dinitrophenylaziridine (2.09g) was refluxed in glacial acetic acid (10 ml) for 30 minutes. Trituration of the cooled solution with water gave the yellow acetate (92%) which crystallised from aqueous ethanol as yellow needles m.p. 129-130° (Lit.[225] 129-130°).

Hydrolysis of the acetate in boiling 2N-hydrochloric acid (2 hours) afforded 2-(2,4-dinitroanilino) ethanol (7.14b) (95%), m.p. 85-86° as golden yellow flakes on crystallisation from aqueous methanol (Lit.[251] 90-92°).

Polymer of 2,4-Dinitrophenylaziridine (7.15a).

2,4-Dinitrophenylaziridine (1.0g) reacted vigorously when mixed with formic acid (98%; 10 ml). An orange solid deposited when the mixture was heated on a steam bath (30 minutes). An ethanol soluble fraction (0.15g) was identical to 2-(2,4-dinitroanilino)-ethanol formate. The ethanol insoluble polymer was purified by solution in dimethylforamide and reprecipitation by ethanol, and had m.p. 240° (varying according to rate of heating.)

Found: C,45.7; H,3.3; N,19.7.

$(C_8H_7N_3O_4)_n$ requires C,45.9; H,3.4; N,20.1%.

2,2'-Bis-(2,4-dinitroanilino)diethyl ether (7.17)

2,4-Dinitrophenylaziridine (1.0g) was boiled in xylene (40 ml) containing acidic alumina (B.D.H.-Brockman Grade 1) (10g) for 4 hours. The yellow-brown solution was filtered to remove alumina. A yellow

solid (0.2g) crystallised from the xylene. Extraction of the alumina with acetone and dilution with water gave more of the same solid (0.3g). Crystallisation from toluene afforded bright yellow microrosettes of the ether m.p. 178-179°.

Found: C,44.4; H,4.0; N,19.2.

$C_{16}H_{16}N_6O_9$ requires C,44.1; H,3.7; N,19.3%.

Also from 2,4-dinitrophenylaziridine in acetone with acid alumina (0.6g) or in xylene (0.5g) or acetone (0.6g) with basic alumina.

Treatment of 2-(2,4-dinitroanilino)ethanol under comparable conditions resulted in a quantitive return of unchanged starting material. Reaction of 2,4-dinitrophenylaziridine and 2-(2,4-dinitroanilino)-ethanol using identical conditions resulted in recovery of the ether (7.17) (50%) and unchanged 2-(2,4-dinitroanilino)ethanol.

(b) Mineral Acids.

1-(2-Chloroethylamino)-2,4-dinitrobenzene (7.14c)

2,4-Dinitrophenylaziridine (2.0g) in boiling 2N-hydrochloric acid (50 ml) (1 hour) deposited the chloroethylamine (2.2g) which crystallised from toluene-light petroleum (60-80) as yellow needles, m.p. 86-87° (Lit.[251] 87-88.5).

Reaction of the aziridine (7.1a) (2g) with boiling 2N-sulphuric acid (50 ml) (1 hour) afforded a brown solid which recrystallised from dimethylformamide-ethanol. The i.r. spectrum of this solid was identical to that of the polymer (7.15a).

1-(2-Bromoethylamino)-2,4-dinitrobenzene (7.14c)

2,4-Dinitrophenylaziridine (1.0g) was refluxed in xylene (10 ml) containing benzyl bromide (4 ml) for 24 hours. On vacuum distillation of the xylene, a brown oil was left. Chromatographic fractionation of a benzene solution of the oil on alumina gave a yellow band. Evaporation of the yellow band gave the bromoethylamine (55%). Crystallisation of the solid from ethanol afforded yellow microrosettes, m.p. 84-85° (Lit.,[210] 98°).

Reaction with Alcohols.

2-(2,4 Dinitroanilino)ethanol methyl ether (7.19a)

A solution of 2,4-dinitrophenylaziridine (1.0g) in boiling methanol (30 ml) containing 2 N-sulphuric acid (1 ml) deposited a yellow solid after 15 minutes. The methyl ether (78%) precipitated and was crystallised from methanol as yellow needles or prisms, m.p. 145-147° (Lit.,[225] 136-137°).

The same methyl ether was formed (80%) by employing acidic alumina (5g) as the acid catalyst. With 2 N-hydrochloric acid (2 ml) the product was 1-(2-chloroethylamino)-2,4-dinitrobenzene (7.14c) (60%). With toluene-p-sulphonic acid (1g) employed as catalyst the methyl ether (75%) and the toluene-p-sulphonate (7.14a) (15%) were isolated.

With methyl iodide (2 ml) in methanol, the methyl ether formed (80%); with ethyl iodide (2 ml) in methanol the yield of the methyl ether was 75%.

There was no reaction in methanol, or the alkyl iodides alone.

2-(2,4-Dinitroanilino)ethanol ethyl ether (7.19b)

2,4-Dinitrophenylaziridine (1g) was boiled in ethanol (20 ml) containing 2 N-sulphuric acid (1 ml) for 3 hours. The cooled solution precipitated the ether (65%). Crystallisation from ethanol afforded yellow needles, m.p. 86-87° (Lit.[225] 86-87°).

With 2 N-hydrochloric acid the chloroethylamine (7.14c) was isolated (72%), with toluene-p-sulphonic acid (1g), the ethyl ether (30%) and toluene-p-sulphonate (55%) were isolated. With ethanol and methyl iodide (4 ml) (4 hours) the iodoethylamine (7.14d) (55%) formed (m.p. and i.r. spectrum identical to authentic sample.)[252]

With ethyl iodide (4 ml) in boiling ethanol (24 hours) the ethyl ether (75%) formed.

The aziridine (7.1a) was stable in ethyl iodide, or ethanol, alone.

Reactions with Bases.

N-(2-Pyridyl)-N'-(2,4-dinitrophenyl)ethylene-diamine (7.23)

2,4-Dinitrophenylaziridine (1.0g) and 2-amino-pyridine (1.0g) were heated together on a steam bath for 2 hours. The mixture was refluxed in ethanol (50 ml) for 10 minutes. An ethanol insoluble yellow solid was isolated; crystallisation from dimethyl-formamide afforded the yellow ethylenediamine (0.4g) m.p. 246-248°.

Found: C,48.8; H,4.7; N,22.0.

$C_{13}H_{13}N_5O_4H_2O$ requires C,48.5; H,4.7; N,21.9%.

On standing a red solid precipitated from the ethanol solution. It had m.p. 108° eff. (0.8g) but could not be crystallised. Refluxing in 2 N-sodium hydroxide for 24 hours afforded the ethylenediamine (0.75g). This red solid is speculatively assigned the structure of the iminopyridyl isomer (7.22).

Similar treatment of 2,4-dinitrophenylaziridine with pyridine (50%) and 3-aminopyridine (20%) afforded the polymer (7.15a). The i.r. spectrum was identical to the authentic product.

This polymer was also obtained from refluxing the aziridine with pyridine in benzene (5%) for 12 hours, xylene (100%) for 8 hours, toluene (96%) for 24 hours, and methanol (50%) for 48 hours.

Replacement of pyridine with 3-aminopyridine in similar reactions also afforded the polymer on refluxing in toluene (5%) for 24 hours, refluxing in acetone (20%) for 4 hours and refluxing in n-butanol (50%) for 8 hours. Equally analogous reaction with 4-aminopyridine resulted in the polymer (80%) in toluene or n-butanol. No reaction occured in acetone.

Sodium 2,4-dinitrophenoxide.

2,4-Dinitrophenylaziridine (2.0g) was refluxed in 1 N-sodium hydroxide (50 ml) for 1 hour. The deep brown solution was kept below 10° C for 3 days. A brown-yellow precipitate of the phenoxide formed (1.5g), identical to an authentic sample.

Reaction with methanol in triethylamine.

2,4-Dinitrophenylaziridine (1.05g) was refluxed in methanol (10 ml) containing triethylamine (2.0 ml)

for 72 hours. Evaporation of the solvent followed by trituration of the residue with ether gave a brown-yellow solid. N.M.R. analysis of this solid in $CDCl_3$ showed peaks at 0.8τ - 3τ (multiplet) indicative of a 2,4-dinitrophenyl fragment, a singlet at 6.57τ (OMe), a singlet at 7.7τ (methylene protons of ring-opened ether). T.l.c. showed the presence of only two products, the aziridine and the methyl-ether.

Photochemical Stability of 2,4-dinitrophenylaziridine.

The aziridine (2.0g) was dissolved in methanol (1 litre) and photolysed for 48 hours. During this period no reaction took place; the electronic absorption spectrum was unchanged (λ max 327 nm) and t.l.c. showed only one product. After leaving the solution exposed to ambient light for 48 hours, examination of the photolysate (t.l.c.) revealed two products, one of which was the unchanged aziridine. The electronic absorption spectrum showed a peak at 350 nm (identical to authentic methyl ether On concentrating the solution to 50 ml yellow crystals of the methyl ether (25%) (7.19a) precipitated. The filtrate was shown to contain only unchanged aziridine (t.l.c.)

Control solutions of the aziridine in ordinary methanol were kept either in the dark or exposed to ambient light without prior u.v. illumination for 4 days at room temperature. In these solutions no reaction occurred.

A solution of the aziridine (1.0g) in methanol (1 litre) containing 0.1% formic acid (98%) was kept

at room temperature exposed to ambient light for 48 hours. On concentrating this solution to 50 ml the methyl ether (7.19a) precipitated (65%). The filtrate contained only unchanged aziridine (t.l.c.)

Photolysis of this solution prior to concentration did not result in reformation of the aziridine (7.1a).

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