# A STUDY OF THE CHEMISTRY OF NITROGEN MUSTARD BY N.M.R. SPECTROSCOPY

A Thesis Submitted for the Degree of
Doctor of Philosophy

by

Michael James Kebbell

University of Warwick

September 1984

*Dedication*

*To Elizabeth, my wife and friend*

ACKNOWLEDGEMENTS

I wish to thank Dr. B. T. Golding for his constant encouragement and interest throughout this work. I am indebted to Dr. O. W. Howarth for his advice on nuclear magnetic resonance spectroscopy and to Dr. E. H. Curzon for recording some of the spectra presented herein.

I am grateful for the contribution of numerous members of the technical and clerical staff of the Department of Chemistry and Molecular Sciences for their invaluable help in smoothing my path.

I wish to acknowledge financial support from the S.E.R.C. in the form of a CASE award in collaboration with BOC Prochem Ltd. and also the advice of Dr. I. M. Lockhart on syntheses with stable isotopes.

Finally, I thank my wife for tolerating the author during preparation of this manuscript.

## ABSTRACT

This thesis describes the use of high-field n.m.r. spectroscopy, in part combined with stable isotope labelling, to study the solution chemistry of nitrogen mustard (2-chloro-(N-2-chloroethyl)-N-methylethanamine). Reactions with a range of nucleophiles were monitored by $^{1}H$ n.m.r. spectroscopy which demonstrated the generation of N-2-chloroethyl-N-methylaziridinium chloride from nitrogen mustard and step-wise substitution of nitrogen mustard except where polymerisation or hydrolysis occurred. The $^{1}H$ n.m.r. spectrum of the N-2-chloroethyl-N-methyl-aziridinium ion was resolved and fully assigned. The intermediacy of this species in reactions of nitrogen mustard is implied by experiments in which identical products are derived from a given nucleophile when added to either the aziridinium ion or the parent nitrogen mustard. Intermediacy of two successive aziridinium ions in alkylation of nucleophiles by nitrogen mustard was confirmed by examination of the $^{13}C$ label distribution in products arising from alkylation of [2,2'-$^{13}C$]-2-chloro-(N-2-chloro-ethyl)-N-methylethanamine. Applying a consideration of the stereochemistry of ring closure to N-methylpiperazine arising from alkylation of ammonia demonstrated that the observed $^{13}C$ label distribution could only arise if aziridinium ion formation was irreversible. The isotope effect on ring opening of the aziridinium ion was measured under a range of reaction conditions for several different nucleophiles.

Observation of the reaction between nitrogen mustard and methionine was simplified by the use of (*S*)-[$^{13}CH_3$]-methionine to demonstrate rapid formation of sulphonium salts and a slow breakdown to homocysteine and 2-methylthio-N-[2-(methylthio)ethyl]-N-methylethanamine.

The synthesis of [2,2'-$^{13}C$]-2-chloro-(N-2-chloro-ethyl)-N-methylanamine is described and a general synthesis of isotopically enriched nitrogen mustards is suggested based on this route. Efficient syntheses of (*S*)-[$^{13}CH_3$]-methionine, (*S*)-[$C^{2}H_3$]-methionine, (*S*)-α-$^{2}H$-alanine and (*S*)-[1-$^{13}C$]-leucine are also described.


**

CONTENTS

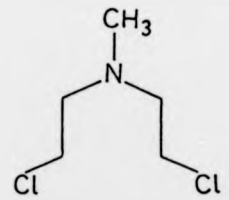
LIST OF ILLUSTRATIONS

# CHAPTER 1

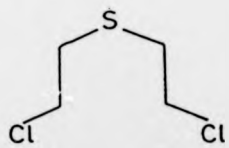
# INTRODUCTION

## 1.1 NITROGEN MUSTARD - AN HISTORICAL INSIGHT

The synthesis of nitrogen mustard (N-2-chloroethyl)-N-methyl-2-chloroethanamine, (1), was first reported in 1931[1] in a description of the synthesis of a piperazinium ion. The trivial name nitrogen mustard was derived by analogy with (S-2-chloroethyl)-2-chloro-1-mercaptoethane (2), which was used as a chemical warfare agent during World War One, under the name "mustard gas". The naming of analogue structures is simplified by use of the trivial names, which are more widely used than the systematic nomenclature and are employed in this thesis as a shorthand form. It is also common to refer to chloroethyl functions as "arms" and compounds with varying numbers of these substituents on nitrogen are described as one-, two-, or three-armed mustards.




(1)




(2)

The long-standing interest in nitrogen mustard (1) centres on its employment as an anti-cancer chemotherapeutic agent[2]. Considering the toxic and vesiccant properties of mustards as a class, any application in a life-preserving role was a surprising innovation. The anti-tumour effect of topical applications of sulphur mustard (2) on experimental animals was reported in the 1930's[3], but at that time clinical use was impractical because mustards were regarded as presenting unacceptable hazards to both staff and patients. During the Second World War the chemistry of nitrogen mustard (1) was examined as part of a programme to acquire information on the behaviour of potential chemical warfare agents. The results of this study were published in 1946 as a series of papers with several authors; these papers are cited as the work of Golumbic *et al.*[4]. Encouraged by the safe-handling of the vesiccant (1) by these workers, Gilman and Philips[2] conducted a series of animal trials with nitrogen mustard before demonstrating beneficial effects in the treatment of Hodgkins' disease in humans. Partial regression of the tumour was observed in human subjects, but on re-growth the tumour was found to have developed a resistance to the drug (1) and early trials extended life-expectancy by only a few weeks. However, the severe side-effects of mustard (1) restricted its use to patients with advanced tumours, so the slightest benefit was most encouraging. In attempts to moderate the toxic side-effects of the drug (1) a large number of modified mustards were synthesised and tested for anti-tumour activity. Adjustment of substituents

on nitrogen was intended to control the reactivity of remaining chloroethyl functions. With the accumulation of sufficient data, rational design[5] of replacements for mustard (1) was envisaged, but the advances which have been made are due more to accident than intent. The control of cancers has proved to be beyond the reach of any approach *via* structural modifications of known drugs. Nitrogen mustard (1) is still in clinical use, but as a minor component amongst a maze of other drugs.

## 1.2 NITROGEN MUSTARDS AS BIOLOGICAL ALKYLATING AGENTS

Nitrogen mustard is an alkylating agent and its use in cancer chemotherapy has led to trials with many other alkylating agents. These compounds now form one of the largest categories in a classification of anti-cancer agents, the divisions being:

alkylating agents
antibiotics
antimetabolites
mitotic inhibitors (spindle poisons).

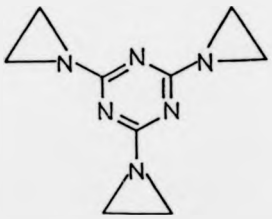
Alkylating agents are generally cytotoxic and so they are often regarded as a last resort for treatment of advanced cancers. The effectiveness of a drug against a particular cancer is determined by the balance between damage caused to cancer cells and that caused to normal cells[7]. Sensitivity of a tumour is controlled by its biochemistry, cell kinetics and location, which are balanced against the distribution and metabolism of a drug. Assessment of the effectiveness of a drug can only reliably be obtained from human

trials, but such trials are permitted only after extensive tests with lower animals. Because these systems do not present identical characteristics to human subjects, interpretation of results in terms of efficiency in man is not reliable. However, the use of a series of rapidly growing transplanted tumours, L 1210 leukemia, Walker 256 carcinoma and Yoshida sarcoma, provides a sensitive screen, which does not give false negative results[8]. An examination of the blood of test animals provides information on the likely application of a drug through assessment of the relative inhibition of lymphocytes and neutrophils. A drug effective against lymphoblastic leukemia selectively inhibits lymphocytes; depression of neutrophils indicates potential in the treatment of myeloid leukemia.

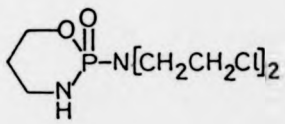
Rapidly dividing cells are regarded as the most sensitive to alkylating agents[9]. Thus, the utility of mustard (1) in treatment of Hodgkin's disease depends on the high proliferation rate of the lymph cells and the deleterious effects of nitrogen mustard are due to extensive damage to bone marrow in particular, with a consequent haematopoietic depression. It has been suggested that alkylating agents also react with non-dividing cells. Repair of alkylation damage prior to division was assumed to account for the lack of cytotoxicity, although non-lethal alkylations were predicted to cause carcinogenic, mutagenic and teratogenic activity. The unpaired DNA of S-phase cells was held to explain their greater susceptibility to alkylating agents, because the absence of hydrogen-bonded structure frees

potential nucleophilic centres. This does not imply a phase specificity, but is an argument for proliferation dependence.

Tumour-inhibitory properties are generally shown only by those mustards with at least 2 alkylating arms which suggested that a form of cross-linking was involved. As a direct result, the cross-linking agent for polymers, triethylenemelamine (3), was given a successful clinical trial[10]. A number of aziridines have been tested as activated forms of nitrogen mustards. Masked nitrogen mustards, the most successful of which is cyclophosphamide (4), have been developed on the assumption that they will be preferentially activated in tumour cells. Thus,



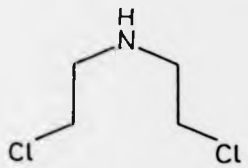
(3)



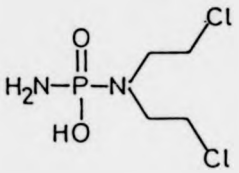
(4)

cyclophosphamide was advanced[11] as a latent form of nor-nitrogen mustard, N-2-chloroethyl-N-2-chloroethanamine (5), which was to be released specifically in tumour cells by virtue of their high phosphoramidase activity. Actually cyclophosphamide is oxidised in liver microsomes and is subsequently degraded to release phosphoramide mustard (6), which is now regarded as the active alkylating agent. Similarly, azobenzenes (e.g. (7)) have been

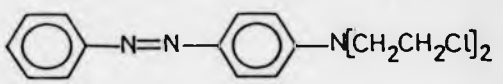
developed as anti-tumour pro-drugs, which are activated by *in vivo* reduction to give p-phenylenediamine mustards as the active alkylators[12].



(5)



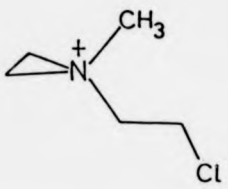
(6)



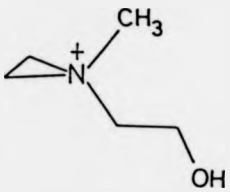
(7)

## 1.3 MECHANISM OF ACTION OF NITROGEN MUSTARDS

Aziridinium ions such as (8) have long been postulated as intermediates in the reactions of mustard (1)[13]. Despite a plethora of evidence for intermediate (8) from, e.g. kinetic studies and its apparent trapping by various reagents, the study described in this thesis is the first to characterise it fully by direct observation in solution. Golumbic *et al.*[4] isolated the N-2-chloroethyl-N-methylaziridinium ion (8) and the N-2-hydroxyethyl-N-methylaziridinium ion (9) as picryl-sulphonate salts during the hydrolysis of nitrogen mustard (1). These salts were characterised by their elemental composition, but the empirical formulae are the same as those of the piperazinium dimers (10) and (11), which are among the predicted end products of the reaction. Picrylsulphonate salts of ions (8) and (9) were

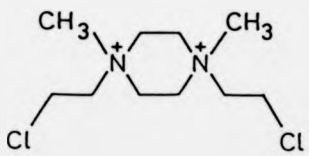


(8)

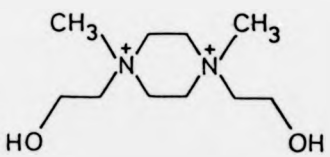


(9)

distinguished from the corresponding salts of (10) and (11) by their reaction with thiosulphate. Dissolution of the picrylsulphonate of (8) in aqueous HCl allowed isolation of the picrylsulphonate of parent mustard (1) demonstrating reversible formation of intermediate (8).



(10)

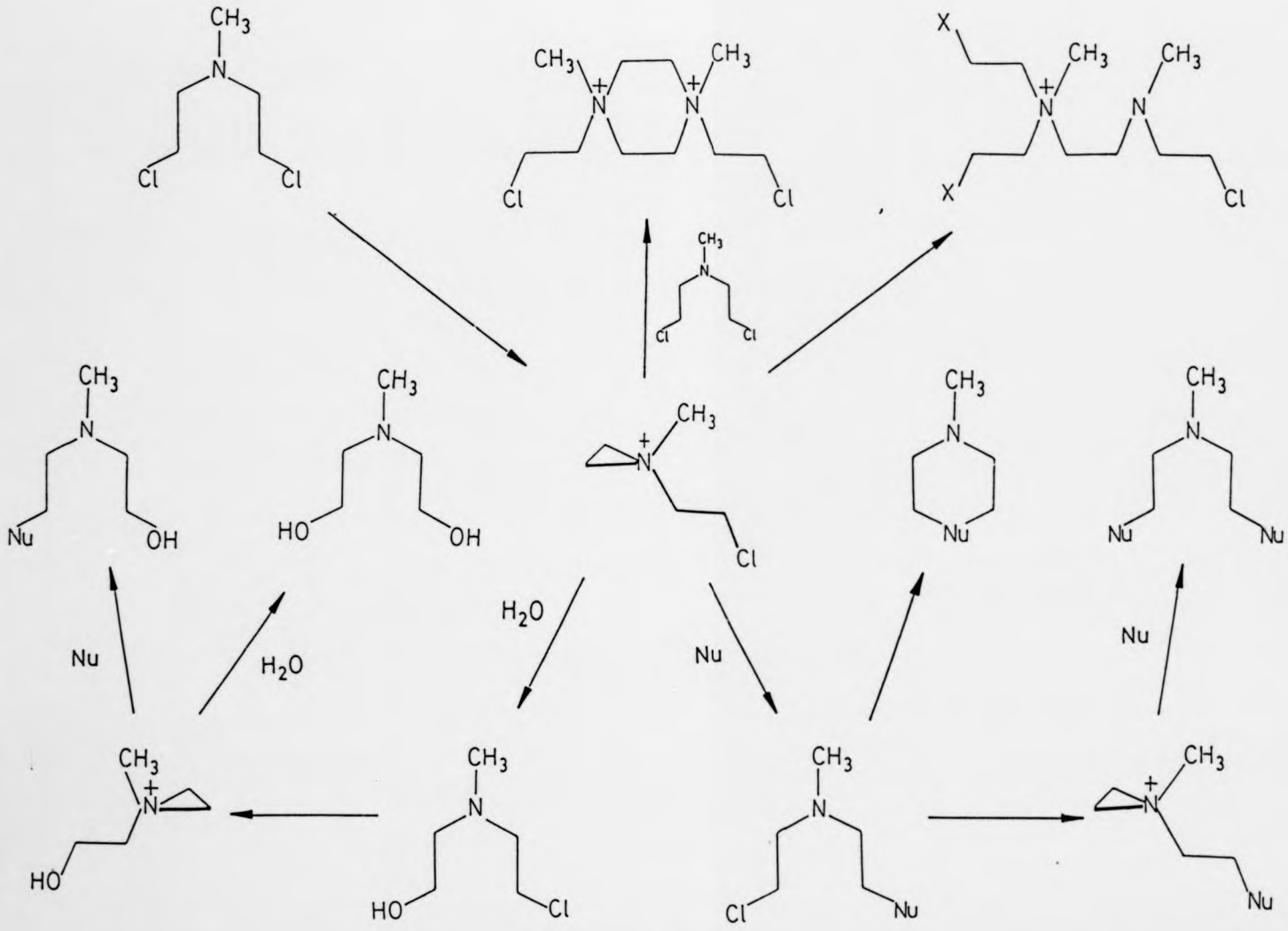


(11)

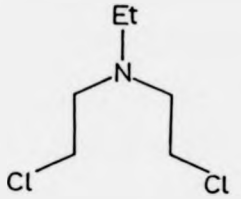
A mercurichloride salt of aziridinium ion (8) was claimed as the product of adding mercuric chloride to an aqueous solution of mustard (1) which had been kept at 25°C for 45 minutes[14]. For this product, only an elemental analysis for mercury was reported, although a melting point depression indicated that it was not a mercury salt of nitrogen mustard. These workers were uncertain of the formation of a second aziridinium ion, e.g. (9) in the

hydrolysis of (1) and invoked a primary carbonium ion formed in a fast reaction.

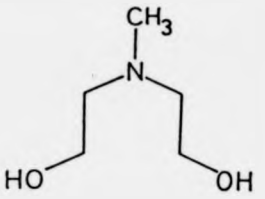
Kinetic measurements by conductimetry pointed unambiguously to aziridinium ion intermediacy in the reactions of aliphatic mustards, but kinetic analysis may only suggest a mechanism and must be regarded as supportive evidence. The study of Bartlett *et al.*[15] showed that the initial rate of reaction of mustard (1) in the presence of either hydroxide or thiosulphate ion in an acetone/water system was no different than in their absence, when dimerisation to piperazinium ions (10) and (11) occurred. Formation of an aziridinium ion is consistent with the observed first order reaction, which was found to be independent of either the nature or concentration of the nucleophile. The ethyl mustard (12) displayed similar kinetic properties[16] and differed chiefly in the greater degree of hydrolysis observed during dimerisation. Bartlett found that addition of 2 mol equiv. of chloride ion reduced dimerisation of mustard (1) to one-seventh of that observed in the control experiment. This demonstrated the reversibility of the aziridinium ion formation and was in accord with the established nucleophilicities of chloride and hydroxide. Working under conditions that are more physiologically appropriate, i.e. low concentration of nitrogen mustard in an aqueous solution of pH 7.4, a second group of workers compared the behaviour of a range of aliphatic mustards and concluded that the rate of cyclisation to aziridinium ions was dependent upon the nitrogen basicity[17].


CH3
N
Cl
Cl
CH3
CH3
N
N
Cl
Cl
X
X
CH3
CH3
N
N
Cl
CH3
N
Cl
Cl
CH3
N
Nu
OH
CH3
N
HO
OH
CH3
N
Cl
CH3
N
Nu
CH3
N
Nu
Nu
Nu
H2O
H2O
Nu
CH3
N
HO
CH3
N
HO
Cl
CH3
N
Cl
Nu
CH3
N
Nu

Scheme 1.1




(12)

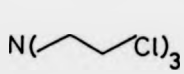



(13)

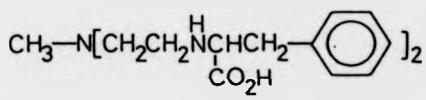
A subsequent publication[18] concentrated on the reactions of aziridinium ions derived from a range of mustards for which thermodynamic parameters were successfully calculated except for nitrogen mustard. Hydrolysis of mustard (1) to N-methyl-2-hydroxyethyl-N-2-hydroxy-ethanamine (13) was found to be complex at pH 9.2 and was explained by side-reactions to form piperazinium dimer (11), although conditions had been chosen to exclude dimer formation. Measurement was also made of the equilibrium constant for the cyclisation of mustard (1) to aziridinium ion (8) in the presence of chloride ion, and using this equilibrium constant it was calculated that dilute solutions (< 0.005 M) of nitrogen mustard at 37°C attain equilibrium at 99.8% conversion into the aziridinium species.

The most complete published study of the reactions of nitrogen mustard is that of Golumbic *et al.*[4]. In addition to identifying hydrolysis products of mustard (1) (see Scheme 1.1), reactions with models for biological nucleophiles were examined. Reactions with a number of amino acids were studied[4] by recording the decrease in the

amount of amino nitrogen after exposure for 20 hours to mustard (1) at pH 8 or pH 9.5. It was concluded that at pH 8 the amino group of alanine reacted to the same extent as the amino groups of more complex amino acids such as threonine. Thus, reaction with other functional groups did not interfere with alkylation of the α-amino function. An exception was noted for histidine, for which a fast reaction with the imidazole function was claimed. Reactions with the thioether group of methionine was claimed for *bis*-(N-2-chloroethyl)-N-2-chloroethanamine (14), but both nitrogen mustard and the ethyl mustard (12) were suggested not to react at all, although this is mysteriously changed to a quantitative reaction in a later reference book[19]. The authors expressed reservations about the kinetic applicability of their work because the experiments were not performed with homogenous solutions and concentrations were unknown, but an attempt was made to show that the chloroethyl function of each of the three mustards had similar reactivity towards a given nucleophile. Reaction with carboxylic acids was not detected for nitrogen mustard but ethyl-mustard (12) was suggested to form esters with acetate and hippurate (*cf.* ref. 17).



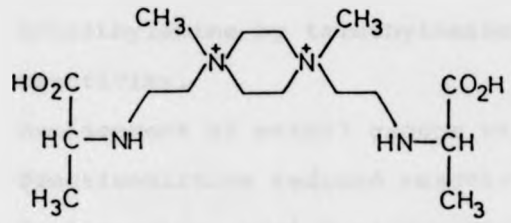
(14)



(15)

A disubstituted product, N-2-(phenylalanyl)ethyl-N-methyl-N-2-(phenylalanyl)ethanamine, (15), was isolated from reaction of mustard (1) with an excess of phenylalanine, but this is the only reaction with a biological nucleophile in which characterisation of the product was reported. In subsequent reactions with a number of potential nucleophiles only the extent of reaction, as determined by competitive reaction with alanine, was reported. According to Golumbic *et al.*[4], the product from the reaction with phenylalanine was unexpected, because analogy with the reaction between nitrogen mustard and alanine, which supposedly gave (N,N'-alanyl-N,N'-methyl) piperazinium dichloride (16), suggested that a similar 1:1 adduct should be formed. The reaction of nitrogen mustard with alanine was not quantitative, so its use as an analytical tool is questionable, as is the structure of the end-product (16) for which no analytical data was advanced. A more complete discussion of the nucleophilic displacements reported in reference 4 is presented, with our own results, in Chapter 4 of this thesis. Reactions with a range of phosphates (e.g. $Na_2HPO_4$, fructose-6-phosphate) were also reported. It was shown that reaction with $Na_2HPO_4$ consumed 1.3 mM of phosphate per mM of mustard (1) at pH 8. These results were overlooked by some later workers examining the alkylation of phosphate groups in DNA.

A second study[20] of nitrogen mustard chemistry attached great importance to the rapid reaction between mustard (1) and hexamethylenetetramine (HMT). These



(16)

workers claim that formaldehyde and ammonia react sluggishly or not at all with nitrogen mustard, and therefore HMT must react intact. Considering the known chemistry of HMT in solution and our results with ammonia as a nucleophile (see Chapter 5), this interpretation appears to be incorrect. More surprising is that this is the only published reference to the reaction between nitrogen mustard and ammonia. Discounting any breakdown of HMT and stating that its presence does not alter the rate of chloride ion liberation from (1), Gurin *et al.* proposed alkylation of HMT with aziridinium ion (8) to form a quaternary salt and subsequent mustard polymerisation with one terminus secured to HMT.

The reactions of nitrogen mustard with cyclic and aliphatic amines, after exposure for 30 minutes, were assessed by thiosulphate titration and general conclusions on the structural requirements of amines for rapid reaction with nitrogen mustard was presented, *viz.*

(1) The nitrogen must be tertiary.

(2) Replacement of methyl groups by larger

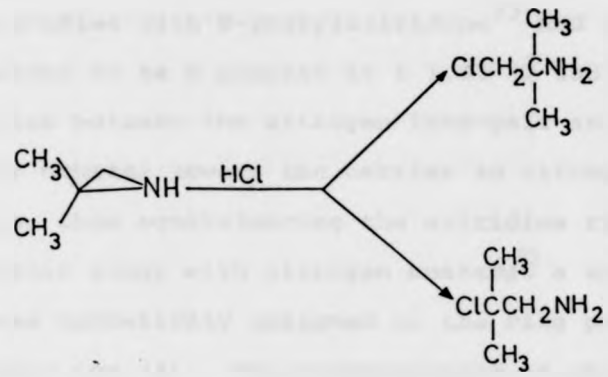
alkyl groups in the aliphatic series (e.g. trimethylamine by triethylamine) reduced reactivity.

(3) Replacement of methyl groups with larger functionalities reduced reactivity.

(4) Cyclic amines were more reactive than acylic amines.

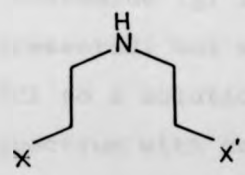
(5) The most effective reagents contained at least two tertiary amine functions separated by methylene groups of unspecified length.

Until the present study, the fundamental chemistry of nitrogen mustard had not been further examined, so that much of the data for interpreting n.m.r. spectroscopic studies of similar alkylating agents and cell components was not available. However, the question of the intermediacy of aziridinium ions in the reactions of mustards (especially aromatic mustards), has frequently been examined. Schatz and Clapp[21] protonated 2,2-dimethylaziridine to give an unsymmetrical aziridinium ion, which was captured by either chloride or hydroxide to yield a mixture of 1- or 2-substituted propylamine derivatives (Scheme 1.2). Three independent $^{1}H$ n.m.r. spectroscopic studies have been published, each concentrating on the hydrolysis of mustards. The earliest study[22] was initiated to determine the composition of variously halogenated mustards. Reactivity of these mustards was also investigated, demonstrating that N-2-fluoroethyl-2-fluoroethanamine (17, $X = X^{1} = F$) was stable in $^{2}H_{2}O$ at neutral pH, whereas N-2-iodoethyl-2-

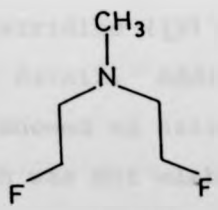


Scheme 1.2

iodoethanamine (17, X = $X^1$ = I) had completely reacted within 6 minutes. Each of the N-2-haloethyl-2-haloethanamines (halo = F, Cl, Br or I) could be distinguished by their chemical shift differences. Treatment of these samples with 1 mol equiv. of base caused cyclisation to an N-2-haloethylaziridine. Pettit *et al.* assigned the



(17)

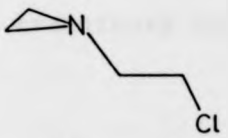


(18)

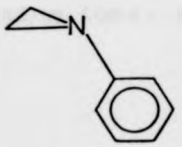
multiplets at δ 1.90 and 2.25 to the aziridine protons. Earlier studies with N-phenylaziridine[23] had shown the ring protons to be a singlet at δ 1.42 (T 298 K), because interaction between the nitrogen lone-pair and a phenyl molecular orbital lowers the barrier to nitrogen inversion, thus equivalencing the aziridine ring protons. In a similar study with nitrogen mustard[22], a small peak at δ 2.78 was tentatively assigned to the ring protons of aziridinium ion (8). The concentration of this species was low throughout and the authors called for a definitive chemical study to be made to identify the end products. The spectra are similar to some presented in Chapter 5, but the chemical shifts disagree. Possibly, Pettit's use of an external standard is to blame; the signals of aziridinium ion (8) are pH independent.

The n.m.r. study by Levins and Papanastassiou[24] compared the rates of hydrolysis of N-2-fluoroethyl-N-methyl-2-fluoroethanamine (18) and fluoro-mustard (17) with their chloro analogues. These authors concluded that, although solvolysis of (17) and (18) was much slower than that of the chlorinated materials, they all shared a common aziridinium ion mechanism, in contrast to suggestions that (17) and (18) solvolysed by an $S_N2$ mechanism[25]. A series of spectra showing conversion of N-2-chloroethyl-2-chloro-ethanamine (5) into N-2-chloroethylaziridine (19) were presented, but were not discussed in detail. Addition of HCl to a solution of aziridine (19) showed an aziridinium ion spectrum with complex coupling, which was not elaborated on. Addition of HCl to solutions of N-phenylaziridine (20) in

methanol caused polymerisation and aziridinium species were not detected. This study presents graphically data indicating changing concentrations during the reactions (1) → (8) → (10), but no spectra are shown and the chemical shifts of (1), (8) and (10) are not listed.



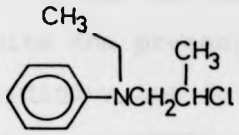

(19)



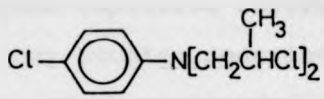

(20)

Sowa and Price[26] identified aziridinium ion (8) and (19) in solutions of mustards (1) and (5), respectively, which had been treated with $NaHCO_3$. They presented data indicating that in systems buffered with $NaHCO_3$ the conversion of nitrogen mustard into aziridinium ion (8) was complete within 60 minutes and that the concentration of (8) at no stage exceeded 57% of the initial concentration of mustard (1). The aziridinium ring protons of (8) were reported as a doublet (J = 2.4 Hz). The reactions of N,N-dimethyl-2-chloroethanamine and N,N-diethyl-2-chloroethanamine with sodium thiosulphate were characterised by n.m.r. spectroscopy and the aziridinium intermediates were identified, but this reaction was not carried out with (1).

The intermediacy of aziridinium ions in reactions of aromatic mustards is frequently denied[27]. Aziridinium ions react rapidly with $Na_2S_2O_3$, but thiosulphate titrations during hydrolysis of aryl mustards indicate negligible aziridinium ion concentration. Aromatic mustards capable of forming unsymmetric aziridinium ions (e.g. (21) and (22)) underwent hydrolysis without rearrangement. This evidence does not exclude the transitory existence of aziridinium ions, but common

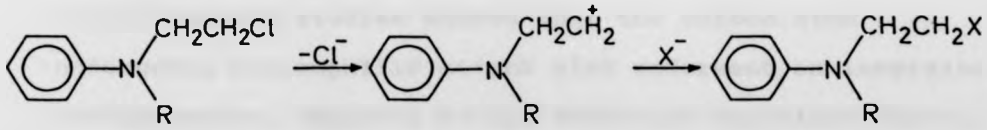


(21)



(22)

opinion assumed that aromatic mustards react *via* primary carbonium ions (Scheme 1.3). The intermediacy of

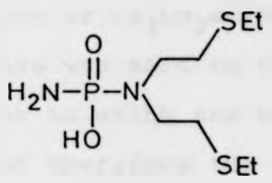


Scheme 1.3

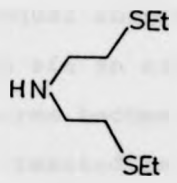
aziridinium ions was implied in a report by Benn *et al.*[28] in which aromatic mustards, specifically deuteriated in their chloroethyl arms, were solvolysed. The ratio of deuterium distribution between C-1 and C-2 of the substituted N-ethyl arms of products showed a slight preponderance towards the C-2 position, which was interpreted as a secondary isotope effect in the attack of solvent on a symmetrical intermediate. However, the reaction was not directly monitored, and so reversible aziridinium ion formation, prior to reaction *via* either an $S_N1$ or $S_N2$ pathway, was not excluded, nor was such a pathway discussed despite the presentation of a scheme depicting reversible aziridinium ion formation (NB in our studies described in Chapter 4, reversible aziridinium formation was definitely excluded).

Crist and Leonard[29] studied reactions of complex aziridinium ions and proposed an equilibrium between cyclic intermediates and carbonium ions when the latter had a stable, tertiary structure and the attacking nucleophile was weak. They suggested cationic polymerisation of epoxides as an analogous situation in which ring-opening should be even easier than with aziridinium ions. However, stereochemical studies showed that the carbon atom undergoing nucleophilic attack also underwent an inversion of configuration, implying an $S_N2$ mechanism and discrediting a carbonium ion route, which should proceed with a loss of configuration due to bond rotation in the ion. It is not sound to extrapolate this result to indicate an $S_N2$ mechanism with aryl mustards whilst nothing is known about

the nature of a possible carbonium ion. Incomplete charge separation in the incipient ion or intimate ion pair effects may direct the attack of an incoming nucleophile so that an aziridinium intermediate is never attained.



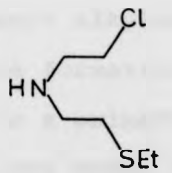

(23)




(24)

A study of the reactions of [2,2'-$^{2}H_{2}$]-N,N-*bis*(2-chloroethyl)phosphordiamidic acid (6a) with ethanethiol claimed to demonstrate the intermediacy of an aziridinium ion by identifying the location of deuterium in the reaction product[30]. These workers found that alkylation of ethanethiol with phosphoramide mustard (6a) gave the di-substituted product (23) and a similar disubstituted product (24) in which the P-N bond of the parent mustard had been cleaved. Under the conditions employed (pH 7.4) cleavage of the P-N bond to give N-2-chloroethyl-2-chloro-ethanamine (5) produces an inferior alkylating agent to the parent phosphoramide mustard. The cleavage product (5) was shown to form small amounts of mono-substituted product [2-chloro-N-2(ethylthio)ethyl ethanamine (25)] and no di-substituted product at pH 7.4. Thus, all the di-substituted

material, N-2-(ethylthio)-ethyl-2(ethylthio) ethanamine (24), in the reaction of phosphoramide mustard with ethanethiol was held to arise from P-N bond scission after alkylation by intact phosphoramide mustard. Repeating the study with the deuterated mustard (6a) gave a product with a mass spectrum interpreted as showing loss of $CH_3CH_2SC^2H_2$ and $CH_3CH_2SCH_2$ in equal abundance. This was said to demonstrate alkylation *via* an aziridinium ion in which the carbons of the ethyl arms become equivalent and therefore the incoming nucleophile reacted in equal proportions at the deuterio and the protio methylene of the aziridinium ring. On fragmentation in the mass spectrometer therefore, equal amounts of nucleophile bonded to either deuterio or protio methylenes were observed. The results with a deuteriated isomer of the phosphoramide mustard P-N bond cleavage product (5a) were also consistent with aziridinium ion intermediacy during alkylation of ethanethiol.



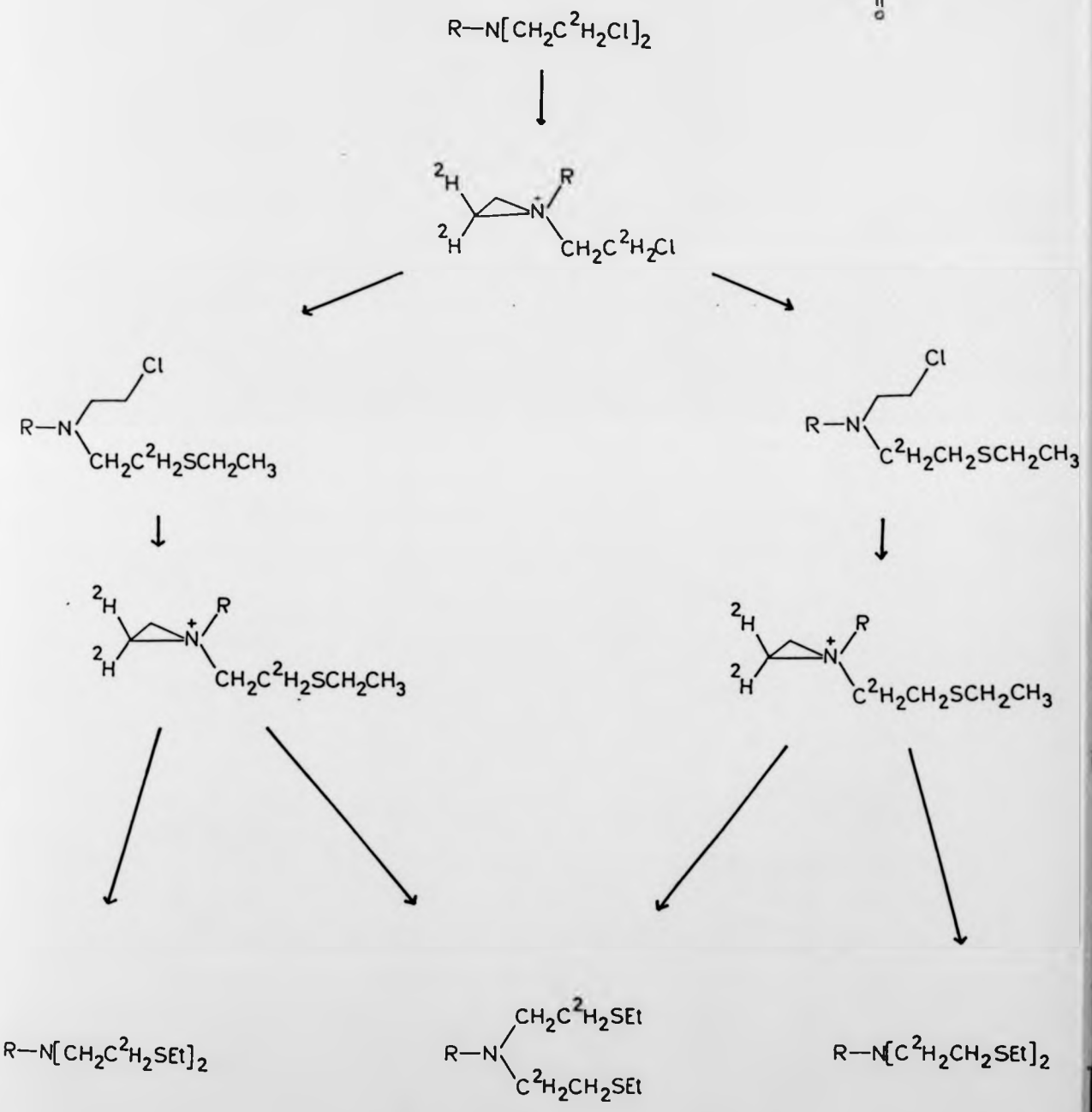

(25)

Identification of the products of these reactions was by gas chromatography/mass spectrometry, which was not of course capable of direct observation of the reactions. Hence, the study with mustard (5) was necessary to

demonstrate that the di-substituted product (24) arose from alkylation of ethanethiol by intact phosphoramide mustard with subsequent P-N bond scission and not by scission prior to alkylation by (5). Alkylation of ethanethiol by specifically deuterated phosphoramide mustard (6a) to give di-substituted products, if it proceeds *via* sequential aziridinium ions, gives rise to 4 different combinations of the deuterium label, two of which are equivalent (see Scheme 1.4). With subsequent P-N bond scission, six different products, in two separate sets of three, are possible if this reaction proceeds *via* sequential aziridinium ions. These products and their yields were not separately determined in this study. The mass spectral evidence presented is consistent with aziridinium ion formation, but only a fraction of the information available from the work is published. Separate determination of each product would not have precluded interpretation of the results as either fast, reversible aziridinium ion formation prior to direct alkylation of the intact mustard, or a similar reversible formation of aziridinium species prior to alkylation *via* a primary carbonium ion. However unlikely these routes may seem, they can only be excluded by direct and continuous observation of the reaction. The suggested explanation for the lack of reactivity of mustard (5), namely deprotonation of its aziridinium ion to give N-2-(chloroethyl)aziridine, could also be confirmed by a technique of direct observation such as n.m.r. spectroscopy. When published (1976) the study of Colvin *et al.* resolved argument over the active metabolite of cyclophosphamide.

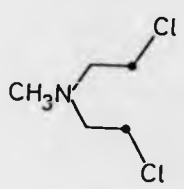
Scheme 1.4 Alkylation of ethanethiol by [2,2'-$^2H_2$]-phosphoramide mustard.

(R = $H_2N$-P(=O)(OH)-)

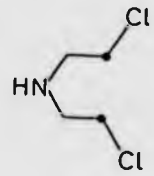


## Isotopically Labelled Nitrogen Mustards

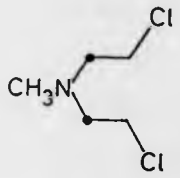
• = $^{13}C$



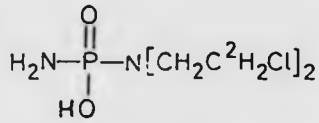
(1a)



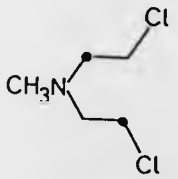
(5a)



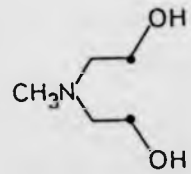
(1b)



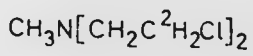
(6a)



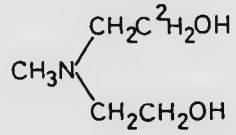
(1c)



(13a)

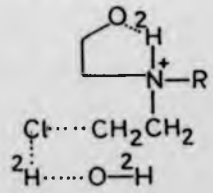


(1d)



(13b)

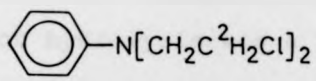
FIG 1.1



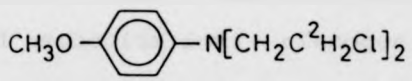
Following microsomal oxidation to 4-hydroxycyclophosphamide the drug decomposed to give phosphoramide mustard and acrolein; subsequent P-N bond cleavage of phosphoramide mustard released the known mustard (5). Demonstration of the superior alkylating ability of phosphoramide mustard was therefore an important observation.

The concept of mustard alkylations proceeding *via* a carbonium ion mechanism originates in mis-interpretation of early studies with aromatic mustards[31]. The early methods of detecting aziridinium ions (e.g. the instantaneous thiosulphate titre) failed to detect such intermediates in the reactions of aryl nitrogen mustards. Although it was noted that the transitory existence of aziridinium ions was not excluded, common opinion held that there was no aziridinium ion intermediacy and that the reaction proceeded *via* a primary carbonium ion. This belief was refuted by Benn *et al.*[28] who presented two pieces of evidence. The first was complementary to early work with mustards capable of forming unsymmetric aziridinium ions, but which did not rearrange on hydrolysis. It was shown that two mustard isomers, capable of forming the same unsymmetric aziridinium ion, gave the same solvolysis product. Thus, one mustard rearranged and the other did not, but the 1:1 mixture of solvolysed mustards resulting from statistical distribution of the nucleophile between the carbons of the aziridinium ion ring was not observed. This was rationalised by postulating preferential attack at the more highly substituted ring carbon. The second piece of evidence utilised aniline mustards specifically

deuterated at the 2-position of the chloroethyl arms. Reaction of [2,2'-$^2H_2$]-N,N-*bis*-(2-chloroethyl)amino-benzene (26) with potassium p-thiocresolate gave a di-substituted product in which the original label distribution in the side-chain, as determined by $^1H$ n.m.r. spectroscopy, was retained whereas hydrolysis or acetolysis yielded products with the deuterium label approximately equally distributed between the methylene units of the side chain. With more reactive mustards, e.g. [2',2"-$^2H_2$]-N,N-*bis*(2'-chloroethyl)-4- methoxyamino-benzene (27), a mixture of 1- and 2-deuteriated products was observed with all three nucleophiles. To explain these results two competing pathways were postulated:



(26)



(27)

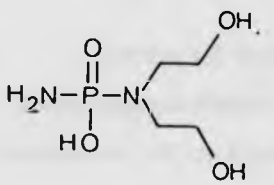
direct $S_N2$ displacement and reaction *via* an aziridinium ion. The reactions were not continuously monitored and reversible aziridinium ion formation cannot be excluded. Given that an alternative pathway had been recognised, this was a serious omission. The routes suggested in this publication were only partially proven: aziridinium ion formation was demonstrated but subsequent capture by a nucleophile was not. Aziridinium ion formation was

presented as a reversible reaction in the Scheme accompanying the work, but no account of this was taken and the study does not provide the compelling evidence for reactions *via* aziridinium ions which it claims.

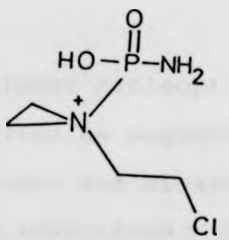
An interesting aspect of the study was the observation of a secondary deuterium isotope effect in the reactions of deuteriated mustard (27), in contrast to the findings of Colvin *et al.* with mustard (6a). The observation of a secondary isotope effect reflects carbonium ion character in the transition state. Thus, such an effect would indicate that the aryl nitrogen mustards pass through a transition state in which a carbon atom bears significant positive charge, in accord with the observations of Bardos *et al.*[32]. These workers measured the alkylating ability and hydrolysis of aryl nitrogen mustards and concluded that the transition state for hydrolysis was a "solvated carbonium ion - nitrogen dipole" which was arrived at *via* an intermediate "which is comparable to the transition state for the formation of an aziridinium ion". This suggests that the two carbon atoms of a potential aziridinium ion ring never became quite equivalent and that to speak of either an aziridinium ion pathway or a carbonium ion pathway is an oversimplification.

A study of the fundamental alkylation chemistry of phosphoramide mustard (6) which employed a direct observation technique has recently been published[33,34]. Zon and his co-workers exploited the high sensitivity and 100% natural abundance of phosphorus to follow hydrolysis and alkylation reactions of mustard (6) by $^{31}P$ n.m.r. spectroscopy.

This method had the advantage of eliminating the synthetic work required to incorporate an n.m.r. sensitive nucleus, but the phosphorus atom in phosphoramide mustard is distant from the site of alkylation and so may be an insensitive guide. With only one $^{31}P$ nucleus present, a different signal was anticipated for each species in solution. The simplicity of the n.m.r. spectra was well suited to kinetic work, but was unhelpful when characterising the species involved. The pH dependent competition between hydrolysis of the mustard (6) to N,N-*bis*-(2-hydroxyethyl)-phosphordiamidic acid (28) and hydrolysis of the P-N bond was studied and the half-life of mustard (6) established under various conditions. The rate of disappearance of phosphoramide mustard (6) was unaffected by the presence of an excess of thioethanol, sodium 2-mercaptoethylsulphonate, thiourea or bovine serum albumin, which is consistent with rate-limiting formation of aziridinium ion (29). In the presence of a 1 M concentration of chloride ion the reactivity was reduced by up to 3-fold; the exact variation was dependent on the counter ion and was greatest for $Mg^{2+}$, least for $Li^{+}$.

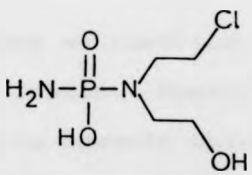



(28)




(29)

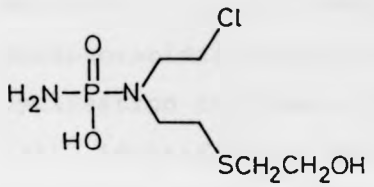
A transitory intermediate was observed in the hydrolysis of mustard (6) at pH 7.4. This was the dominant species in the early stages of hydrolysis and was assumed to be the aziridinium ion (29). This is a reasonable assumption but the $^{31}P$ chemical shift difference between the present mustard (6) and derived aziridinium ion (29) was only 0.7 p.p.m., which seems inconsistent with the placement of a positive charge adjacent to the phosphorus. Attempts to use $^{13}C$ n.m.r. to further characterise (29) were unsuccessful and $^{1}H$ n.m.r. was apparently not considered. Kinetic evidence to link the reactive intermediate with aziridinium ion (29) included the anticipated decrease in half-life with increasing pH, which was not anticipated for the fully hydrolysed mustard (28). However, this was also consistent with a half-reacted phosphoramide mustard (30).



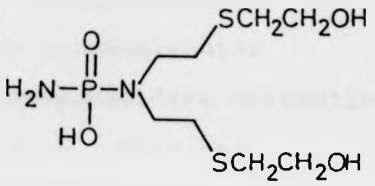

(30)

Buffered reactions with sulphur nucleophiles, e.g. 2-mercaptoethanol, were interpreted as sequential displacement of chloride such that mono- and di-substituted species, e.g. (31) and (32) could be identified. This was the first report of the observation of sequential substitution of a bifunctional mustard and is mirrored

in the results presented in Chapter 4 of this thesis. However, the reaction between nitrogen mustard (1) and thiourea reported in that chapter is not, according to the observations of Zon *et al.* reproduced in the chemistry of phosphoramide mustard (6). The authors argue that the $NCH_2CH_2SC(=NH)NH_2$ function of alkylated thiourea is labile compared to the products of alkylation of other sulphur nucleophiles. This is not the case when the alkylating agent is nitrogen mustard, although

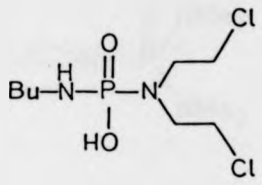


(31)

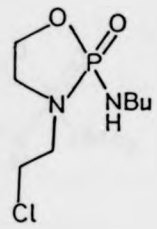


(32)

the product possesses an identical grouping to that which Zon suggests to be labile. Possibly, aziridinium ion (8) is much more reactive towards thiourea than is aziridinium ion (29). This is in accord with the relative alkylating abilities reported by Colvin *et al.*[30], yet (29) is reported[35,*cf.*36] to alkylate guanosine 5'-monophosphate, whereas we found that (8) did not. In view of the widely different therapeutic indices of the two parent mustards, (1) and (6), similar alkylating ability would be surprising, although formal similarities are strongly suggestive of it.

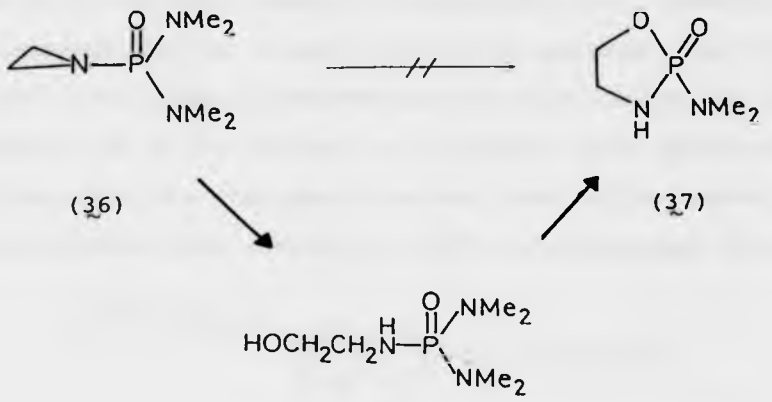


(33)



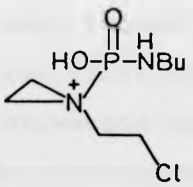
(34)

Zon's studies with substituted phosphoramide mustards, e.g. N'-*n*-butyl-N,N-*bis*-(2-chloroethyl)-phosphoramidic acid (33) indicated intramolecular cyclisation to give a 1,2,3-oxazaphospholidine derivative (34), identified by mass spectrometry. This was suggested to arise from intramolecular O-alkylation of the aziridinium ion (35) and was held to be analogous to O-alkylation of phosphodiester linkages in DNA. According to Eschenmoser[37] and to Baldwin[38], and by our own observations (Chapter 5), such an intramolecular process should not occur: it represents a "front-side" $S_N2$ substitution and is sterically forbidden. Reaction of 1-aziridinylbis(dimethylamino)phosphine oxide (36) to give the oxazaphospholidine (37) proceeds *via* an aziridine-ring opened intermediate, 2-hydroxyethylphosphorotriamide (38) (Scheme 1.5). A similar route was rejected for the reaction (33) → (34), because the oxazaphospholidine (34) was formed very rapidly and the analogous reaction was known to be slow. However, the steric requirements of aziridinium ion (35) preclude a direct intramolecular reaction and so the ring-opening route appears the more likely.



(36) (37) (38)

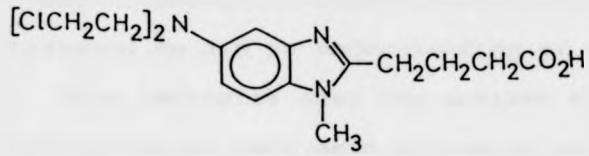
Scheme 1.5



(35)

Recently, $^{1}H$ n.m.r. spectroscopy was used to study the hydrolysis of a complex mustard, γ-[1-methyl-5-*bis*(β-chloroethyl)amino-2-benzimidazolyl]butyric acid (39), used clinically under the trade name of cyclostasan. This paper states that "the mode of action of nitrogen mustard is caused by elimination of a chlorine atom ...

to give a highly reactive carbonium ion". Evidence was presented for an unusual mechanism whereby the first hydrolysis step proceeded *via* an aziridinium ion and the second *via* a carbonium ion. Spectra were presented to illustrate the changes observed when dilute solutions of cyclostasan were heated at 70°C in unbuffered $^2H_2O$ solution.




(39)

The concentration of cyclostasan and two hydrolysis products, derived by replacement of chloride with hydroxyl functions, were fitted to a kinetic model which assumed two consecutive first order reactions. Using an Arrhenius plot, activation energies for the two transition states were determined, the second being significantly less than the first. An aziridinium ion was assumed as the first transition state, but the lower activation energy of the second transition state was explained by postulating a deuterium bonded system (Fig. 1.1). The authors argue that the stability of this deuterium bonded configuration makes a second aziridinium ion very unlikely, but their own estimate of errors in the Arrhenius plot suggests that the second activation energy is not accurately known. The deuterium bonded structure proposed would be attainable with any

hydrolysing mustard in $^{2}H_{2}O$, but has never previously been suggested.

The reactions of cyclostasan were observed in a p$^{2}$H range of 0.5-4.0 to minimise chemical shift changes due to the release of acid. The $^{1}$H n.m.r. spectrum of the chloroethyl arms of cyclostasan under these conditions was reported as being non-first order and too complex for observation to aid an understanding of the reaction pathway. This indicates that the mustard nitrogen was protonated giving an ABCD spin system in accord with our observations of nitrogen mustard at low pH values. Reactions of cyclostasan were followed by monitoring changes in the chemical shift of the benzene ring protons which presented a comparatively simple ABX system. It was recognised that these resonances were not as suitable as those of the chloroethyl arms for studying the hydrolysis. The spectral simplification gained by working at pH values above the pKa of the mustard nitrogen was offset by a large decrease in solubility.

The species arising from hydrolysis were assumed to be those derived by replacing Cl with OH. No account was taken of possible dimeric products even though these were indicated by a disparity between the amounts of free chloride and deuterons detected. Identification of end-products was not experimentally determined and full use was not made of the $^{1}$H n.m.r. technique.

A substantial body of evidence implies that the reactions of nitrogen mustards proceed *via* aziridinium

ions. However, much confused discussion abounds in the literature. The use of modern techniques, especially n.m.r. spectroscopy in identifying reaction products supports the intermediacy of aziridinium ions. Until now, however, even those workers who have directly monitored reactions of mustards have failed to characterise spectroscopically the intermediate aziridinium ions.

## 1.4 INTERACTION OF ALKYLATING AGENTS WITH MACROMOLECULES

### 1.4.1 Interaction of Alkylating Agents with Nucleic Acids

The common assumption[40] of DNA as the primary target for mustard alkylation explains some experimental observations, e.g. the higher sensitivity of proliferating tissues compared to those at rest and the relationship between mutagenicity and carcinogenicity. The very high molecular weight of DNA gives a more favourable ratio of alkylating agent to substrate than does the supposition of a protein target and cross-linking of DNA strands explains the higher efficiency of bifunctional alkylating agents compared to their monofunctional counterparts. However, tumours of greatly different sensitivity appear to experience a similar extent of DNA alkylation and in whole animal experiments good correlation of alkylation of cellular DNA and sensitivity of that cell to alkylation is not observed. Until this correlation is

established there remains a doubt as to the importance of DNA alkylation.

The first report of alkylation of guanine was that of Young and Cambell[41] who observed a reaction between sulphur mustard (2) and nucleoproteins. Guanine N-7 was later identified as the major site of alkylation in DNA[42]. Other DNA alkylation sites were reported as adenine N-1, N-3 and N-7, with adenine N-3 alkylation reaching 25% of that of guanine N-7. In RNA, the principal minor site of alkylation is N-1 of adenine which approaches 33% of the reactivity of guanine N-7. This difference in reactivity is consistent with the Watson-Crick model for DNA. The reactivity of bases within the double helix of the DNA molecule is limited by hydrogen bonding as illustrated by Ludlum[43], who demonstrated a reduced reactivity of adenosine N-1 in poly A poly U compared with poly A. Similarly, Lawley and Brookes[44] demonstrated that the only alkylation site in nucleic acid pyrimidines is cytosine N-3, but in DNA and double stranded RNA this site is again blocked by hydrogen bonding. The pKa of guanine in aqueous solution is less than that of either adenine or cytosine. Thus, preferential alkylation on guanine requires that nucleophilic centres in adenine and cytosine are not available.

A related possible mechanism for the mutagenicity of alkylating agents has been reported[45]. Alkylation on N-7 of 9-substituted guanines resulted in formation of a zwitterion by loss of a proton from N-4, thus precluding the normal hydrogen bonding to cytosine through this

proton and weakening the usual base pairing. A negative charge on N-4 allows base pairing with thymine within the DNA helix and this anomalous behaviour was advanced as a cause of mutagenicity. Formation of an analogous zwitterion was demonstrated[46] for both 7-methylguanosine and 2'-deoxy-7-methylguanosine and the same base-pairing anomaly was postulated. A follow up report[47] indicated the subsequent ring-opening of 7-methylguanosine in neutral solution, which was greatly accelerated at basic pH values. The reaction was monitored by $^{1}H$ n.m.r. spectroscopy of a developing resonance at $\delta$ 8.15, characteristic of a formylamino group which would arise by hydrolysis of the imidazole ring.

A consensus of several reports[44,45,46,48] indicates that 7-alkyldeoxyguanosines (models for alkylated DNA) eliminate the sugar moiety by acid catalysed hydrolyses at pH 7 to leave a 7-alkylguanine. Ring opening was a base catalysed phenomenon observed above pH 8.5. A model for alkylated RNA, 7-alkylguanosine, showed no loss of the sugar residue at pH 7 and at alkaline pH values ring fission was much slower than for the corresponding 7-alkyldeoxyguanosine. 7-Methylguanosine in intact methylated RNA was less reactive[49] than the model compounds showing no breakdown at neutral pH and slower ring fission in alkaline solution. After alkylation with nitrogen mustard (1) DNA has been shown[50] to undergo a slow weight loss ascribed to acid catalysed $\beta$-elimination of deoxyribose residues and resultant chain cleavage. This hydrolysis of the glycosyl bond in alkylated DNA was alkyl group

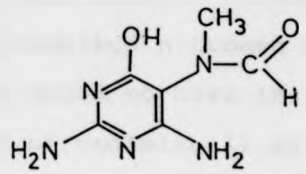
dependent, e.g. ethylated DNA was more stable than DNA treated with a methylating agent or sulphur mustard. Under pseudo-physiological conditions therefore only long-lived alkylated RNA species would be expected to show significant decomposition due to ring fission, whereas extensive cleavage of the glycosyl linkage of 7-alkylguanine residues in DNA occurs.

Minor site alkylation at N-1 of adenine did not give appreciable elimination of the base from DNA, but at neutral pH 1-methyldeoxyadenosine underwent a methyl group migration to give a 6-methylamino-derivative[44]. Methylation of N-3 of adenine resulted in a release of 3-methyladenosine from alkylated DNA at a rate which was 5 times faster than for 7-methylguanine[51]. Thus, alkylation of neither the major nor principal minor alkylation sites in RNA give rise to a significant loss of base from the nucleic acid, but methylation of both the major and principal minor alkylation sites of DNA leads to base elimination at neutral pH.

Recent work has examined the fate of ring opened bases *in vivo*. Subsequent to ring fission, N-7 alkylated guanine is retained in DNA as demonstrated with, e.g. nitrogen mustard[52], and this may be relevant to observation of mutagenic and carcinogenic effects. 7-Methylguanosine has a long half-life in intact DNA ($t_{\frac{1}{2}}$ ~ 150 h at neutral pH) which was assumed[53] to show that there was no enzymic mechanism for the removal of alkylated bases from DNA. Indeed, RNA polymerase from *Escherichia coli* was shown[54] to

transcribe a synthetic co-polymer of uridine and 7-methylguanine *in vivo* without miscoding, although errors did result if the polymer contained methylcytosine. The conclusion that the base pairing properties of guanine and 7-methylguanine were similar was in contrast to the earlier proposition that this modification was mutagenic. However, subsequent to ring fission, alkylated guanine of DNA which had been exposed to nitrogen mustard was retained and this ring-opened alkylated base was advanced as an explanation for mutagenicity. An enzyme was subsequently found in *E. coli* cell extracts and rodent liver extracts[55] which is capable of eliminating the imidazole ring opened form of N-7 substituted guanine from DNA. Work with the DNA glycolase enzyme showed that only double stranded DNA was a good substrate and that intact 7-methylguanine was not cleaved from alkylated DNA. For effective cleavage the imidazole ring was opened by alkaline hydrolysis generating denatured DNA which was re-annealed and neutralised. Unusual bases in the DNA sequence, such as uracil or 3-methyladenosine were unaffected[56,57], but these are known to have their own DNA glycolases. So far the requirement for an alkyl group on the ring opened guanine is not established and alkylation may not be necessary for enzymic recognition of the substrate.

Analysis of the products of alkaline ring fission of N-7 alkylated guanines have variously reported several poorly defined products[58] or the formation of a single derivative[59], 2,6-diamino-4-hydroxy-5-N-methyl-formamidopyrimidine (40). It has been shown that the



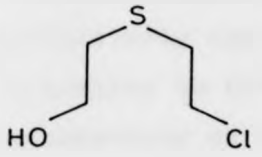
(40)

imidazole function of adenosine can be opened by hot alkali without prior alkylation. Analogous hydrolysis of guanosine may explain the observation of multiple products. Compounds comparable to (40) have also been reported after exposure of guanylic acid solutions to ionising radiation[60]. Subsequent work[61] has identified (40) as the major product from alkaline hydrolysis of 7-methylguanine, a second component arising from de-formylation of (40). These results were confirmed by h.p.l.c., but the possibility that minor species were lost during work-up procedures would not be excluded. The authors demonstrated that the use of an ammonium formate buffer during ion exchange separation of the reaction mixture equilibrated the two products; the formamidopyrimidine (40) contained de-formylated product after storage, which may explain reports of multiple products. It appeared that the enzymic substrate was the formylated species which has an enolic relationship to 7-methylguanine and was suggested as a source of anomalous base-pairing and hence mutagenicity. The proposed equilibrium offers an explanation for the

observations of Kohn *et al.*[52] on the alkylation of DNA with $^{14}$C-methyl labelled nitrogen mustard. Interstrand cross-links were found to have increased stability (as measured by loss of radiolabel) at pH values where ring-opening of 7-alkylguanines is predicted. DNA, from *E. coli* grown on [8-$^{14}$C]guanine, in the presence of an oxidising agent lost an amount of $^{14}CO_2$ proportional to the number of interstrand crosslinks. Thus, de-formylation to give $^{14}$C-formic acid was followed by oxidation to $^{14}CO_2$. The data was presented as independent evidence for N-7 alkylation of DNA guanine and correlation of alkylation with loss of $^{14}CO_2$ demonstrated that at least one end of interstrand crosslinks was anchored to guanine N-7.

An early study[62] with mono- and di-functional sulphur mustards suggested that the only difference between them was the capability of the latter to di-alkylate in either an inter- or intrastrand sense. Such crosslinking is now regarded as fundamental to the cytotoxic character of alkylating agents. Interstrand crosslinking was first suggested by physical measurements on DNA treated with di-functional alkylating agents, e.g. reversible denaturation was observed when DNA was treated with nitrogen mustard implying a bridge which maintained the relative positions of the two constituent strands. Studies on the correlation of degree of alkylation and toxicity in bacteriophage systems emphasise the greater biological activity of di-functional alkylating agents. Assay of viable phage immediately after exposure to an alkylating agent provides a measure of the efficiency of alkylation. The superiority

of di-functional alkylating agents was reproduced in bacteriophage containing single stranded DNA, which may indicate an important role for intrastrand crosslinks. Immediate inactivation of the phage has been attributed to[63,64] the alkylation event, but subsequent increases in activation suggest repair mechanisms such as de-purination reactions.




(41)

$H_3CO_2SOCH_2CH_2CH_2CH_2OSO_2CH_3$

(42)

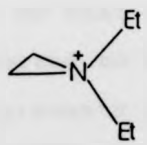
The T-7 bacteriophage has often been used for studies with alkylating agents because it has a small, relatively easily studied DNA molecule. Working with sulphur mustard (2) and S-2-chloroethyl-2-hydroxy-1-mercaptoethane (41) it was shown[62] that at the mean lethal dose of the bifunctional mustard (2) there were 1.3 moles of di-(guanin-7-yl ethyl)sulphide and 7 moles of monoalkylated product per mole of DNA polymer. The mean lethal dose of the monofunctional mustard (41) represented 280 moles of monoalkylated product per mole of DNA. Estimation of the crosslinks showed that approximately a quarter were interstrand alkylations, but the majority were intrastrand events. Incubation of phage treated with mustard (2) gave no further inactivation, but similar

treatment of phage with monoalkylated DNA caused progressively greater inactivation. This was related to depurination with loss of about seven purines becoming lethal.

Bacteriophage T7 DNA was also used to compare alkylation by nitrogen mustard with that of ethyl methanesulphonate and the difunctional analogue 1,4-dimethanesulphonylbutane (42) (busulphan or myleran)[65,66]. Qualitatively the results for the monofunctional agent are similar to those obtained for (41) above. Curiously the behaviour of busulphan (42) followed that of the monoalkylating agent; no interstrand crosslinks were detectable and there was a steady increase in lethality on further incubation of the sample. Intrastrand crosslinks were not excluded and may be responsible for the observed difference in human response to busulphan and ethyl methanesulphonate. Nitrogen mustard alkylation gave complex results. On incubation of the phage for varying times lethality was observed to increase to a maximum shortly after exposure, but a decrease to a minimum after two days was followed by a further increase. The decrease in lethality was associated with a loss of crosslinks probably due to depurination of one end of the diguaninyl bridge and was not associated with host cell reactivation. Random depurination was advanced as an explanation for the eventual increase in lethality. These workers also reported interstrand crosslinking by diepoxybutane which confounded their argument for the lack of such behaviour with busulphan. The rigid Watson-Crick structure of

DNA precluded crosslinking with a four carbon bridge, but if it was possible for diepoxybutane then this structure cannot be as rigid as the model suggests.

Price *et al.*[67] studied the relative reactivities of monofunctional analogues of nitrogen mustard in their alkylation of DNA components. Short exposure of DNA to N,N-diethylaziridinium chloride (43) yielded only N-7 alkylated guanine, but increased reaction times allowed isolation of N-6 alkylated adenine. This was assumed to be the rearrangement product of N-1 alkylation. The longer exposure resulted in considerable denaturation of the DNA and control experiments with denatured DNA over short reaction times gave equal yields of alkylated guanine and adenine. Comparison with monomeric guanosine showed a fifty-fold greater reactivity of guanine in helical DNA and a seven-fold greater reactivity in denatured DNA. Conversely, adenine in native DNA was least reactive and in denatured DNA was estimated to react five-fold faster than in poly A. Interestingly, in addition to 7-alkylguanine a 7,9-dialkylated species was isolated after imidazole ring opening; this species has not been reported in other studies.



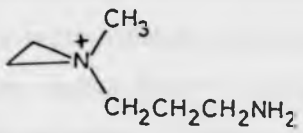

(43)

### 1.4.2 Interaction of Alkylating Agents with Biological Macromolecules other than Nucleic Acids

The purine and pyrimidine bases of nucleic acids are not the sole targets for alkylation within these macromolecules. It has variously been reported that alkylating agents react primarily with the phosphate groups of DNA[68] and that alkylation of the phosphates was negligible[44]. Phosphate esterification is now established, but its importance is uncertain: mono-functional alkylating agents should be quite as effective as their difunctional analogues in forming phosphate esters, yet as previously noted there is a marked difference in the clinical effectiveness of the two classes. Additionally, esterification of phosphate groups would lack base specificity making chain scission more random than is observed. An estimation of the extent of phosphotriester formation has been made on DNA treated with radio-labelled methyl methanesulphonate[69]. Judging that all of the $^{14}$C-methyl groups bound to purines would be heat labile, the radioactivity remaining after heating was assigned to stable phosphodiesters. Statistically the phosphotriesters stand an equal chance of breaking any of the three bonds to give a stable product so that one-third of the radioactive methyl groups are expected to be lost and the measured radioactivity then represents only two-thirds of the total phosphate alkylation. Allowing for this loss and for 3% of heat-stable alkylation on cytosine and adenine N-1, the authors arrived at a figure for phosphate alkylation

of 5 to 18½% of the total alkylation. According to a study on the hydrolysis of phosphate esters[70], however, there is not an equal probability of each of the bonds in the phosphotriester breaking. This work suggested that the small alkyl group would be preferentially lost, consequently formation of phosphotriesters would not lead to DNA chain breakage.

A study of the alkylation of DNA by nitrogen mustard at 0°C suggested[71] the existence of a labile intermediate, possibly conforming to transalkylation from a phosphotriester to form an N-alkyl bond (*cf.* ref. 68). This view, although not uncommon at the time, has not been substantiated although the concept of electrostatic interaction between negatively charged phosphate groups and the aziridinium cation may explain the experimental observations. This electrostatic interaction was used[72] to explain the kinetics of alkylation of guanine in DNA. The ionised phosphate units of the sugar-phosphate backbone would also reinforce the nucleophilicity of the phosphate. As the reaction proceeds the rate enhancement is lost due both to charge neutralisation arising from phosphotriester formation and from protonation of the alkylating agent's nitrogen function, subsequent to alkylation. The electrostatic argument is supported by a decrease in the rate of RNA alkylation with the increasing $Na^+$ concentration for the diethylaziridinium ion ($R^+$), (43), but not for the N-(aminopropyl)-N-methylaziridinium ion ($R^{2+}$) (44). Increasing concentrations of $Na^+$ would readily displace



(44)

the diethylaziridinium ion from ion pair association with the tRNA, but the double cation would be more stable. A ready analogy is the observation that $Mg^{2+}$ associates more strongly than $Na^{+}$ with RNA.

There is no doubt that alkylating agents react extensively with the protein constituents of cells, e.g. key enzymes. However, DNA is generally favoured as the important target with respect to cytotoxicity, principally on the stoichiometric grounds outlined above. In complex biochemical systems such as animals it is perfectly possible that undiscovered enzyme systems with a role central to replication exist. Should this be the case then this hypothetical enzyme system might be a better candidate for alkylation than DNA by virtue of greater chemical reactivity. It has been suggested[73] that the coenzyme NAD is the target for lethal alkylations within tumour cells. The sensitivity of glycolysis to a wide range of alkylating agents has been demonstrated . Tumour cells depend more heavily on anaerobic glycolysis than do healthy cells and suffer a depression in their NAD level when alkylated thereby interfering with glycolysis during the conversion of 3-phosphoglyceraldehyde

to 1,3-diphosphoglyceric acid. Selective depression of NAD has been demonstrated at an alkylation level which did not interfere with other sensitive sites[74], but under similar conditions other workers have demonstrated alkylation of DNA without affecting the NAD[75]. Interference with glycolysis is not universal in cells exposed to alkylating agents. Therefore, it is unlikely that cytotoxic effects are exerted through interference with NAD. However, this example illustrates the possibility of killing cells *via* protein alkylation.

Nitrogen mustard was known to prevent polymerisation of deoxyhemoglobin S (Sickle cell hemoglobin) in concentrations, which caused insignificant changes in physical parameters such as oxygen affinity[76]. Work with radiolabelled mustard indicated alkylation of a peptide containing the first eight amino acids of the β peptide chain. Of the constituent amino acids, the β2 histidine was considered to be the most likely alkylation site, but the label was lost during hydrolysis of the peptide. Therefore, no definite assignment could be made. Direct analysis by n.m.r. spectroscopy avoids the degradation step and is an excellent method for determining the alkylation site. Accordingly, Roth *et al.* claimed to have demonstrated alkylation of the β2 histidine residue by an n.m.r. method[77]. Proton n.m.r. spectra were presented which showed the gradual loss of intensity of a resonance, with increasing concentration of nitrogen mustard (1). By comparison with a mutant hemoglobin in which the β2 histidine was replaced with arginine, the disappearing

resonance was attributed to the histidine and from the chemical shift the suggested assignment (as the C-2 H) is reasonable. However, the poor quality of the spectra reflected in a gradual broadening of the resonances with increasing mustard concentration, could support the suggestion that the histidine resonance has become so broadened as to be undetectable. In such a complex system all the observed resonances cannot be assigned, so the lack of an explanation for changes in other resonances is not surprising. However, with the 20-fold molar excess of nitrogen mustard employed a very great deal of information would have been available from the alkylating agent's signals. Unfortunately, these were not discussed. The problems of spectral interpretation encountered in this study could be avoided by the use of a specifically labelled nitrogen mustard such as the one described in Chapter 3 of this thesis.

A natural abundance $^{13}C$ n.m.r. study of the alkylation of the histidine residue of hen egg white lysozyme with iodoacetate overcame the problem of product assignment[78]. There are 28 non-protonated aromatic carbons in lysozyme, most of which were assigned before the study commenced. Using N-acetyl-L-histidine as a model, the effect of pH variation on the chemical shift of C-1 of the amino acid and its three carboxymethylated derivatives was examined. Alkylation of N-acetyl-L-histidine gives four non-protonated resonances in the aromatic region of the $^{13}C$ n.m.r. spectrum. One of the resonances is due to unreacted amino acid, one to N-2, N-4 dicarboxy-

methylhistidine and the others to N-2 and N-4 carboxymethylhistidine. Recording a series of spectra over a pH range of 4-10 affords four titration curves. One of these corresponds to that found for N-acetyl-L-histidine, while another is pH insensitive and therefore arises from the di-alkylated species which retains no titratable functions. The N-2 and N-4 monoalkylated products could be distinguished both by their pK values and by their relative yields.

Examining the aromatic chemical shifts of alkylated lysozyme over a wide pH range yielded only two titration curves, one of which was for the intact enzyme. The second titration curve closely followed that of N-4 carboxymethylthistidine and was assigned on that basis. It is clear from the spectra that only one product is obtained, the identification of which is achieved by an enviably straightforward procedure which is emphasised by the choice of $^{13}C$ n.m.r.

Lysozyme is a comparatively small molecule and is thus readily studied. Recently a study of the reaction of lysozyme with ethylenimine (aziridine) was published[79]. The authors distinguished four singly modified products, each of which was found by product analysis to have been alkylated on a carbonyl group. Alkylation of the enzyme in the presence of a non-nucleophilic substrate failed to produce the most labile of the four products suggesting that this target was in the active site. Two of the products were 2-aminoethyl esters of glutamate-35 and aspartate-52,

while the remaining derivative was not fully characterised. The enzyme derivatives alkylated at glutamate-35 and aspartate-52 retained their binding affinity for normal substrates, but failed to show any turnover, implying that these sites were essential for enzyme activity. The labile product might have been characterised had a direct observation technique been employed; alkylation sites were determined by sequence analysis.

Both the difunctionality of effective alkylating agents and the reactivity of nucleophilic protein sites are combined in the formation of DNA-protein crosslinks. Many workers have reported this kind of crosslink[80], but until recently their nature had not been studied. On treating L1210 cells and nuclei with nitrogen mustard (1), Kohn *et al.*[81] were able to show that a subset of nuclear proteins were bound to DNA through alkylated purine residues. Protein was released from the complex by mild acid hydrolysis at a rate comparable with the elimination of 7-alkyl guanines under similar conditions. Alkaline pre-treatment prevented the release of protein by acid hydrolysis, again consistent with guanine N-7 (base-catalysed opening of the imidazole ring prevents the acid hydrolysis of alkylated guanine). The authors argued that the span of the nitrogen mustard (7.5 Å) restricts the choice of protein to be crosslinked and therefore it must be closely associated with guanine. Gel electrophoresis of protein from the DNA-protein complexes obtained from alkylation of either nuclei or whole cells showed similar bands. Thus, a fixed relationship between DNA and protein probably exists under both conditions.

Other work by these authors showed that protein-protein crosslinking was slower than that between DNA and protein. With nitrogen mustard there were several protein-DNA crosslinks for each DNA-DNA interstrand crosslink. This behaviour may be of great significance in explaining the actions of (1) on the cell.

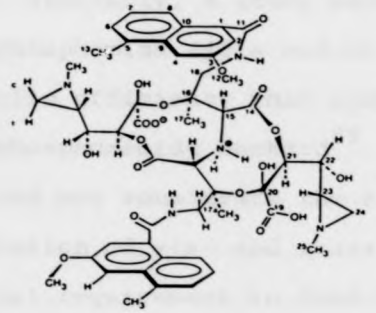
## 1.5 RATIONAL DESIGN OF ANTI-CANCER DRUGS

Following the observation that nitrogen mustard (1) was an effective anti-tumour drug, significant effort has been expended in producing modified nitrogen mustards. The results are very disappointing and of all the many variations on the mustard theme few have entered clinical use. Most workers reasoned that altering the substituents on nitrogen would affect the lipophilicity of the resultant drug (and hence its distribution within the patient) and would alter the reactivity of the remaining alkylating functions. For example, the use of steroidal carriers for the *bis*- chloroethylamine moiety improves diffusion through the blood brain barrier[82]. Replacement of the methyl group of (1) with an aromatic substituent reduces the mustard's basicity, hence decreasing its reactivity, whereas inclusion of an aziridine function was hoped to increase reactivity in tumour cells which were more acidic than host cells.

The only clear information to emerge from this work is the general requirement for at least two alkylating arms. Because of the poor return on the time taken in synthesising the compounds to be tested this modification approach has been largely abandoned, except by certain Russian groups. Instead the enormous variety of chemicals available in Nature has been exploited in systematic screening of plant extracts and microbial fermentation products.

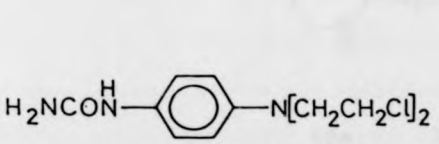
Carzinophilin A (45) is secreted by a *Streptomyces* microbe and has long been known to be an effective anti-cancer drug, although its high toxicity makes its clinical use problematical. Recently the structure of this complex molecule has been published and exhibits several features which rationale design would have suggested[83]. The synthesis of such a structure would have posed considerable problems. The drug has two naphthalene groups capable of intercalating the DNA of its target. A complex cyclic peptide holds the naphthalene groups at the appropriate distance to insert between base pairs, but the configuration of this peptide is claimed to match the pitch of the DNA helix. Once in position, there are two aziridine rings which, following protonation, are capable of alkylating a suitable nucleophile crosslinking the DNA strands. Additionally, Carzinophilin A has a preference for the DNA minor groove, is electrostatically attracted to the phosphate backbone through a charged nitrogen and exhibits a specificity for guanine. The synthetic chemist would be hard pressed to have combined all these features within one molecule.

It has taken thirty years to elucidate the drug's structure.

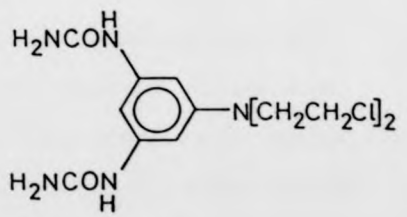


(45)

A certain amount of work on modification of mustards is still published, but attempts are made to ensure that the modifications are not random. Following reports that 4-[*bis*(2-chloroethyl)amino]phenylurea (46) was effective in causing regression of established tumours, synthesis of a *bis*-urea derivative (47) was



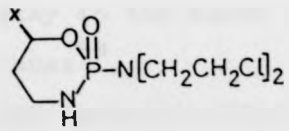
(46)



(47)

undertaken[84]. Although the synthesis was successful, its nine steps and non-quantitative yields do not make economic sense. Despite promising indications in tests

with experimental tumours, there are no apparent advantages over similar established drugs, e.g. chlorambucil. Similarly, a study with 5-fluoro- and 5-chlorocyclophosphamide (48 a and b) did not show any greater metabolic efficiency than cyclophosphamide in the yield of phosphoramide mustard[85]. Hence the substituents did not accelerate the β elimination step. However, resolution of *cis*- and *trans*-isomers did confirm a stereochemical requirement in that the *cis*-isomer was inefficiently metabolised compared with cyclophosphamide, but the *trans*-isomer was metabolised with comparable efficiency. In common with several similar synthetic studies, the authors were unable to make sensible use of their n.m.r. spectra because of the complex coupling exhibited in such systems.



48a X = F
48b X = Cl

The most promising approach to rational design of mustard-like drugs lies in the use of quantitative structure-activity relationships (QSAR). This has recently been applied to aniline mustards and has accorded results which account for the mechanism of action in terms of the electron density on the

mustard nitrogen and lipophilicity as measured in an octanol water system[86]. However, the model failed to account for the unusually high activity of chlorambucil. QSAR is strongly dependent on the biological test methods against which physicochemical parameters are plotted and these tests are necessarily simplifications of the clinical situation. However, a recent QSAR study of aniline mustards, which shows that there is not a direct relationship between mutagenicity and carcinogenicity, suggested that alkylation of DNA *via* aziridinium ions was competing with microsomal epoxidation of the aromatic ring followed by alkylation[87]. The epoxide pathway was advanced to explain the insignificance of electronic terms in the theoretical model employed and is consistent with shortcomings in the Ames test for carcinogens. In the intact animal, activated mustards may be epoxidised and scavenged within the liver, whereas less reactive mustards could stay in the blood stream to be absorbed by sensitive tissues[88].

The QSAR approach offers an opportunity for designing nitrogen mustards with the correct structural features for alkylating DNA, while simultaneously optimising the therapeutic index. To achieve this aim, information on mustard interactions with their chemical environment is required so that the theoretical models can be improved. High resolution n.m.r. spectroscopy combined with stable isotope labelling has the potential for rapid non-destructive direct observation of drug behaviour. This thesis sets out to demonstrate that

the technique of n.m.r. spectroscopy combined with stable isotope labelling can be usefully employed in the study of mustard chemistry.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 MATERIALS

### 2.1.1 Solvents

All solvents used were of AnalaR or equivalent grade unless cited to the contrary. Standard procedures were employed for purification and drying of other solvents which were stored in securely stoppered containers under dry nitrogen and sealed with Parafilm. Chloroform and deuteriochloroform were filtered through basic alumina immediately prior to use, to remove HCl and ethanol.

### 2.1.2 Chemicals

Chemicals were of the AnalaR grade where possible and were frequently purified before use, by standard procedures. Nitrogen mustard hydrochloride was twice recrystallised from acetone and was stored desiccated, as was also the $^{13}C$-labelled drug. Labelled precursors were obtained from BOC Ltd. Prochem, Deer Park Road, London, SE19 3UF and were used as received.

## 2.2 INSTRUMENTATION

### 2.2.1 N.M.R. Spectra

All spectra, except those obtained on a Bruker WH400 instrument, were recorded by the author. $^{1}H$ n.m.r. spectra were recorded on the following instruments:

(a) Bruker (model WH90) 90 MHz $^{1}H$ n.m.r. spectrometer,

or (b) Perkin Elmer (model R34) 220 MHz $^{1}H$ n.m.r. spectrometer,

or (c) Bruker (model WH400) 400 MHz $^{1}H$ n.m.r. spectrometer.

Peaks are designated by their relative chemical shift ($\delta$) in parts per million, followed in brackets by their relative integral (e.g. 2H) to denote number of hydrogen atoms, their multiplicity (s = singlet, d = doublet, t = triplet, q = quartet and m = multiplet), and the spin-spin coupling constant, J, in Hertz, where appropriate. Spectra were recorded at 220 MHz unless otherwise stated. Solutions in $^{2}H_2O$ were referenced to the methyl resonance of TSS ($(CH_3)_3SiCH_2CH_2CO_2^-$) as zero delta; solutions in organic solvents used TMS as a reference at zero delta.

$^{13}C$ n.m.r. spectra were recorded on instruments (a) or (c) above, operating at 22.63 MHz and 100.26 MHz, respectively. Peaks are assigned as for $^{1}H$ n.m.r. spectra. The spectra were referenced to 1,4-dioxan ($\delta$ = 67.4) unless indicated to the contrary. The spectra were run with broad band $^{1}H$ decoupling and only showed singlets, except as noted.

All n.m.r. spectra were assigned by the use of reference tables, comparison with n.m.r. spectra of model compounds or authentic materials or by noting intensity enhancements on the addition of authentic material.

### 2.2.2 Optical Rotations

Optical rotations were measured on a Bendix NPL automatic polarimeter (model 143D) using a 1 cm pathlength cell. Before each measurement the temperature was noted and the instrument was calibrated with a standard sucrose solution so that values are expressed as specific rotations, $[\alpha]_D^{^oC}$.

### 2.2.3 Gas Liquid Chromatography (g.l.c.)

G.l.c. analyses were performed using a Pye-Unicam (model 204) flame ionisation gas chromatograph with nitrogen as the carrier gas. Comparison with authentic materials and co-injections were attempted where possible.

### 2.2.4 Ultra-Violet (u.v.) Spectra

Ultra-violet spectra were recorded with a Pye-Unicam (model SP800) ultra-violet spectrophotometer.

### 2.2.5 Mass Spectra

Electron impact mass spectra were recorded with a Kratos MS80 instrument. Peaks are quoted as m/z, followed by their percentage of the base peak, which is indicated as 100%. The molecular ion is designated as $M^+$.

### 2.2.6 Measurement of pH

Solution pH values were determined using a Corning PT1-5 digital pH meter equipped with a Pye-Unicam 405 combined electrode, or for small samples, an $EL28_A$ combination electrode. Quoted pH values are direct readings and have not been adjusted for $p^2H$ in $^2H_2O$ solutions. Before use, the pH meter was calibrated with pH 7.0 buffer and another buffer in the region of the anticipated pH value.

## 2.3 METHODS

2.3.1 Solutions in organic solvents were dried using $Na_2SO_4$ which had been dried at 110 $^oC$ for a minimum of 48 h. Evaporation under reduced pressure refers to removal of bulk solvent at 20 $^oC$ and *ca*. 20 mmHg using a rotary evaporator, unless noted.

Glassware was dried by storage at 110 $^oC$ overnight and cooling in a desiccator over silica gel. Glassware used in dissolving metal reductions was treated as above and was then flame-dried, with cooling under dry nitrogen.

2.3.2 Solutions of sodium methoxide in methanol were prepared under dry nitrogen from anhydrous methanol and freshly cut sodium. The sodium was cleaned by brief immersion in anhydrous methanol before dissolving in the bulk methanol. Titration of aliquots against aqueous standard HCl solutions (prepared from BDH Ltd. cvs ampoules) was performed using phenolphthalein as indicator.

2.3.3 Thin layer chromatography was carried out analytically on pre-coated aluminium backed plates (Merck, Kieselgel $60F_{254}$, cat. no. 5554) or preparatively on glass plates coated with Merck Kieselgel G (type 60, cat. no. 7731) in 0.25 mm layers. Samples were applied as solutions in the eluting solvent. Eluting systems used were:

(1) Merck 5554 plate, 880 ammonia absolute ethanol 25:75, 1:3 [after development, plates were air-dried, sprayed with ninhydrin (BDH aerosol or 0.5% solution in butanol) and heated to visualise spots].

(2) Merck 5554 plate or preparative plate, ethyl-acetate/methanol 4:1.

Solvents were freshly made up and are quoted as volume/volume ratios. Standards were included when available, frequent use of co-application being made. Plates were used within a few weeks of purchase (Merck), and home-made plates were dried at 110 $^{o}C$ to activate them before use.

Free SH was detected and determined by Ellman's test. Aliquots (0.5-1 $cm^3$) of liquid ammonia reactions were evaporated in a nitrogen stream, dissolved in 1 $cm^3$ of pH 8 *tris* buffer and treated with Ellman's reagent (100 µl of a 4 $mg/cm^3$ solution) (Fig. 2.1). Free SH was indicated by a yellow colour which was quantified by spectrophotometric observation at 400-420 nm.

### 2.3.4 Ion Exchange Chromatography

Ion exchange columns were prepared as a slurry of 50 g of CG120 type I resin in water in a 2 cm OD column. Before introduction of the sample the column was equilibrated with the eluting solvent. Chromatograms were always performed with the resin in the $H^+$ form.

### 2.3.5 Sorensen's buffer for reactions monitored by n.m.r. spectroscopy.

Citric acid monohydrate (38 mg) and disodium phosphate dihydrate (290 mg) were made up to 10 $cm^3$ with $^2H_2O$ to form a 0.1 M buffer solution of pH 7.2.

## 2.4 HANDLING PROCEDURE FOR TOXIC MATERIALS

All of the mustards used are known carcinogens and severe vesicants. Reaction products other than those arising from hydrolysis, which have been shown to be unexceptionally reactive, must be assumed to share the harmful characteristics of the parent mustards. Azide

derivatives may also be explosive. Contaminated glassware was soaked overnight in an ammonia bath (1:1 water/.880 ammonia). Disposable gloves, worn when handling vesicant solutions were similarly decontaminated before incineration. Reactions and manipulations of mustards were performed within a tray which was periodically soaked with ammonia solution to control spillages. Reaction mixtures were disposed of by ammonia treatment. Spills on gloves, etc., were lavaged with 5% sodium thiosulphate solution which was always kept available and was freshly prepared.

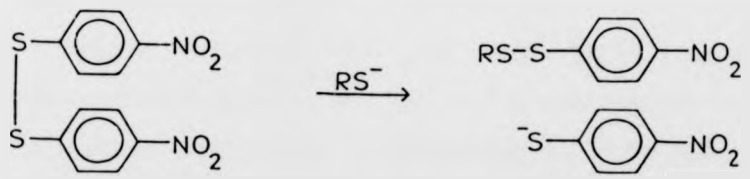


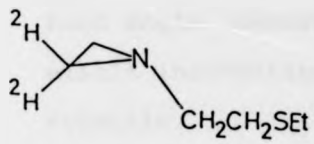

Figure 2.1

# CHAPTER 3

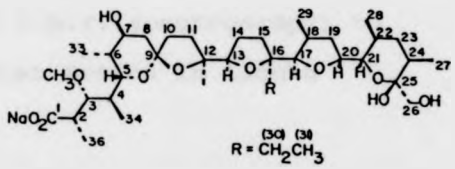
# SYNTHETIC PROCEDURES

## 3.1 INTRODUCTION

In 1951 $^{14}C$-methyl labelled nitrogen mustard was employed to determine the distribution of the drug in normal and leukemic mice. This showed loss of label as $^{14}CO_2$, $^{14}C$ metabolites in the urine and a preferential fixation in the lymph nodes, adrenals, thymus and brain of leukemic mice compared with the controls. Lack of chemical characterisation of the site of alkylation is a general feature of such early radiolabelling studies, the drug distribution being of prime interest. More refined work has utilised materials labelled with stable isotopes to imply aziridinium ion intermediacy in the alkylation step. Colvin *et al.*[30] employed [2,2'-$^2H_2$]-N-(2-chloroethyl)-2-chloroethanamine (5a) and a gas-chromatographic/mass-spectral technique, purportedly to show that reaction with ethanethiol proceeded *via* aziridinium ion intermediates (19a) and (49) to give N-2-(ethylthio)ethyl-2-ethylthioethanamine (24). However, the possibility of a gratuitous, fast pre-equilibrium formation of aziridinium species (19a), product being formed by direct displacement of chloride from (5a), was not considered. Furthermore, the yield of dithioether (24) was not given.




(49)

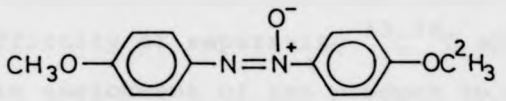



(50)

Fourier transform n.m.r. spectroscopy has been used to study numerous reaction pathways and in conjunction with specific labelling with stable isotopes it is a technique well suited to study the chemistry of nitrogen mustard in solution. Two recent examples of the technique follow. In a biosynthetic study[89] combinations of $^{13}C_1$- and $^{13}C_2$-labelled acetate, propionate and succinate were fed to cell cultures producing the polyether antibiotic monensin A (50). This produced a series of partially $^{13}C$-labelled antibiotics in which the biosynthetic origin of all the carbon atoms in monensin A was established. Furthermore, the complete assignment of its $^{13}C$ n.m.r. spectrum was made possible. Making use of the difference in chemical shifts for carbon atoms attached to $^{16}O$ or $^{18}O$ produced from [1-$^{13}C$,$^{18}O_2$]-propionate or [1-$^{13}C$,$^{18}O_2$]-acetate, the origin of six of the oxygen atoms in the antibiotic was deduced. Cultures grown in an atmosphere of $^{18}O_2$ gave monensin A for which the remaining ether oxygens could be assigned.

Boden *et al.*[90] achieved selective deuteriation of the benzene nucleus and side-chains of some 4,4'-

bisalkoxyazoxybenzenes (e.g. (51)) and used a C-C-$^2$H bond angle (determined by $^2$H n.m.r. spectroscopy) to elicit information on molecular motion in liquid crystals.



(51)

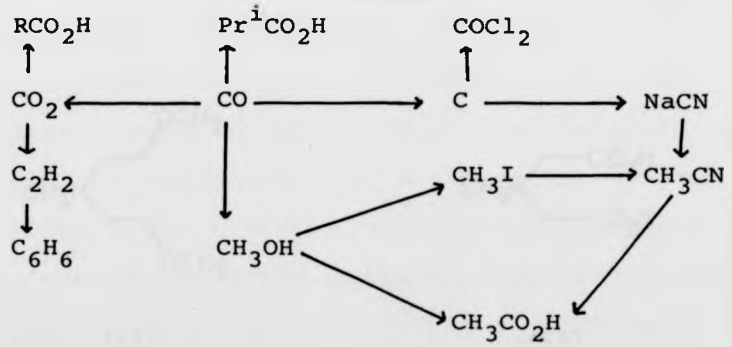
## 3.2 SYNTHESIS OF ISOTOPICALLY LABELLED MOLECULES

Efficient isotopic labelling ideally requires label introduction late in a synthetic sequence and in a high yielding step. However, labelling in the carbon skeleton frequently means building onto the labelled moiety from the outset, although with complex molecules an increase in the overall yield may be achieved by convergent synthesis. Extensive investment of time to optimise reaction conditions or to proceed from the most simple labelled reagent may not represent an overall economy, especially when labour is expensive or time is short. Syntheses for molecules so common that they are not otherwise produced on a laboratory scale are frequently devised when elaborating isotope, but practical difficulties often dictate purchase from a commercial source. The specialised apparatus required for certain reactions,

e.g. the catalytic reduction of $CO_2$ to MeOH exceeds the cost of isotope on the scale used.

Separation of $^{13}C$ is achieved by cryogenic distillation of carbon monoxide using efficient columns which are maintained in careful equilibrium so that the heavier $^{13}CO$ may be removed at the base. Species containing different oxygen isotopes are also fractionated and the difficulty of separating $^{13}C^{16}O$ and $^{12}C^{18}O$ limits the isotopic enrichment of the product to about 95 atom % $^{13}C$. Higher enrichments may be achieved by scrambling the various species catalytically before a further distillation. After separation, the carbon monoxide is converted into a range of intermediates (Scheme 3.1) which are supplied commercially.

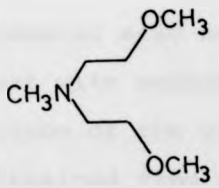
Scheme 3.1



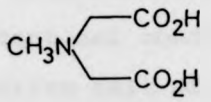

## 3.3 SYNTHESIS OF [2,2'-$^{13}C_2$]-N-(2-CHLOROETHYL)-N-METHYL 2-CHLOROETHANAMINE (1a)

Nitrogen mustard can be produced by alkylation of methylamine with 2 mol equivs. of ethylene oxide and chlorinating the resultant diol (13). This route has

been used for preparing $^{14}C$-methyl labelled mustard and from the viewpoint of specific labelling is viable only for labelling the methyl group and nitrogen atom of (1). Deuteriated mustards have been prepared by reducing N-carboxymethyl-N-methylglycine dimethyl ester (52) with lithium aluminium deuteride and chlorinating the resultant diol. $^{2}H$ N.m.r. spectroscopy does not possess the resolution to distinguish easily the products of the solution chemistry of nitrogen mustard and therefore a synthesis was devised for nitrogen mustard bearing carbon labels directly bonded to chlorine, i.e. attached to the potential leaving groups. Monitoring the fate of the labelled atoms directly by $^{13}C$ n.m.r. spectroscopy should be feasible. Location of $^{13}C$ atoms in products of reactions could demonstrate the intermediacy of aziridinium ions.



(52)



(53)

Scheme 3.2 shows the route used to make the title compound. This incorporates the isotope in the first step but the bromination of acetic acid is a clean, high-yielding reaction from which [1-$^{13}C$]-bromoacetic acid was readily obtained pure by distillation. Dibromoacetic acid was not observed in the reaction

mixture. Formation of carboxymethyl-N-methylglycine (53) from chloroacetic acid and methylamine is an established procedure[91]. However, chlorination of acetic acid to give chloroacetic acid is not so experimentally convenient as bromination to yield bromoacetic acid and the chlorination procedure also gave dichloroacetic acid. Therefore, the established procedure for making acid (53) was adapted to allow for the greater reactivity of bromoacetic acid towards halide displacement. Instead of treating the halogeno-acid with 2 mol. equivs. of base and then adding the amine, the acid was neutralised and the second mol. equiv. of base was added with the amine. This method avoided exposing the bromoacetic acid to an excess of hydroxide and no displacement of bromide by hydroxide was observed. Attempts to use methylamine as the sole base species gave a mixture of N-methylglycine and the di-acid (53). Chloroacetic acid and bromoacetic acid were each allowed to react with methylamine under identical conditions; a comparison of the yields of the barium salt of di-acid (53) obtained from these experiments showed a marked advantage in using bromoacetic acid. Later work demonstrated a large variation in the yield of barium salt. This was reduced by precipitation of the $BaCl_2.2H_2O$ used from water with ethanol and by careful drying prior to use. However, this step could be avoided by separating the di-acid (53) from the reaction mixture by the use of a column containing a strong acid ion exchange resin and elution with water. Monitoring reactions between bromo-

acetic acid and methylamine by $^{1}H$ n.m.r. spectroscopy showed that all of the halogeno-acid was invariably consumed, but some N-methylglycine was formed and this was present in early fractions from the ion-exchange column.

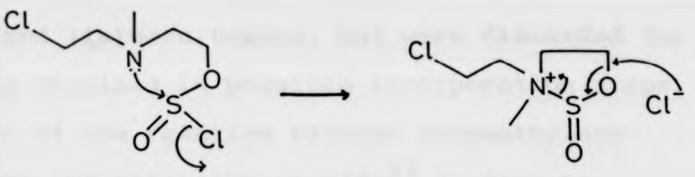
Esterification of di-acid (53) was achieved by heating in methanolic HCl. It was necessary to dry the resultant N-carboxymethyl-N-methylglycine dimethyl ester (52) hydrochloride before treating with sodium methoxide. Reduction of the di-ester (52) with lithium aluminium hydride gave N-2-hydroxyethyl-N-methyl-(2-hydroxy)ethanamine (13).

It was anticipated that the chlorination of diol (8) might proceed *via* intermediate aziridinium ions and yield a mixture of labelled mustards, (1a), (1b) and (1c). It had been shown that chlorination of [1-$^{13}$C]-(methylthio)ethanol gave a 1:1 mixture of [1-$^{13}$C]- and [2-$^{13}$C]-1-chloro-2-(methylthio)ethane irrespective of the chlorinating agent employed[92]. This was interpreted as the consequence of neighbouring group participation by sulphur giving a cyclic thiiranium ion which was captured by chloride. The lone pair of the nitrogen atom of intermediates derived in the chlorination of diol (13) could effect an analogous intramolecular displacement to form an aziridinium ion. In practice, reaction of diol (13) with thionyl chloride in either benzene or chloroform gave specifically [2,2'-$^{13}C_2$]-N-(2-chloroethyl)-N-methyl-2-chloroethanamine (1a). Alkyl chlorosulphites can be isolated from mixtures of thionyl chloride and alcohols. If chlorosulphites are intermediates in the conversion

Fig. 3.3 Reaction of 2-hydroxy-(N-2-hydroxyethyl)-N-methylethanamine with thionyl chloride.

Path A


Path B




Path C

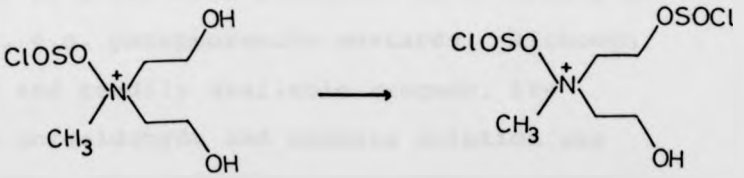
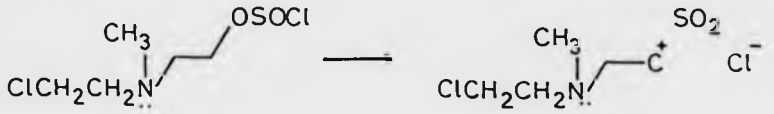
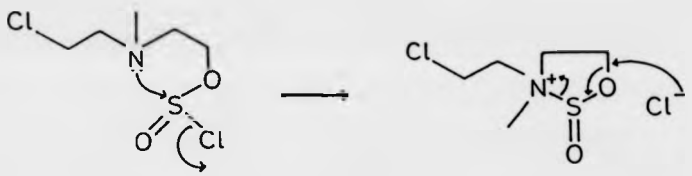


Fig. 3.3 Reaction of 2-hydroxy-(N-2-hydroxyethyl)-N-methylethanamine with thionyl chloride.

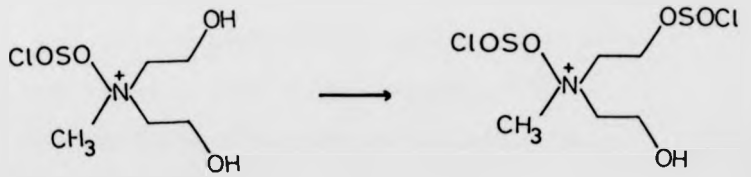
Path A




Path B



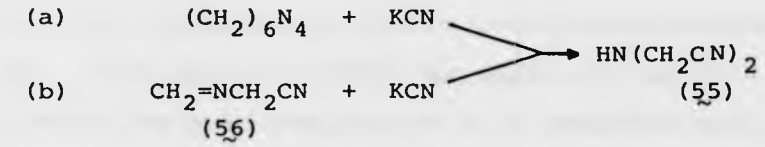

Path C



of diol (13) into the labelled mustard (1a) the polarised C-O bond is expected to encourage formation of an aziridinium ion (Scheme 3.3, path A). Formation of the cyclic intermediate (54) and cleavage of this as shown (3.3B) ensures retention of label specificity. Alternatively, quaternisation of the mustard nitrogen would occupy the lone pair and preclude aziridinium ion formation. Reaction of the quaternary compound with thionyl chloride could lead *via* an alkyl chlorosulphite to specifically labelled mustard.

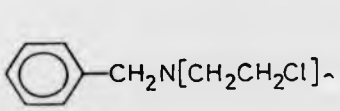
Other approaches to the synthesis of specifically labelled mustard (1a)were tested, but were discarded due to poor yields obtained in possible incorporation steps. Thus, a report of the reaction between hexamethylene-tetramine (HMT) and potassium cyanide[93] leading to N-cyanomethyl-1-cyanomethanamine (55) was investigated because $K^{13}CN$ is a readily available source of carbon label (Scheme 3.4a). The mustard (5a) derived from di-nitrile (55) is a suitable precursor of a variety of other mustards, e.g. phosphoramide mustards. Although HMT is a cheap and readily available reagent, its synthesis from formaldehyde and ammonia solution was examined to explore the option of introducing a label through either of these reagents. By a standard method HMT was produced in only 45% yield. More seriously, di-nitrile (55) could not be isolated in more than 20% yield, so a promising idea was abandoned.

Scheme 3.4

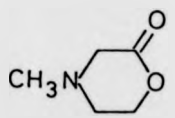



In another approach, the reaction between 2-aza-3-cyanoprop-1-ene (56) and cyanide was investigated (*cf.* Scheme 3.4b), but rejected due to poor yields.

For a possible general synthesis of labelled mustards, benzylamine was condensed with 2 mol. equivs. of bromoacetic acid to give N-benzyl-N-carboxymethylglycine from which N-benzyl-N-(2-chloroethyl)-2-chloroethanamine (57) was obtained in a similar manner to that described for mustard (1a). Hydrogenolysis prior to chlorination would give N-2-hydroxyethyl-2-hydroxyethanamine, a useful intermediate for entry into a range of N-substituted mustards.




(57)




(58)

To distinguish between the two chloroethyl arms of nitrogen mustard, a scheme was devised for selectively deuteriating one arm as in structure (1d) This material

would be useful for studying aspects of the reaction of nitrogen mustard with ammonia. Oxidation of diol (13) with silver carbonate on Celite gave N-methylmorpholin-2-one (58). Time did not permit the completion of this synthesis in which the next step was to be a reduction with $LiAl^2H_4$ to give (13b).

## 3.4 SYNTHESIS OF LABELLED AMINO ACIDS

### 3.4.1 Introduction

One of the original objectives of this project included the synthesis of some labelled amino acids for work not initially connected with nitrogen mustard chemistry. The intended experiments (metabolism of amino acids in whole animals) were not performed, but the amino acids prepared were all considered as substrates for alkylation by nitrogen mustard. Studies with [$^{13}CH_3$]-S-methionine were especially rewarding. The classical synthetic methods for amino acids produce racemates, whereas some modern methods have concentrated on the problem of asymmetric synthesis. Current methods for preparing amino acids mostly proceed from an α-keto-acid by one of three routes:

(a) conversion into a dehydroamino acid and hydrogenation aided by an optically active catalyst;

(b) reduction of a Schiff's base derived from an α-keto acid derivative and an optically active amine; and

(c) reduction of a Schiff's base obtained from an amine and an optically active α-keto acid derivative.

An illustration of one of these procedures is the variable temperature hydrogenation of a chiral Schiff's base derived from the ethyl ester of α-ketopropionic acid and (*S*)-phenylalanine using Raney nickel modified with

histidine[94]. Low temperature hydrogenation gave (*S*)-alanine of 60% optical purity. There was observed a decrease in optical purity to zero at 17°C and then an increase to give a maximum 43% optical purity of (*R*)-alanine at 50°C. The authors explain this as a rotation about the C-N bond to give 2 configurations, one of which predominates according to temperature, with hydrogen being delivered from the least hindered side.

Our synthetic targets were [2-$^{2}$H]-alanine, [1-$^{13}$C]-leucine and [$^{13}CH_3$]-methionine as optically pure (*S*)-isomers. Before describing how these labelled amino acids were prepared, current methodology for preparing amino acids and opportunities for introducing isotopic label(s) *via* these syntheses, are reviewed. For the preparation of a pure optical isomer of an amino acid two approaches are possible. The classical approach was to synthesise the racemate of an amino acid by, e.g. Strecker or Gabriel synthesis, Curtius or Hofmann degradation, alkylation of acetamido-malonate or oxidation of an amino-alcohol. The racemate was resolved by fractional crystallisation of diastereoisomeric salts formed on reaction with an optically active agent. More recently, enzymic methods of resolution have been employed, e.g. selective destruction of one enantiomer of an N-acetyl amino acid by hog kidney acylase. The second approach is to use an asymmetric synthesis of one enantiomer of an amino acid, analogous to the biosynthesis of amino acids.

Current Methods

(1) Corey's hydrazonolactone route (Scheme 3.5)

Corey has synthesised both enantiomers of the chiral precursor[95]. Thus, the hydrazonolactone arising from condensation with an α-keto ester may be obtained in either chirality. This molecule has limited conformational mobility so that reduction adds hydrogen from the least hindered side of the α-proton, i.e. from *cis*- to the C-2 proton of the indoline moiety. A methyl substituent as R' allows the liberation of amino acid which is 96-99% optically pure; also, the chiral reagent is regenerated.

(2) Addition of a nitrile to a chiral borane, e.g. di-isopinocamphenylborane.

Reaction with HCN and solvolysis of the boron complex yields an α-aminonitrile which is hydrolysed to the amino acid. The optical purity, e.g. 12.4% for valine from 2-methylpropionitrile, is poor compared with other enantioselective routes[96].

(3) Template reactions

The conformational rigidity of certain metal complexes makes them useful templates for chiral syntheses. The rhodium complex shown in Fig. 3.1 readily catalyses the hydrogenation of N-acyldehydroamino acids to the corresponding (*R*)-N-acylamino acids in high yields with excellent optical purities[97].

Scheme 3.5 Corey's amino acid synthesis.

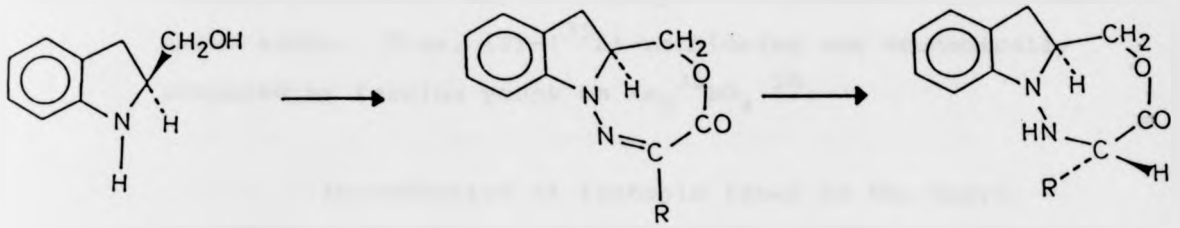
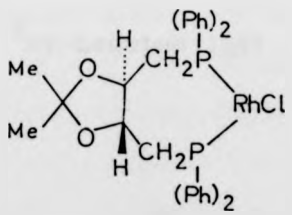



Fig. 3.1 Rhodium complex used as a template in the synthesis of amino acids.



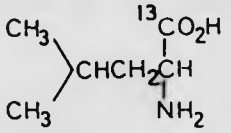
(4) Biochemical methods

Biological organisms, both healthy and nutrient demanding mutants, may be employed to synthesise and label amino acids. Thus, (*S*)-[$^{35}$S)-methionine was economically prepared by feeding yeast on $Na_2{}^{35}SO_4$ [98].

Introduction of isotopic label in the above procedures is conjectural, but labelling of the α-keto ester in 1, the HCN in 2 or the N-acyldehydroamino acid in 3 are all feasible. Exchangeable labels, e.g. deuterium introduced during a reduction step, may be lost during work-up procedures so proper account of this must be taken. The older synthesis of racemic amino acids are well provided with examples of isotope incorporation. Frequently simple, readily available compounds such as $K^{13}CN$ or $^2H_2O$ are employed and no modification of the established procedure is required.

## 3.4.2 Representative Syntheses of Labelled Amino Acids

### 3.4.2.a (*S*)-[1-$^{13}$C]-Leucine (59)

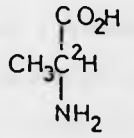


(59)

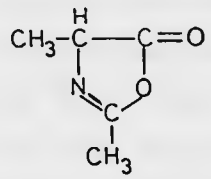
The Strecker synthesis is the most common procedure for carboxyl-labelled amino acids but is rarely employed in an unmodified form. Our method for the title compound is unusual in the use of aqueous ammonia to absorb $H^{13}CN$ liberated from $K^{13}CN$. Liberation of HCN is usually avoided, but was safely performed by protecting the outlet of an otherwise enclosed apparatus with NaOH traps to absorb any HCN not captured by the ammonia. Titration of the ammoniacal cyanide solution allowed the efficiency of this method to be assessed (93% of expected HCN detected by $AgNO_3$/KCl titration). Reaction with the aldehyde proceeds *via* cyanide addition to an iminoaldehyde and is the low yield step in Strecker procedures. We achieved an overall yield of 40% which corresponds to the anticipated yield of the stage of nitrile formation. A similar synthesis of [1-$^{13}$C]-methionine from 3-(methylthio)-propanal utilising an ethanol-ammonia solution of KCN gives a 60% yield and would bear investigation for leucine[99].

Acetylation and enzymic resolution with Acylase I gave 45% (*S*)-leucine from unlabelled material. It was anticipated that racemisation of the predominantly (*R*)-N-acetyl leucine and further enzymatic resolution of the racemate would be worthwhile; three such resolutions of 45% yield each would give a total recovery of *ca.*78% of the acetyl leucine as (*S*)-leucine.

3.4.2.b (*S*)-α-$^{2}$H-Alanine (60)



(60)

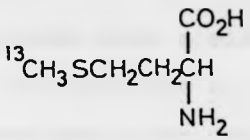


(61)

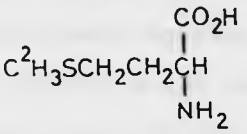
Conventional methods for α-deuteriation of amino acids, such as, Al(III)/pyridoxal-catalysed exchange in $^{2}H_{2}O$, are time-consuming and require large excess of isotope. We have devised a procedure that exploits the acidity of the α-proton in the azlactone derived from N-acetylalanine (61). Attempts to combine acetylation with deuteriation by reacting alanine with deuterioacetic acid and acetic anhydride gave a mixture of N-acetyl and α-deuterio-N-acetylalanine as well as unreacted alanine. Therefore N-acetylalanine was prepared and purified before exchanging the α-proton in a further step. Its amidic N-H and carboxyl H were exchanged by three evaporations from a small volume of $^{2}H_{2}O$. This was followed by treatment with $CH_{3}CO^{2}H$ at 60 °C. However, $^{1}H$ n.m.r. spectroscopy showed no deuterium incorporation under these conditions. Addition of acetic anhydride and heating for a further hour at 60 °C gave essentially complete deuteriation, as evidenced by $^{1}H$ n.m.r. spectroscopy. Repeating this preparation on a larger scale gave a product that was resolved with Acylase I to give

(*S*)-α-$^{2}$H-alanine (42.5% based on N-acetylalanine; 85% of theory). In principle (*R*)-α-$^{2}$H-N-acetylalanine recovered from this resolution could be racemised and hence, recycled. However, this would exchange the label unless the racemisation were performed in $^{2}H_2O$. This route benefits from its incorporation of label at a late stage and is especially economic in its utilisation of isotope.

3.4.2.c (*S*)-[$^{13}CH_3$]- and (*S*)-[$C^{2}H_3$]-Methionine (62 a & b)



(62a)



(62b)

The ready reduction of methionine to the thiolate anion of homocysteine makes labelling with iodomethane facile, but previous work has often produced racemised or contaminated products. Reduction with lithium in liquid ammonia followed by alkylation with iodomethane (monitored by estimating homocysteine) and crystallisation of the product from aqueous ethanol was established to be superior to the previous reduction with sodium. The addition of ammonium chloride as employed by Dolphin *et al.*[100] was omitted so that it did not co-crystallise with the methionine. However, we observed partial racemisation with this method, probably due to removal of

the α-proton by lithium amide in the reaction mixture. Addition of 1 mol. equivalent of ammonium chloride prevented this racemisation and did not contaminate the product when worked up in the usual way. Stoichiometric reduction of the methionine requires 3 mol. equivs. of lithium, one each for neutralising the ionised carboxyl group, removing a proton from the quaternised amine function and one for effecting the thioether reduction. Invariably as much as an extra 1 mol. equiv. was required when monitoring the reduction over a period of time to ensure complete reduction. Attempting the reduction with a single dose of 3.5-4 mol. equivs. of lithium left the blue colour of unreacted metal in ammonia solution undischarged. Alkylation of the thiolate anion was monitored by testing an aliquot of the solution with Ellman's reagent. This procedure was extremely reliable and represents the use of a cheap, optically pure starting-material ('chiral pool') for the preparation of a valuable, optically active product.

## 3.5 EXPERIMENTAL

### 3.5.1 Synthesis of [2,2'-$^{13}C_2$]-N-(2-Chloroethyl)-N-methyl-2-chloroethanamine

#### [1-$^{13}$C]-Bromoacetic Acid, 54 atom %

To a mixture of [1-$^{13}$C]-acetic acid (3.6 g), 91.1 atom %, acetic acid (2.4 g), acetic anhydride (1 drop) and purified red phosphorus (35 mg) was added bromine (18.7 g)

dropwise over a period of 4½ hours, whilst boiling under reflux with the exclusion of atmospheric moisture. Heating was continued for a further 1½ hours, the mixture cooled, treated with water (20 $cm^3$) and allowed to stand. The resulting solution was distilled under reduced pressure to give [1-$^{13}$C]bromoacetic acid as a white solid (10.56 g, 76%) b.p. 110-111 $^{o}$C at 30 mmHg, m.p. 30 $^{o}$C, $\delta_H$ ($^2H_2O$): 3.87 (s, $CH_2$).

[1,1'-$^{13}$C]-N-Carboxymethyl-N-methylglycine (53)

[1-$^{13}$C]Bromoacetic acid (10.56 g) in water (20 $cm^3$) was cooled in an ice-salt bath and treated with 6.65 M NaOH (11.35 $cm^3$) at such a rate that the temperature did not exceed 12 $^{o}$C. A mixture of 6.65 M NaOH (11.35 $cm^3$) and $MeNH_2$ (4.2 $cm^3$ of 28% w/v aqueous solution) was added with cooling to maintain a maximum temperature of 15 $^{o}$C before standing at room temperature for 3 days. A boiling solution of $BaCl_2.2H_2O$ (9.7 g in 30 $cm^3$ $H_2O$) was added with vigorous shaking, the resulting suspension refluxed for 30 minutes and a heavy deposit of the barium salt of (53) removed by filtration (6.86 g, 63% after drying). The salt was treated as a boiling aqueous suspension with 2.73 M sulphuric acid (9.25 $cm^3$) over 45 minutes, cooled and filtered through Celite. Boiling water extracts (2 x 15 $cm^3$) of the Celite plug were combined with the filtrate, evaporated under reduced pressure to *ca.* 15 $cm^3$ and crystallised overnight at 4 $^{o}$C after addition of an excess of methanol to give [1,1'-$^{13}C_2$]-N-carboxymethyl N-methylglycine (2.8 g, 51% from $MeNH_2$) $\delta_H$ ($^2H_2O$): 3.02 (s,

$CH_3$), 4.0 (s, 2 x $NCH_2$).

[1,1'-$^{13}$C]-N-Carboxymethyl N-Methylglycine Dimethyl Ester

The solution obtained by passing excess dry HCl through a suspension of N-carboxymethyl-N-methylglycine (2.8 g) in dry methanol, was boiled under reflux for 2 hours with exclusion of atmospheric moisture. Rotary evaporation gave a thick oil which was taken up in dry methanol, rotary evaporated, and pumped at high vacuum (0.05 mmHg) for 30 minutes. Dissolution and evaporation was repeated twice more, finally drying under high vacuum for 2 hours. The free base was liberated from its hydrochloride by addition of 3.57 M sodium methoxide solution in methanol (5.25 $cm^3$) followed by dropwise addition until a single drop caused a sharp rise in pH (to pH 8). After filtration through Celite and rotary evaporation, residual NaCl was removed by dissolving in diethyl ether, filtration, rotary evaporation and pumping under vacuum (0.05 mmHg) to leave a clear viscous oil of N-carboxymethyl N-methylglycine dimethyl ester (2.54 g, 77%). $\delta_H$ ($C^2HCl_3$): 2.5 (s, NMe), 3.45 (s, 2 x $CH_2$) and 3.7 (s, 2 x Me).

[2,2'-$^{13}$C]-N-(2-Hydroxyethyl)-N-methyl-2-hydroxyethanamine

To a stirred solution of $LiAlH_4$ (0.695 g) in dry ether (30 $cm^3$) at -18 $^oC$ and under a static pressure of dry nitrogen, was added dropwise over 1 hour a solution of N-carboxymethyl-N-methylglycine (2.54 g) in diethyl ether (30 $cm^3$) maintaining the temperature below -5 $^oC$. The suspension was boiled at reflux for 2 hours and then

the excess of $LiAlH_4$ was destroyed by successive addition of water (0.7 $cm^3$), 10% NaOH (1 $cm^3$) and water (2.1 $cm^3$). The product, a sticky white solid, was transferred to a small Soxhlet thimble, extracted overnight with diethyl ether, evaporated, dried over $Na_2SO_4$ and distilled to give [2,2'-$^{13}$C]-N-(2-hydroxyethyl)-N-methyl 2-hydroxy-ethanamine as a colourless liquid, b.p. 138-142 $^{o}$C at 20 mmHg: 1.5 g, 86%, $\delta_H$ ($^2H_2O$): 2.38 (s, $CH_3$), 2.71 (t, 2 x $CH_2N$, J = 12.2 Hz) 3.71 (t, 2 x $CH_2OH$, J = 12.2 Hz).

[2,2'-$^{13}$C]-N-(2-Chloroethyl)-N-methyl-2-chloroethanamine

To thionyl chloride (2.11 g) in benzene (3 $cm^3$), boiled under reflux in a closed apparatus connected to a static nitrogen supply, was added over 1 hour [2,2'-$^{13}C_2$]-N-(2-hydroxyethyl-N-methyl-2-hydroxyethanamine, 54 atom % (0.1841 g) and N-2-hydroxyethyl-N-methyl-2-hydroxyethanamine (0.8186 g) as a suspension in benzene (2 $cm^3$). The resulting mixture was refluxed for a further 2 hours and cooled overnight to yield a mass of off-white crystals. These were washed with petroleum ether (b.p. 40-60 $^{o}$C) and recrystallised from acetone to give [2,2'-$^{13}C_2$]-N-(2-chloroethyl)-N-methyl-2-chloroethanamine hydrochloride as white crystals: 1.175 g, 73%, $\delta_H$ ($C^2HCl_3$): 2.38 (s, $CH_3$-N), 2.82 (t, $CH_2$-N), 3.57 (t, J $^{13}$C-$^{1}$H 151.0 Hz, 0.1 x 3 H, $^{13}CH_2$-Cl, and 0.9 x 3 H, t, $^{12}CH_2$-Cl).

### 3.5.2 Synthesis of N-Benzyl-N-2-chloroethyl-2-chloroethanamine

Preparation of N-benzyl-N-carboxymethylglycine

To a stirred, cooled (5 $^{o}$C) solution of chloroacetic acid (4.72 g) in water (8 $cm^3$) was added 6.65 M NaOH (15 $cm^3$) at such a rate that the temperature did not exceed 10 $^{o}$C. Immediately upon completing the addition, benzylamine (2.37 g) was introduced maintaining the temperature at or below 20 $^{o}$C by occasional immersion in an ice bath. The clear solution was left at room temperature for 3 hours and then shaken with a boiling aqueous solution of $BaCl_2.2H_2O$ (6.4 g in 20 $cm^3$ $H_2O$), whereupon a dense white precipitate of the barium salt of the title compound separated. This was filtered off and dried over silica gel: 5.85 g, 68%. The barium salt was treated with 2.73 M sulphuric acid (3.3 $cm^3$) and boiled under reflux for 30 minutes. After filtration through Celite and rotary evaporation of the combined filtrate and Celite-washings, N-benzyl-N-carboxymethylglycine crystallised: 2.72 g, 52% from benzylamine.

Preparation of N-benzyl-N-carboxymethylglycine dimethyl ester

A suspension of N-benzyl-N-carboxymethylglycine (2.7 g) in methanol (20 $cm^3$) was saturated with dry HCl to give a clear solution and then boiled under reflux for 2 hours with the exclusion of atmospheric moisture. On cooling, the solution was evaporated, re-dissolved in methanol, evaporated again and pumped under vacuum (0.05 mmHg)

for 30 minutes. This procedure was repeated twice more to ensure that all free HCl was removed from the sticky oil. This was treated with one mol. equivalent of 3.57 M sodium methoxide in methanol (3.62 $cm^3$) and then made alkaline by dropwise addition of more base. Methanol was removed by rotary evaporation, the residue was taken up in diethyl ether and filtered through Celite. The filtrate was concentrated to leave the title ester as a clear oil: 2.1 g, 69%.

N-Benzyl-N-2-hydroxyethyl-2-hydroxyethanamine

A stirred solution of $LiAlH_4$ (0.423 g) in ether (30 $cm^3$) was treated with N-benzyl-N-carboxymethylglycine dimethyl ester (2.1 g) in ether (30 $cm^3$) under a nitrogen atmosphere at a rate which kept the temperature below -7 $^oC$. This suspension was boiled under reflux for 2 hours, cooled and excess $LiAlH_4$ was destroyed by sequential addition of water (0.5 $cm^3$) 10% NaOH (0.75 $cm^3$) and water (1.5 $cm^3$). The resulting off-white solid was Soxhlet-extracted overnight with ether. Drying with $Na_2SO_4$, evaporation and distillation gave the title compound as a clear mobile liquid: 1.19 g, 74%, b.p. 140-142 $^oC$ at 0.4 mmHg.

N-Benzyl-N-2-chloroethyl-2-chloroethanamine

Under a static nitrogen atmosphere thionyl chloride (1.64 g) was brought to reflux in chloroform (3 $cm^3$) and a solution of N-benzyl-N-2-hydroxyethyl-2-hydroxyethanamine (1.19 g) in chloroform (3 $cm^3$) was added over *ca.* 1 hour. Reflux was maintained for a

further 2 hours, the solution was cooled and evaporated to leave an amorphous white solid which was recrystallised from water to give N-benzyl-N-2-chloroethyl-2-chloroethanamine hydrochloride as fine white needles: 0.91 g, 63%, m.p. 147-148 $^{o}$C (lit. m.p. 148-150), $\delta_H$ ($^2H_2O$): 3.64 (t, $C\underline{H}_2$-N-Bz), 3.98 (t, $C\underline{H}_2Cl$), 4.53 (s, $\underline{C}H_2$-$C_6H_5$), 7.54 (s, $C_6H_5$).

### 3.5.3 Preparation of *Rac*-[1-$^{13}$C]Leucine

A solution of Na$^{13}$CN (2.0 g, 90 atom %) in water (15 cm$^3$) was added to 2.17 M sulphuric acid (10 cm$^3$) in an apparatus equipped with a long nitrogen inlet and a lagged splash-head, leading *via* a condenser and collecting flask to a Dreschel bottle containing 0.880 ammonia solution (12 cm$^3$) at 0 $^{o}$C. The Dreschel outlet reached the atmosphere *via* a train containing 5 M sodium hydroxide traps to absorb unreacted HCN. Heating was initiated during the addition to maintain a vigorous reflux for *ca.* 1 hour, when the bulk of the liquid had distilled. Absorbed HCN in the ammonia trap was determined by silver nitrate titration (93% of theory). The stirred cyanide solution was treated with an excess of 3-methylbutanal (9.77 g) and stirring was continued for *ca.* 4 days when off-white crystals of 2-amino-3-methylcyanopropane were removed by filtration. This was hydrolysed by refluxing for 1½ hours with 48% aqueous HBr (150 cm$^3$). After separation of organic residues, HBr was expelled by passage of nitrogen and the resultant oil taken up in water (25 cm$^3$). The

solution was adjusted to pH 14 with 1 M NaOH (~ 150 $cm^3$) before heating (for 3 hours), whilst passing nitrogen, until the vapour reached pH 8. A white crystalline solid separated on standing to give after recrystallisation and drying, [1-$^{13}$C]sodium leucinate: 2.53 g, 40%, $\delta_H$($^2H_2O$, pH 8.5 with $Na_2CO_3$), 0.90 (m, 2 x $CH_3$), 1.41 (m, $C\underline{H}_2CH$), 1.65 (m, $C\underline{H}(CH_3)_2$), 3.25 (m, $C\underline{H}$-$CO_2^-$).

Preparation of [1-$^{13}$C]-(*S*)-leucine from sodium leucinate

[1-$^{13}$C]sodium leucinate (2.4 g) was warmed with glacial acetic acid (25 $cm^3$) to give an even dispersion. The mixture was then boiled for 4 minutes and cooled to room temperature. Cautious addition of acetic anhydride (4.1 $cm^3$) followed by vigorous boiling (3 minutes) gave a clear, yellow solution. After standing at room temperature for 2 hours, this was concentrated (35 $^oC$, 20 mmHg) to constant weight. The residue was dissolved in water (20 $cm^3$), and pumped (20 $^oC$ at 20 mmHg) for 3 hours whereupon it crystallised. Decolourisation with charcoal and reprecipitation from a minimum of boiling acetone gave an oily product of *rac*.-[1-$^{13}$C]-N-acetylleucine which crystallised with scratching: 1.98 g, 66%, $\delta_H$ ($^2H_2O$): 0.9 (m, 2 x $CH_3$-CH), 1.65 (broad m, $C\underline{H}(CH_3)_2$ and $CH_2$·CH), 2.01 (s, $C\underline{H}_3C$=O), 4.31 (t, CH-$CO_2H$).

The *rac*.-[1-$^{13}$C]-N-acetylleucine was dissolved in water (150 $cm^3$), adjusted to pH 7.2 with ammonia, made up to 200 $cm^3$ and treated with 20 mg of Acylase I. Incubation in a water bath at 37 $^oC$ for 20 hours gave a reaction mixture whose $^1H$ n.m.r. spectrum showed that

*ca.* one-half of the N-acetyl group had been hydrolysed and therefore the reaction was deemed to be complete. Acetic acid (10 $cm^3$) and charcoal were added, the solution was filtered through Celite and evaporated to leave a clear oil. Treatment with an excess of boiling ethanol gave a cloudy solution which deposited fine white crystals on standing at 4 $^oC$. The [1-$^{13}$C]-L-leucine was recovered by filtration, washed with ethanol (2 x 10 $cm^3$) and with ether (2 x 10 $cm^3$) and dried: 0.51 g, 74% of theory, $[\alpha]_D^{22}$ = +15.2 in 50% HCl, lit. $[\alpha]_D^{26}$ = 15 in 6 M HCl.

### 3.5.4 (*S*)-[2-$^2$H]Alanine

*Rac.*-Alanine (3.31 g) was suspended in acetic acid (30 $cm^3$) and boiled for 1 minute with the exclusion of atmospheric moisture. The briefly cooled solution was treated with acetic anhydride (30 $cm^3$), boiled for 2 minutes and left at room temperature for 1½ hours before removing the solvent (35 $^oC$, 20 mmHg). Water (20 $cm^3$) was added and the evaporation repeated 3 times, giving a solid product which was recrystallised from ethyl acetate. On standing at 4 $^oC$ for 12 hours, large white crystals of *rac.*-N-acetylalanine were removed by filtration, washed with ether and dried over silica gel: 2.6 g, 53%, $\delta_H$ ($^2H_2O$): 1.42 (d, $\underline{C}H_3$-CH), 2.17 (s, $CH_3C$=O), 4.43 (q, $C\underline{H}$-$CO_2H$).

*Rac.*-N-acetylalanine (2.2 g) was added to a mixture of acetic anhydride (10 g) and deuterioacetic acid

22 g) and placed in an oil bath at 60 °C for 1 hour when a $^{1}H$ n.m.r. spectrum suggested complete α-deuteration. Removal of solvent (50 °C, 20 mmHg; then 30 °C, .05 mmHg) induced crystallisation of *rac.*-[α-$^{2}H$]-N-acetylalanine which was resolved without further purification.

The crystals were dissolved in water (150 $cm^3$) and adjusting the pH to 7.2 with ammonia, Acylase I (20 mg) was added and the mixture incubated in a water bath at 35 °C for 24 hours. A $^{1}H$ n.m.r. spectrum suggested that the reaction was complete. The reaction mixture was treated with acetic acid (10 $cm^3$) and charcoal (1 g), filtered, concentrated (30 °C, 20 mmHg) and crystallised from boiling ethanol (100 $cm^3$). On standing at 4 °C for 18 hours feathery crystals of (*S*)-[α-$^{2}H$]alanine were recovered by filtration and air dried: 0.64 g, 85% of theory. $[\alpha]_D^{20}$ = +13.5°, C = 4.57 in 5 M HCl, lit. $[\alpha]_D^{20}$ = +14.0°, C = 6.0 in 1 M HCl. $\delta_H$ ($^{2}H_2O$) 1.40 (s, $C\underline{H}_3$-$C^{2}H$), 3.72 (q, $C\underline{H}CO_2H$). The integration ratio of the two signals suggests that deuteriation proceeds to 75%. Exchange of acidic protons in $^{2}H_2O$ prior to treatment with deuterioacetic acid would increase the level of isotope incorporation.

### 3.5.5 Preparation of (*S*)-[Methyl-$^{13}C$]methionine

Using flame-dried apparatus, liquid ammonia (*ca.* 200 $cm^3$) was distilled from sodium (*ca.* 0.25 g) on to *S*-methionine (4.46 g) over 1 hour under dry nitrogen. During 20 minutes flattened lithium shot (0.67 g) was

added in several portions to the refluxing solution, maintaining a positive pressure of dry nitrogen. The resulting blue solution was stirred, and after 2 hours the blue colour had been discharged. An aliquot of the reaction mixture was removed and evaporated, the residue in $^{2}H_{2}O$ was examined by $^{1}H$ n.m.r. spectroscopy to show unreacted methionine (MeS at $\delta$ 2.1) and additional portions of lithium were added (up to 1 mol. equivalent was necessary) until examination by $^{1}H$ n.m.r. spectroscopy showed the absence of methionine. Addition of ammonium chloride was followed by a first portion of [$^{13}C$]-iodomethane (4.4 g from B.O.C. Ltd. Prochem), 91 atom % $^{13}C$. The reaction was boiled under reflux for 2 hours, when an aliquot was withdrawn and tested with Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid)). Free thiol was detected and further iodomethane (0.29 g) was added and the reaction mixture boiled under reflux for another hour. A repeat Ellman's test still indicated the presence of free thiol and a further portion of labelled iodomethane (0.27 g) was added. After refluxing for an hour, no free thiol was detected and the solvent was removed by allowing the reaction vessel to warm up overnight under an atmosphere of nitrogen. The off-white residue was dissolved in water (100 $cm^{3}$) and was evaporated to dryness under reduced pressure (50 $^{o}C$, 20 mmHg). This procedure was repeated twice. The solid residue was taken up in water (200 $cm^{3}$) and the pH of the solution was reduced to 5.6 by the addition of 2 M hydrochloric acid. Evaporation to *ca.* 15 $cm^{3}$ gave white crystals, which redissolved on

boiling and were re-precipitated by the addition of boiling ethanol (300 $cm^3$). The crystalline solid deposited by storage at -20 $^oC$ for 12 hours was collected at the pump and was washed with ice-cold ethanol/water (5 $cm^3$), ethanol (2 x 5 $cm^3$), and with ether.

Recrystallisation from ethanol and drying gave (*S*)-[methyl-$^{13}C$]methionine (3.61 g, 81%, 91 atom %, $^{13}C$) as a white crystalline solid, m.p. 273-277 $^oC$, single spot by t.l.c. $R_f$ 0.45, no detectable free thiol (Ellman's test), ammonium ions (Nessler's reagent) or lithium ions (flame test). $[\alpha]_D^{22}$ = +21.7$^o$ (C 0.84, 5 M HCl) (*cf.* authentic (*S*)-methionine used as starting material: $[\alpha]_D^{22}$ = +22.0$^o$ (C = 0.095, 5 M HCl)). $\delta_H$ ($^2H_2O$): 2.1 (d, J $^{13}C$-$^1H$, 138.6 Hz, 0.9 x 3 H, $^{13}CH_3S$; and 0.1 x 3 H, s, $^{12}C\underline{H}_3S$), 2.28 (m, $C\underline{H}_2$-S), 2.7 (m, $C\underline{H}_2$-CH), and 4.22 p.p.m. (t, C<u>H</u>).

<u>Preparation of N-cyanomethyl-2-cyanomethanamine from hexamethylenetetramine (HMT)</u>

To a mixture of HMT (0.72 g) and KCN (2.0 g) in water was added 1 M HCl (3.1 $cm^3$) dropwise over 2 hours. The solution was stirred for 4 hours and stood for 8 hours when the colour had changed from pale-yellow to brown. The solution was filtered and the filtrate extracted with ethyl acetate (3 x 5 $cm^3$). Decolourisation of the filtrate with charcoal and filtration through Celite followed by evaporation gave a pale yellow residue of the title compound: 0.55 g, 19%.

Preparation of 2-aza-3-cyanoprop-1-ene

Aqueous sodium cyanide (4.9 g in 15 $cm^3$ of $H_2O$) was added to an ice cold solution of ammonium chloride (5.4 g) and 38% aqueous formaldehyde (16.2 g) at such a rate that the temperature did not exceed 5 $^oC$. Half-way through the process, simultaneous addition of glacial acetic acid (3.8 $cm^3$) was begun maintaining efficient magnetic stirring throughout. After the additions were complete the ice-bath was removed and stirring continued for 1½ hours. Filtration removed a fine white precipitate which was shaken with water and filtered again to give the title compound as a micro-crystalline solid: 3.29 g, 48% from NaCN.

Preparation of N-cyanomethyl-2-cyanomethanamine from 2-aza-3-cyanoprop-1-ene

This was carried out in an analogous manner to the preparation of N-cyanomethyl-2-cyanomethanamine from HMT and KCN to give the title compound in 14% yield.

Comparative preparation of N-carboxymethyl-N-methylglycine (barium salt from 1-bromoacetic acid and 1-chloroacetic acid

To a cooled, stirring solution of the halogeno-acid in water (10 $cm^3$) a 1 mol. equivalent of NaOH was added at such a rate that the temperature did not exceed (a) 7 $^oC$ for $BrCH_2CO_2H$ and (b) 9 $^oC$ for $ClCH_2CO_2H$. A mixture of a second mol. equivalent of NaOH and a one-half mol. equivalent of $MeNH_2$ was added during 15 minutes with cooling to maintain the temperature below (a) 18 $^oC$

for $BrCH_2CO_2H$ and (b) 17 °C for $ClCH_2CO_2H$. The reaction mixtures were left at room temperature for 14 hours without stirring. Each solution was then shaken with boiling aqueous $BaCl_2.2H_2O$ (1.05 mol. equivalents in 10 $cm^3$ water) and heated at reflux for 40 minutes. Filtration and drying gave the barium salt of N-carboxymetnyl-N-methylglycine: (a) 73% from $BrCH_2CO_2H$ and (b) 63% from $ClCH_2CO_2H$.

Attempted one-step preparation of *rac.*-[α-$^2$H]-N-acetylalanine

*Rac.*-alanine (0.1 g) was taken up in $^2H_2O$ and evaporated to dryness. The exchanged alanine was heated with deuterioacetic acid (1 g) and acetic anhydride (0.5 g) for *ca.* 5 minutes with the exclusion of atmospheric moisture. After standing at room temperature for 4 hours the mixture was evaporated to leave a clear oil. Water (10 $cm^3$) was added and evaporated (30 °C, 20 mmHg) on three occasions. The residue was treated with boiling ethyl acetate, the solvent was removed and an aliquot of the residue taken up in $^2H_2O$ for examination by $^1$H n.m.r. spectroscopy. This analysis showed that acetylation had proceeded to the extent of 61% and that this material had incorporated deuterium at 78%.

Studies on the synthesis of N-carboxymethyl-N-methylglycine

General Method

Bromoacetic acid (1.00 g) was weighed into a wide-necked sample vial, dissolved in water (2.5 $cm^3$)

and cooled in an ice-salt bath. Stirring was commenced and one mol. equivalent of NaOH added slowly through a parafilm seal before removing the cooling bath. Immediately a mixture of one mol. equivalent of $MeNH_2$ and one mol. equivalent of NaOH was rapidly added, cooling only when the temperature approached 30 $^{o}C$. After standing without stirring for 2 hours an aliquot (0.25 $cm^3$) was removed, combined with $^{2}H_2O$ (0.25 $cm^3$) and TSS and the $^{1}H$ n.m.r. spectrum recorded.

Experimental Sequence

The method described above was repeated, and $^{1}H$ n.m.r. spectra were recorded at 2, 4 and 15 hours. Addition of authentic N-carboxymethyl-N-methylglycine, bromoacetic acid and methylamine identified each of these in the 2 hours spectrum. After 15 hours only resonances of the title compound and methylamine remained, thus near quantitative conversion was anticipated. However, treatment of the refluxing reaction mixture with a boiling solution of $BaCl_2.2H_2O$ (1.05 mol. equivalents in 2 $cm^3$ $H_2O$) gave only a 35% yield of the barium salt of N-carboxymethyl-N-methylglycine after 40 minutes at reflux, cooling and filtration.

Experiment 2. The reaction was repeated under more dilute conditions (bromoacetic acid dissolved in 10 $cm^3$ $H_2O$). A $^{1}H$ n.m.r. spectrum of an aliquot recorded after 3 hours, showed a clean reaction giving only the title compound. Isolation of the barium salt as before gave a 37% yield.

Experiment 3. The bromoacetic acid was neutralised to test the stability of its sodium salt. Over a period of 21 hours there was no change in the $^{1}H$ n.m.r. spectrum of the mixture compared to the spectrum recorded directly after mixing.

Experiment 4. Performed as experiment 1 but oven drying of $BaCl_2.2H_2O$ at 110 $^{o}C$ increased the yield of barium chelate to 62%.

Experiment 5. As above, the $BaCl_2.2H_2O$ being recrystallised from water by ethanol precipitation and dried at 110 $^{o}C$ for 24 hours before use. Yield of barium salt of N-carboxymethyl-N-methylglycine 73%.

# CHAPTER 4

# HIGH-FIELD $^{1}H$ N.M.R. SPECTROSCOPIC STUDIES OF THE REACTIONS OF NITROGEN MUSTARD

## 4.1 INTRODUCTION

The advent of super-conducting magnets has allowed a tremendous increase in the power of nuclear magnetic resonance instrumentation. This power is usually assessed as the frequency of the signal required to induce a proton to resonate in the applied magnetic field. Using conventional electromagnets the most powerful magnetic field induces resonance at 100 MHz, but with super-conducting magnets field strengths corresponding to *ca.* 200 MHz are common-place and even more powerful instruments are available up to *ca.* 7-900 MHz. Using a 220 MHz continuous wave instrument we have found that nitrogen mustard chemistry can be conveniently monitored at (biologically meaningful) concentrations of around 0.1 M.

As indicated in Chapter 1, only one comprehensive study of nitrogen mustard reactions with biological nucleophiles has been published, appearing in the late 1940's when the authors were handicapped by limited analytical techniques compared to those available today. These workers[4] obtained good analytical data for all the mustard hydrolysis products, as well as the N-2-chloroethyl-N-methylaziridinium ion (isolated as its picrylsulphonate), but with the exception of phenylalanine, no attempts were made to characterise the products of

reactions with model compounds for biological nucleophiles. Our preliminary findings with such species, e.g. methionine, for which Golumbic *et al.* state that "the tendency (of nitrogen mustard to form a sulphonium salt with methionine) is so small as to be questionable", were at variance with their results and so we re-examined the hydrolysis of (1). This was not straightforward even with the modern techniques available to us, and it is a considerable tribute to Golumbic *et al.* that we found that their work on hydrolysis appears to be correct.

Early n.m.r. studies of nitrogen mustard by three independent groups were discussed in Chapter 1[22,24,26]. Performed with the instrumentation of the early 1960's it is not surprising that none of these groups managed to characterise properly the derived aziridinium ion. However, the major difference between these studies and ours pertains to the life-time of nitrogen mustard solutions. Under conditions which generate nitrogen mustard base from the hydrochloride in $^2H_2O$ none of these papers reports its near quantitative conversion into the N-2-chloroethyl-N-methylaziridinium ion (8) which we have observed. Furthermore, their estimates of the life-time of (8) disagree significantly with our own measurements. The most complete of these studies[22] acknowledges a requirement for further definitive work to assign the end-products after neutralisation, which is what we have attempted to do.

There are certain drawbacks to using n.m.r. spectroscopy for the study of the chemistry of nitrogen mustard, the chief of which is that to make sensible

comparisons between spectra one has to ensure that they are both recorded at known, and preferably identical, pH values. The $^{1}H$ n.m.r. N-methyl resonance of nitrogen mustard is observed at *ca.* δ 2.6 in the free base and at *ca.* δ 3.03 in the protonated species. Thus, if the pH is such that the nitrogen mustard is partially protonated, a weighted average signal will be observed somewhere between these two extremes. In a system where a number of different N-methyl resonances are expected this pH shift can bedevil structural assignments. Therefore a buffer system was used to minimise such pH dependent shifts and to maintain the reactivity of basic functions during the reaction.

Many buffers contain either a potential nucleophile or sufficient organic material to obscure interesting regions of the spectrum and so the choice of buffer was limited. Prompted by the observation that the aziridinium ion was unreactive towards acetate, Sorensen's citrate-phosphate buffer was an automatic choice, because it was the only common buffer which operated in the required pH range and contained only a small quantity of an organic component. The constitution of this buffer is described in Chapter 2; at pH 7.2 a 0.5 $cm^3$ sample contains only 1.9 mg (9.89 μmol) of citric acid. Working at high magnetic field strengths there is a heating effect in aqueous ionic solvents due to ion transportation. Although this is at its most severe when the $R_f$ decoupler is activated (in recording decoupled $^{13}C$ n.m.r. spectra) the added difficulty of correctly phasing ionic solutions led us to use a low ionic strength

buffer regardless of the nucleus we were observing. In practice, this meant that the 'buffer' acted really as a reagent, doing little more than neutralising nitrogen mustard hydrochloride. Significant pH drops were not observed because most of the nucleophiles used were present in excess and were themselves more basic than the buffer, e.g. amino acids exist largely as zwitterions at pH *ca.* 7 and can scavenge $H^+$ by protonating the carboxyl group. Thus, the maximum observed pH decrease was from pH 7.2 to pH 6.6. All measurements were direct readings from a digital pH meter equipped with a combination electrode and are uncorrected for $p^2H$ (i.e. add 0.4 to obtain $p^2H$).

Because nitrogen mustard is a bifunctional alkylating agent conditions of nucleophile concentration were chosen to permit substitution at each chloroethyl arm without using such high nucleophile concentrations that intra-molecular cyclisation would be swamped. The protocol adopted was to dissolve 2.5 mol. equivalents of nucleophile in 0.5 $cm^3$ of buffer, record the $^1H$ n.m.r. spectrum of the nucleophile at pH 7.2 (if appropriate), remove the sample to a vial containing one mol. equivalent (*ca.* 10 mg) of nitrogen mustard hydrochloride, mix, return to the n.m.r. tube and commence recording spectra as soon as possible thereafter. There was little point in attempting to commence observation immediately on mixing, because the nitrogen mustard base came out of solution and took *ca.* 1 minute to re-dissolve. This was a quite adequate interval during which to commence observation with the Perkin Elmer R34

continuous wave instrument and was just sufficient time (with practise) using the Bruker WH90 instrument. The latter spectrometer operates in the FT mode, which gives a spectrum over the entire sweep-range in a single pulse. Therefore, there is no time-lag between recording (and integrating) resonances, which is particularly useful for fast reactions. Attempts to use this instrument's kinetic package to study kinetics of mustard reactions were unsatisfactory because of inadequate resolution.

## 4.2 NON-FIRST-ORDER SPIN SYSTEMS

A number of nitrogen mustard-derived compounds exhibit $^1H$ n.m.r. spectra with complex splitting patterns from which neither chemical shifts nor coupling constants may be directly read.

An examination of $^1H$ n.m.r. spectra of reactions performed in citrate-phosphate buffer shows a quartet centred at $\delta$ 2.56 for the methylene groups of the citrate component (Fig. 4.1). The two protons in each of the methylene groups are chemically like paired atoms. A member of each pair may not be exchanged with the other by a rotational symmetry operation and although they are constitutionally equivalent, they are diastereotopic and this gives rise to two superimposed AB spin systems. Designating the lines as $f_1$ to $f_4$ and specifying the centre, $Z = \frac{1}{2}(\nu_A + \nu_B)$ then it can be shown[101] that $\nu_A = Z - \frac{1}{2}\nu_0\delta$ and $\nu_B = Z + \frac{1}{2}\nu_0\delta$, where $\nu_0\delta$ is the chemical shift difference ($\nu_0\delta = \sqrt{(f_2 - f_3)(f_1 - f_4)}$). Evidently

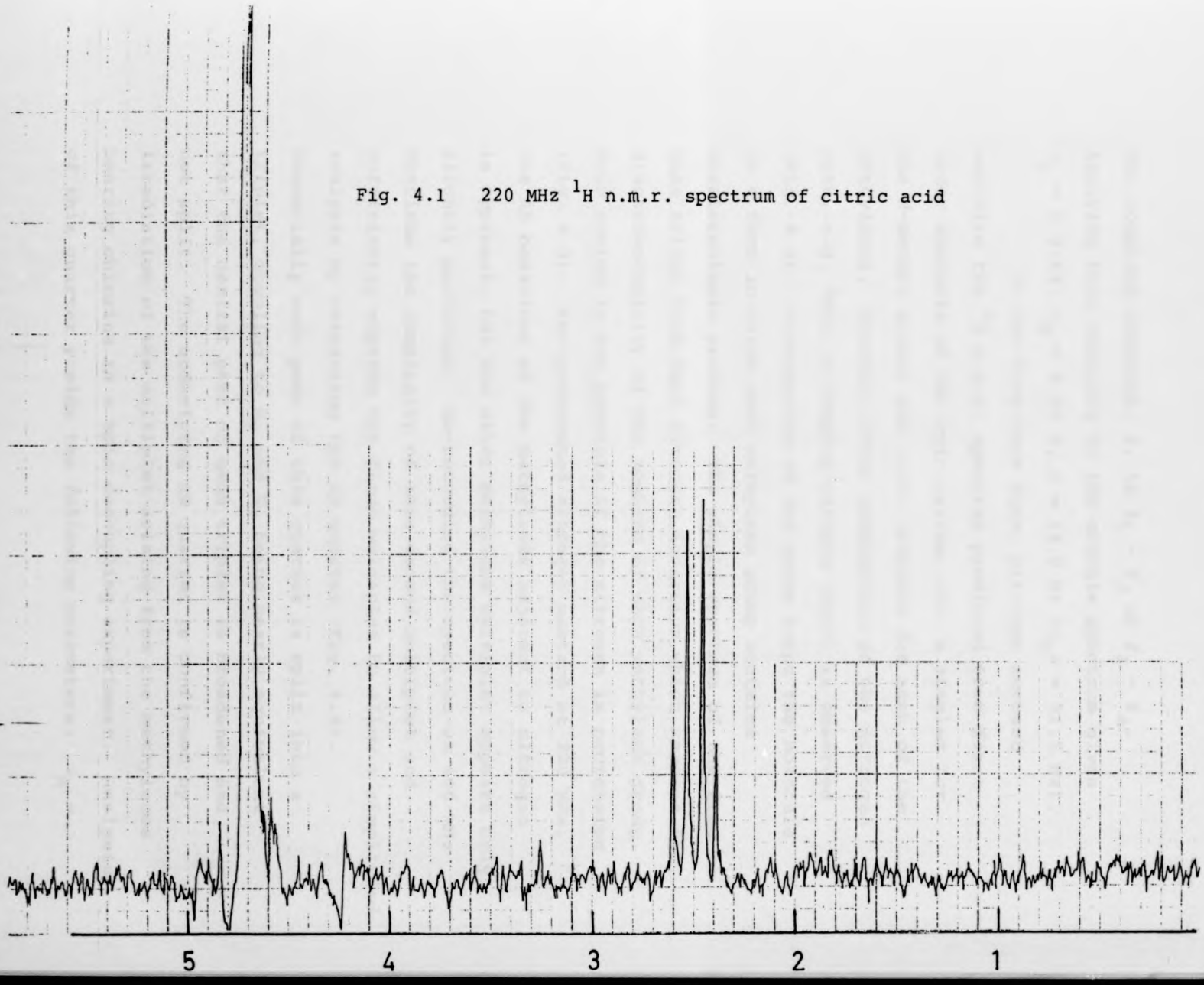



Fig. 4.1 220 MHz $^{1}H$ n.m.r. spectrum of citric acid

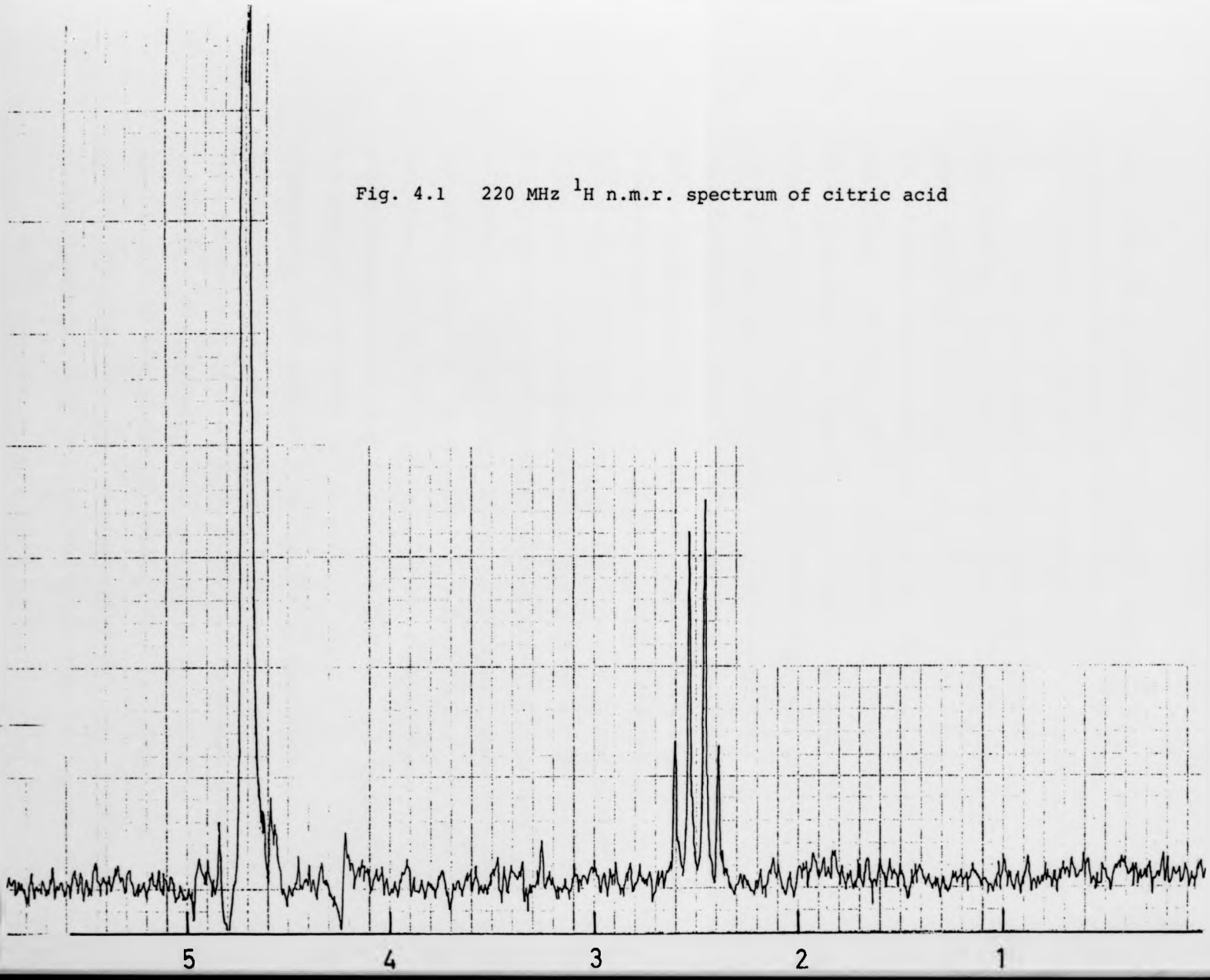



Fig. 4.1 220 MHz $^{1}H$ n.m.r. spectrum of citric acid

the coupling constant, J, is $f_1 - f_2$ or $f_3 - f_4$. Applying this analysis to the citrate spectrum gives $\nu_A$ = δ 2.63, $\nu_B$ = 2.49 δ, J = 13.5 Hz ($\nu_0\delta$ = 31.5 Hz).

In the free base form, nitrogen mustard exhibits the $^1$H n.m.r. spectrum predicted from first-order analysis of the spin system, *viz.* a singlet for the N-methyl signal and 1:2:1 triplets for each of the methylenes. However, after protonation of the nitrogen with, e.g. $^2$HCl, a complex pattern (ABXY) is observed (Fig. 4.2). Protonation of the amine traps the molecule in a form in which each methylene group contains diastereotopic protons. The simple spectrum of the free base arises from fast nitrogen inversion which removes the diastereotopicity of the members of each methylene group. Such motion is not possible if the nitrogen is protonated (Fig. 4.3). For protonated nitrogen mustard at 220 MHz, the AB behaviour of the methylenes adjacent to nitrogen is apparent, but the other methylene multiplet appears only slightly perturbed. Re-recording the spectrum at 400 MHz confirms the complexity of this second multiplet and sufficiently expands the first multiplet to allow a simplified analysis by extracting the AB quartet (Fig. 4.4). Essentially each peak of this quartet is split into a triplet, coupling to $H_X$ and $H_Y$ being nearly equivalent that the central peak of each triplet is broadened and not split. The underlying AB quartet is confirmed by irradiation of the multiplet arising from the methylenes bearing chlorine in a spin decoupling experiment. Analysis of this quartet yields the following parameters: $\nu_A$ =

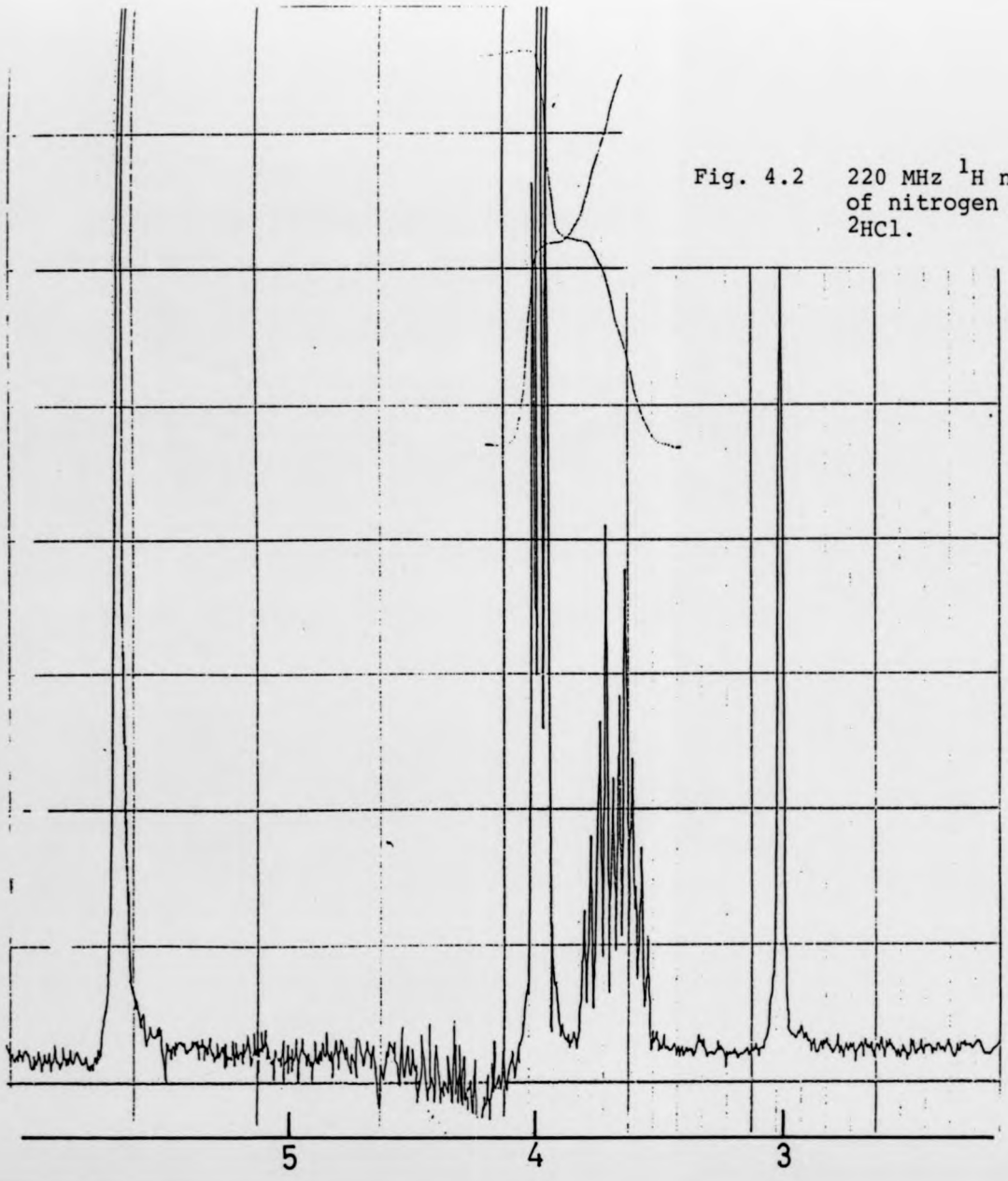
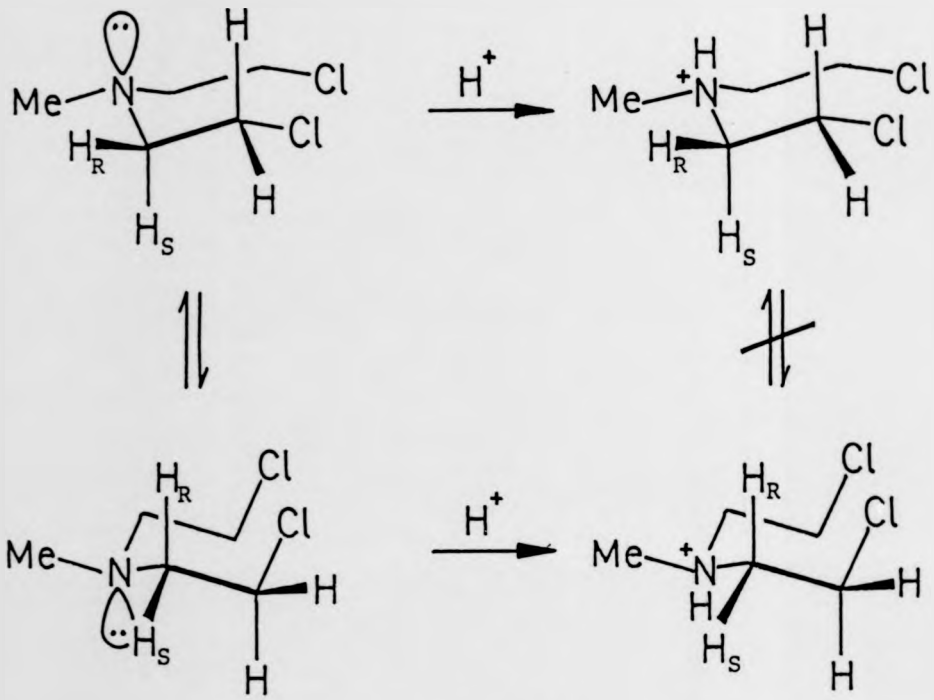



Fig. 4.2 220 MHz $^{1}H$ n.m.r. spectrum of nitrogen mustard in 2 M $^{2}HCl$.

Fig. 4.3

SYMMETRY AND CONFORMATIONAL PROPERTIES OF
NITROGEN MUSTARD (FREE BASE AND PROTONATED FORM)




$pK_a$ 6.45

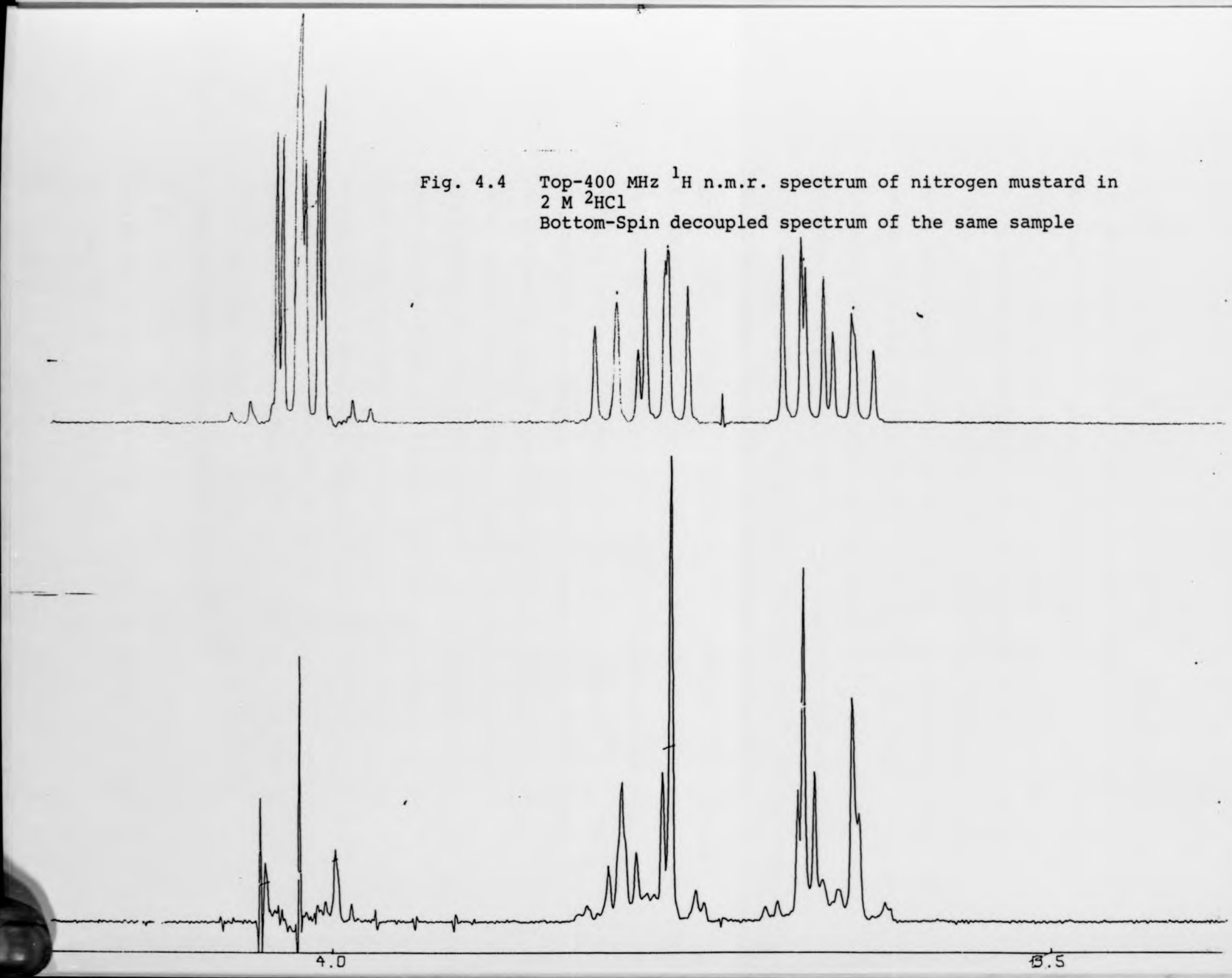



Fig. 4.4 Top-400 MHz $^{1}H$ n.m.r. spectrum of nitrogen mustard in 2 M $^{2}HCl$
Bottom-Spin decoupled spectrum of the same sample

3.657 δ, $\nu_B$ = 3.779 δ, $J_{AB}$ = 13.662 Hz. Splitting of this quartet due to coupling to the other methylene group, gives an average coupling constant $J_{AB-XY}$ = 11.2 Hz. The $^1H$ n.m.r. spectrum of the ring methylenes of the N-2-chloroethyl-N-methyl aziridinium ion is too complex to be resolved at 220 MHz. At 400 MHz the spectrum shows more resonances and in particular the small central feature, which is absent in the lower field spectrum. The aziridinium ring is described as an AA'BB' spin system denoting 2 chemically like, magnetically non-equivalent nuclei separated by a very small chemical shift difference. An AA'BB' spin system is characterised by 2 chemical shifts and 4 coupling constants, in theory giving rise to 48 lines if they can be resolved. Direct analysis can only give approximate answers and so these systems are usually solved by a computer-simulation method[102]. The eigenvalues determined from the calculated spectrum are back-transformed to generate diagonal elements of the Hamiltonian matrix, which yields an improved set of parameters for calculation of a second spectrum. Improved eigenvalues from the second spectrum are fed into the iteration loop again and, if the initial estimate of coupling constants and chemical shifts was sensible, a convergent iteration cycle is established which reaches a condition where further iteration does not significantly alter the parameters. The spectrum calculated from these parameters will be superimposable on the experimental one, i.e. the calculated chemical shifts and coupling constants are characteristic of the observed spectrum, but may not be a unique solution.

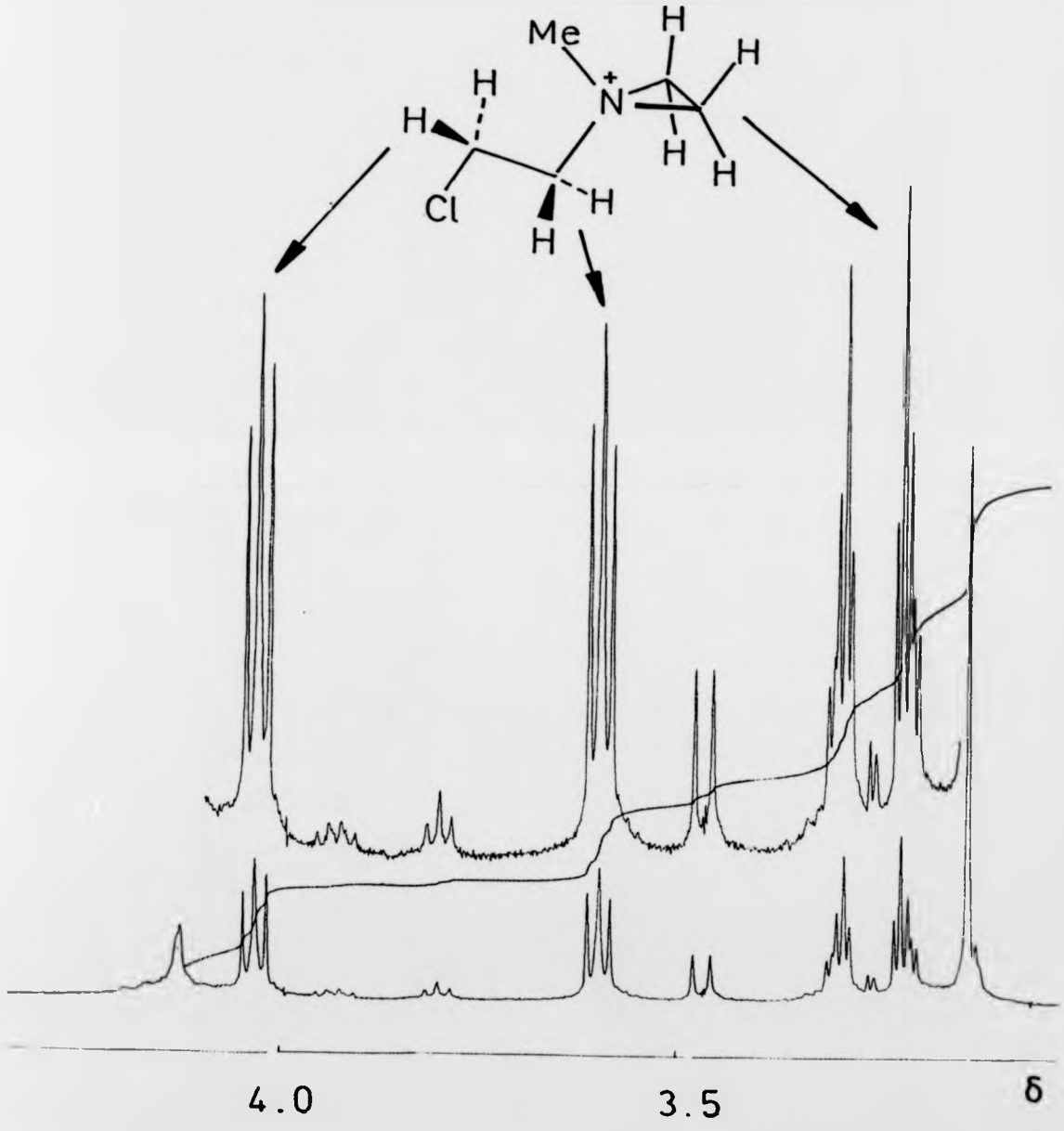
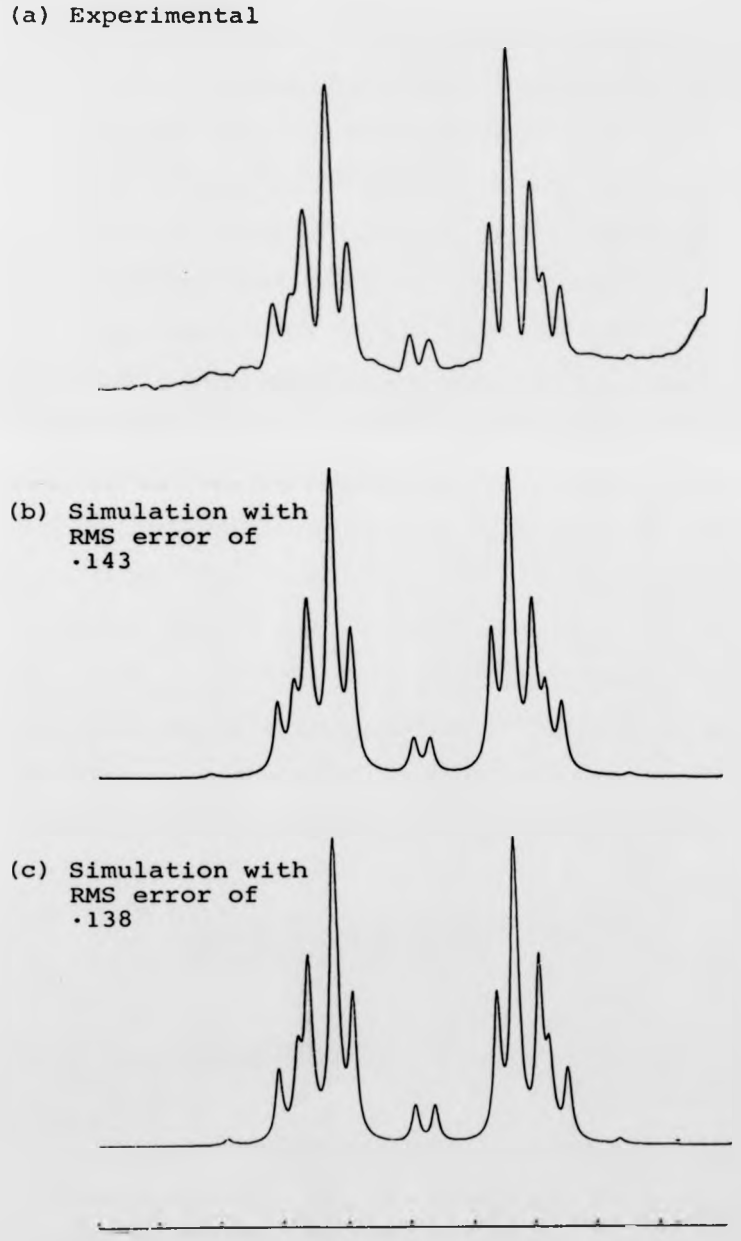



Fig. 4.5 400 MHz $^{1}H$ n.m.r. Spectrum of the Reaction between $MeNH(CH_2CH_2Cl)Cl^-$ and 1 mol equiv. $Na_2CO_3$ after 1.5 h/r.t.

Fig. 4.6 Comparison of experimental and simulated spectra of the N-2-chloroethyl-N-methylaziridinium ion ring.



Following this procedure for the N-2-chloroethyl-N-methylaziridinium ion gave $\delta_A$ 3.36, $\delta_B$ 3.28, $J_{AA'}$ = 6.882 Hz, $J_{AB} = J_{A'B'}$ = -3.599 Hz, $J_{AB'} = J_{A'B}$ = 7.744 Hz, $J_{BB'}$ = 6.863 Hz. An NOE experiment showed that the protons designated $H_A$ (upfield resonance) are *cis* to the methyl substituent. The relative magnitude of the *cis*- and *trans*-coupling constants is the reverse of that observed in other 3-membered ring systems such as cyclopropanes and epoxides. This simulation assigned 17 transitions with an RMS error of 0.143 and in fact excluded a very small peak at 1294 Hz. Repeating the simulation with a set of coupling constants suggested by work described in Chapter 6, all 18 transitions were assigned with an RMS error of 0.135. This yielded the values $\delta_A$ = 3.36, $\delta_B$ = 3.28, $J_{AA'}$ = 9.656 Hz, $J_{AB} = J_{A'B'}$ = -3.66 Hz, $J_{AB'} = J_{A'B}$ = 7.813 Hz, $J_{BB'}$ = 9.605 Hz, i.e. $J_{cis} > J_{trans}$. This is evidently a more satisfying solution and is consistent with the estimated order of *cis*- and *trans*-coupling constants in 3-membered rings. The simulations are compared in Fig. 4.6.

## 4.3 REACTIONS OF NITROGEN MUSTARD WITH NUCLEOPHILES

### 4.3.1 Introduction

The characterisation of the N-2-chloroethyl-N-methylaziridinium ion (8) in solution has allowed us to examine reactions of this intermediate with nucleophiles.

Previous workers were unable to study reactions of this species, although it was isolated as a 2,4,6-trihydroxybenzene sulphonate salt. This salt was fed to *in vitro* cell systems in an attempt to demonstrate greater alkylating ability than with the parent nitrogen mustard. Golumbic and co-workers have re-generated nitrogen mustard from the sulphonate salt of (8) by treatment with HCl. The same researchers have demonstrated that (1) and the 2,4,6-trihydroxybenzene sulphonate salt of the derived aziridinium ion (8) give the same hydrolysis products, but this is the only attempt to demonstrate that (8) lies on the reaction co-ordinate of alkylations with nitrogen mustard.

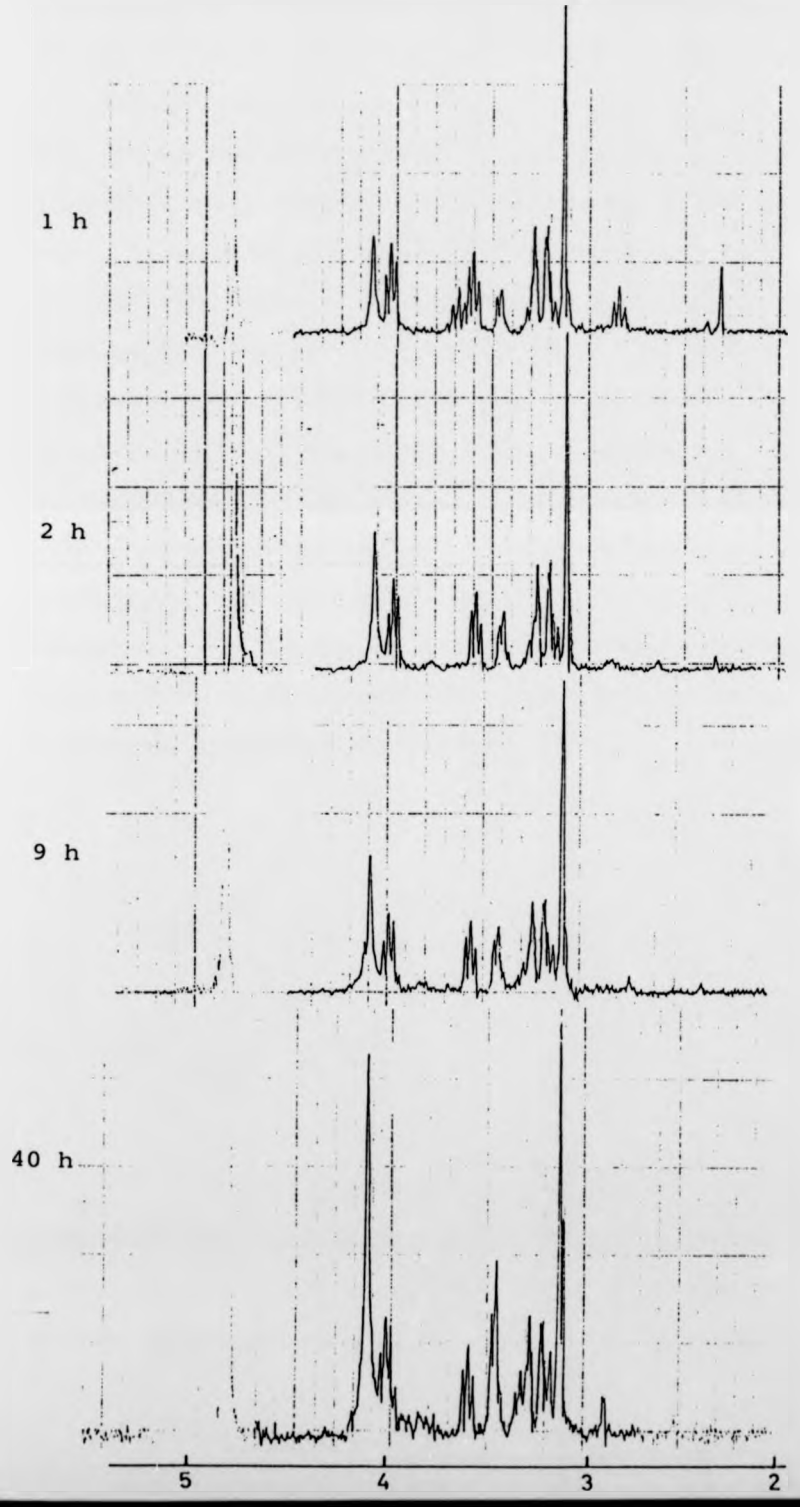
We have used $^1$H n.m.r. spectroscopy to monitor the conversion of aqueous nitrogen mustard to aziridinium ion (8) and the subsequent consumption of the ion by nucleophiles. This has shown that (a) the end-products of reactions with a range of nucleophiles are identical by n.m.r. spectroscopy with the products derived from treating nitrogen mustard with the same nucleophiles, and (b) for at least one nucleophile (2-mercaptoethanol) the same intermediates are observed. Despite advantages in confirming the reaction sequence from nitrogen mustard, observation of (8) did not permit the measurement of reaction kinetics by $^1$H n.m.r. spectroscopy, because it proved impossible to generate solutions containing solely the aziridinium ion. A certain percentage of the aziridinium species was captured by nitrogen mustard to form cyclic dimers (*cis*- and *trans*-(10)). For

observations of reactions by $^{1}H$ n.m.r. spectroscopy, an optimum balance between aziridinium species and dimers was obtained by the use of solutions which still contained significant quantities of nitrogen mustard. Therefore, the exact concentration of the aziridinium ion (8) was not known accurately at the start of a reaction and could be altered during a reaction by its derivation from remaining nitrogen mustard or by its reaction with a nucleophile.

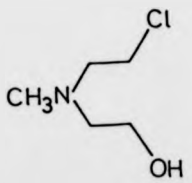
### 4.3.2 Generation of the N-2-Chloroethyl-N-methylaziridinium Ion (8) from Nitrogen Mustard Hydrochloride

Treatment of a solution of nitrogen mustard hydrochloride in $^{2}H_2O$ with 1 mol. equivalent of $Na_2CO_3$ in $^{2}H_2O$ allowed the generation of the N-2-chloroethyl-N-methylaziridinium ion (8) to be monitored by $^{1}H$ n.m.r. spectroscopy (Fig. 4.7). After 2 hours the spectrum showed no nitrogen mustard and was dominated by signals for (8): $\delta_H$ ($^{2}H_2O$) 3.16 (s, $CH_3$-$\overset{+}{N}$), 3.28, 3.36 (AA'BB', 2 x ring $CH_2$), 3.64 (t, J = 12.2 Hz, $CH_2$-$\overset{+}{N}$), 4.08 (t, J = 12.2 Hz, $CH_2Cl$). Traces of piperazinium species (*cis*- and *trans*-10) were detectable after 20 minutes and continued to increase thereafter. After 2 hours, approximately 20% of the starting nitrogen mustard had been converted into dimers and most of the remaining 80% had cyclised to (8). Two N-methyl resonances were also present ($\delta_H$ 2.82 and 2.97), one appearing much faster than the other and losing intensity to it. As this second peak increased, an associated methylene triplet at $\delta_H$ 3.93 became broadened and the

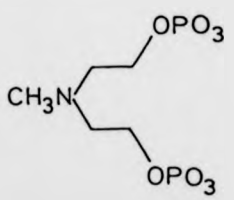
Fig. 4.7 Reaction between nitrogen mustard and $Na_2CO_3$ monitored by 220 MHz $^1H$ n.m.r. spectroscopy.



methyl resonances of *cis*- and *trans*-(10) were distorted. Treatment of nitrogen mustard hydrochloride with an excess of $Na_2CO_3$ very rapidly gave piperazinium dimers and N-2-hydroxyethyl-N-methyl-2-hydroxyethanamine (13). The latter material proved to be identical with the slower forming species observed during the generation of (8). This suggests that simultaneous with nitrogen mustard cyclisation to (8) and the capture of this species by nitrogen mustard to give (10) there occurs the hydrolysis of nitrogen mustard to (8) *via* N-2-chloroethyl-N-methyl-2-hydroxyethanamine (63). Capture of aziridinium intermediates by the latter species would add hydroxyethyl substituted piperazine (11) to the already present (10) explaining the observed distortion in the $^1H$ n.m.r. spectra. Treatment of nitrogen mustard hydrochloride with $NaHCO_3$ caused reactions analogous to those described following treatment with $Na_2CO_3$.



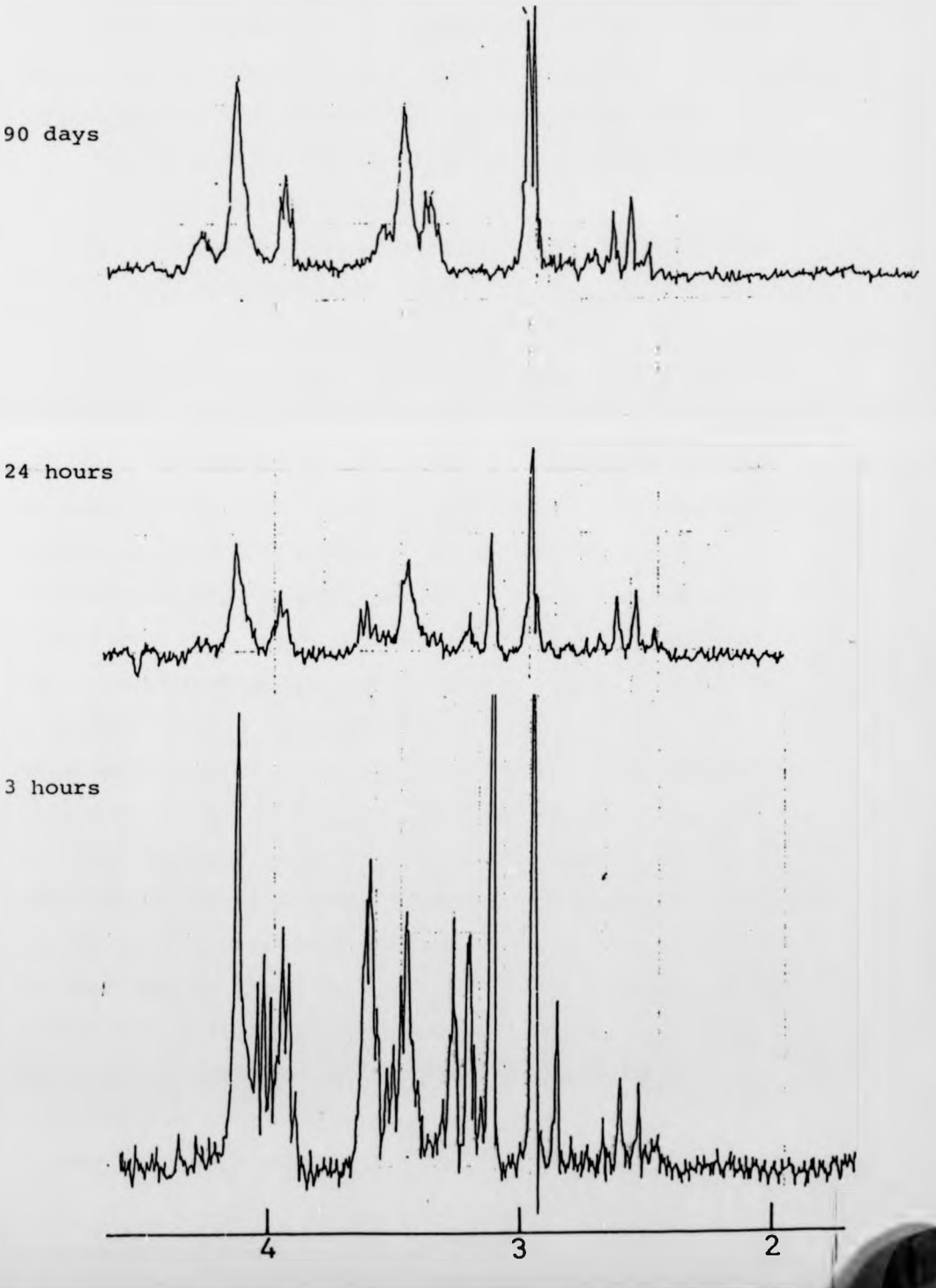

(63)




(64)

It was necessary to check that there was no interference from reactions between the pH 7.2 citrate-phosphate buffer and aziridinium intermeidates. Observations on the effect of sodium acetate on both nitrogen mustard

Fig. 4.8 Reaction of N-2-chloroethyl-N-methylaziridinium chloride with pH 7.2 citrate-phosphate buffer monitored by 220 MHz $^{1}H$ n.m.r. spectroscopy



and its derived aziridinium ion (8) suggested that reaction with the citrate component would not be significant and it was anticipated that reaction with phosphate would not generate a stable product. Cyclic aziridinium intermediates in buffer could be generated in 3 different ways:

(i) treatment of nitrogen mustard hydrochloride with buffer alone;

(ii) treatment of nitrogen mustard hydrochloride with buffer and 1 mol. equivalent of base; or

(iii) addition of buffer to aziridinium ion previously generated by treatment of nitrogen mustard hydrochloride with 1 mol. equivalent of $Na_2CO_3$.

In practice the procedure (iii) was adopted because it was more closely related to reactions without buffer and because relative concentrations of the aziridinium ion (8) and the piperazinium dimers were more favourable than with buffer-mediated generation of (8). On prolonged contact with the buffer (> 48 hours) a product with a mustard-like $^1H$ n.m.r. spectrum was observed together with (10) (Fig. 4.8). The nature of this product is uncertain, but a phosphate ester of nitrogen mustard, e.g. (64) is consistent with the observed spectrum and the behaviour of similar alkylating agents with inorganic phosphate components of buffers as reported by Chang *et al.*[103]. In the presence of an effective nucleophile this material is not observed, which is in accord with the anticipated relative efficiencies of phosphate and chloride as leaving groups[104]. If a phosphate ester is less reactive than

the parent nitrogen mustard, it will not so readily revert to an aziridinium ion, thereby not interfering with the course of a reaction with added nucleophiles. Presumably it is observed in long-term reactions because (a) chloride is diluted in an excess of phosphate and (b) the pH has dropped sufficiently for the amine to be protonated and incapable of cyclisation.

Significantly, such material was not observed in the hydrolysis of nitrogen mustard by aqueous $Na_2CO_3$. At concentrations of $Na_2CO_3$ exceeding 2 M (solution 0.1 M in nitrogen mustard), the sole product was the diol (13). Lower concentrations of $Na_2CO_3$ (*ca.* 1 M) gave a mixture of (10), (11), (13), (65) and (66) illustrating sharp competition between polymerisation and hydrolysis.

### 4.3.3 Reactions between the N-2-Chloroethyl-N-Methylaziridinium Ion and added Nucleophiles

#### (a) Reactions with $Na_2S_2O_3$ (Fig. 4.9)

In the absence of a buffer, there was a very fast, clean reaction to give a di-thiosulphate ester (67) (also known as a Bunte salt) and piperazinium dimers only. Addition of a buffered solution of $Na_2S_2O_3$ to a solution of (8) in $^2H_2O$ gave a similar reaction.

#### (b) Reaction with NaOH (Fig. 4.10)

Addition of hydroxide to a solution of (8) in $^2H_2O$ initiated a series of fast reactions. The $^1H$ n.m.r. spectrum was complex, but all the resolved resonances

Fig. 4.7 Reaction between N-2-chloroethyl-1-N-methyl-aziridinium chloride and $Na_2S_2O_3$ monitored by 220 MHz $^1H$ n.m.r.

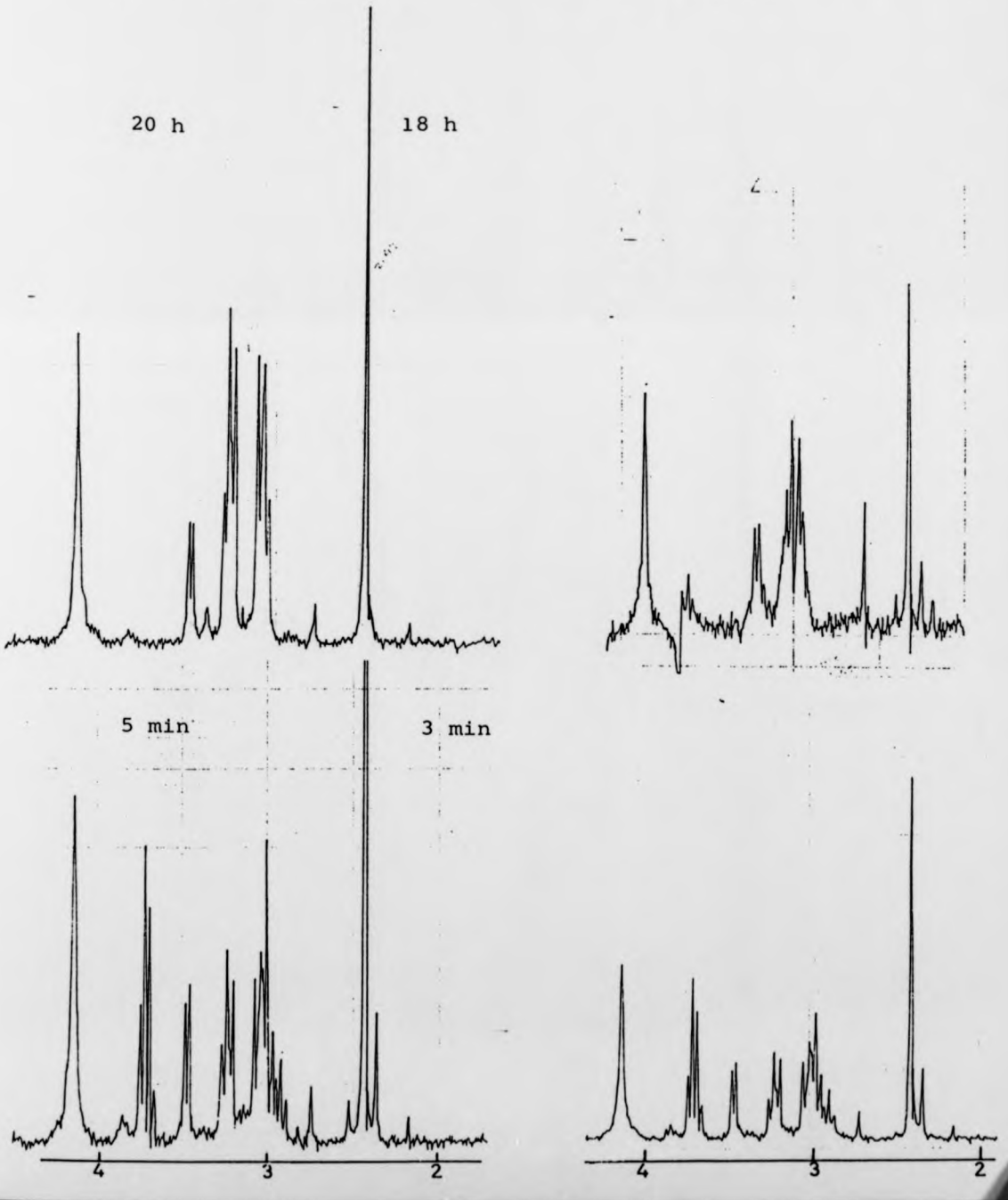
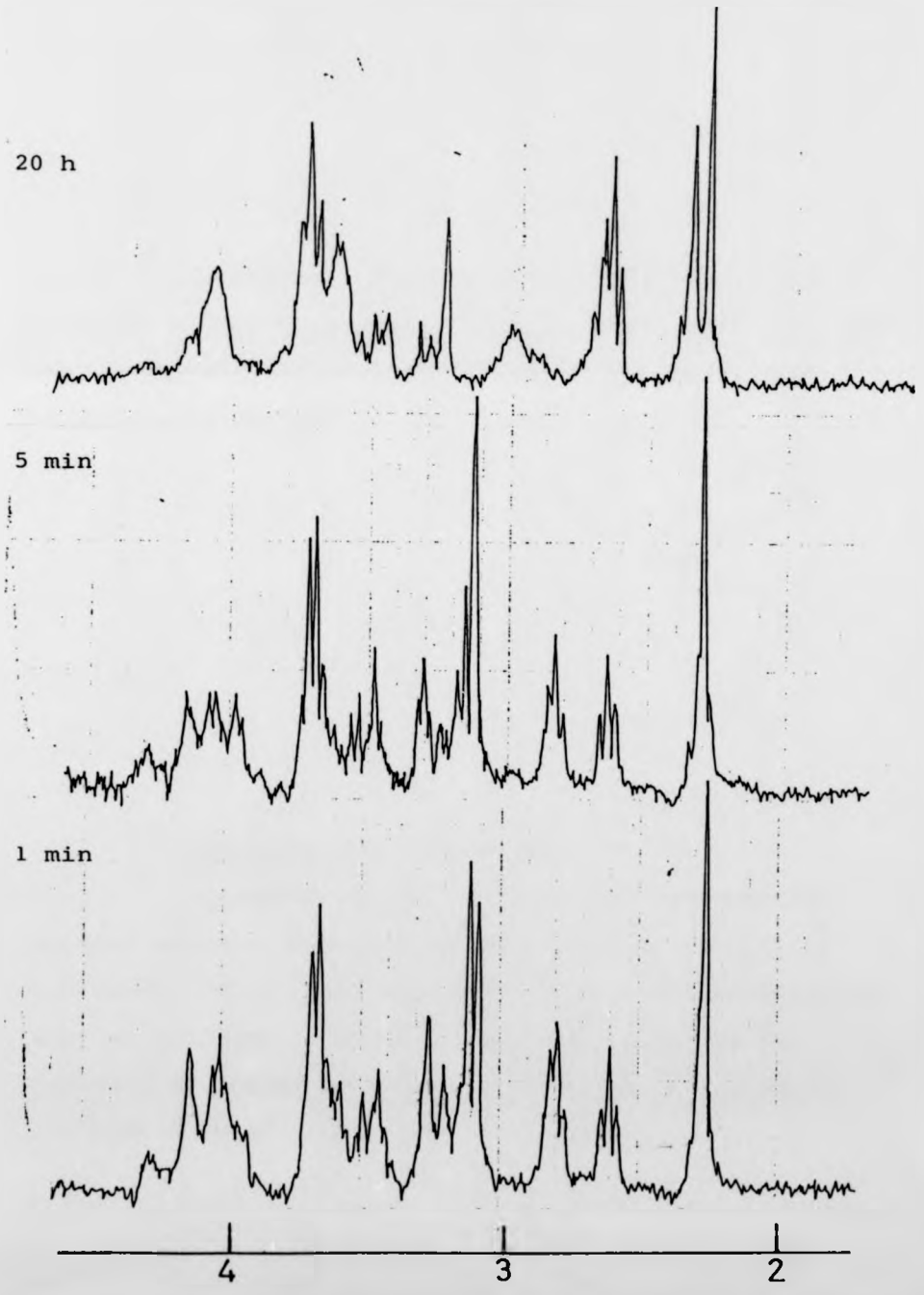
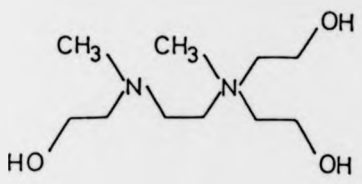
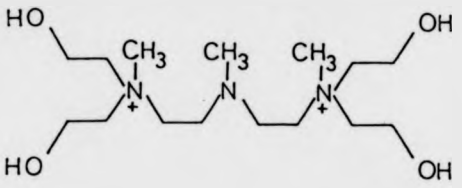


Fig. 4.10 Reaction between N-2-chloroethyl-N-methyl-aziridinium chloride and NaOH monitored by 220 MHz $^{1}H$ n.m.r. spectroscopy.



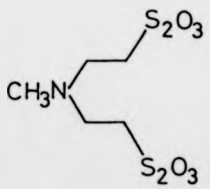


(65)

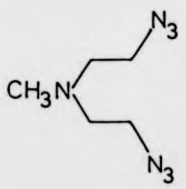


(66)

could be identified. There was very little of (10) present, dominant products being both the diol (13) and the diethylene triamine (66). Minor resonances were attributable to (65).



(67)



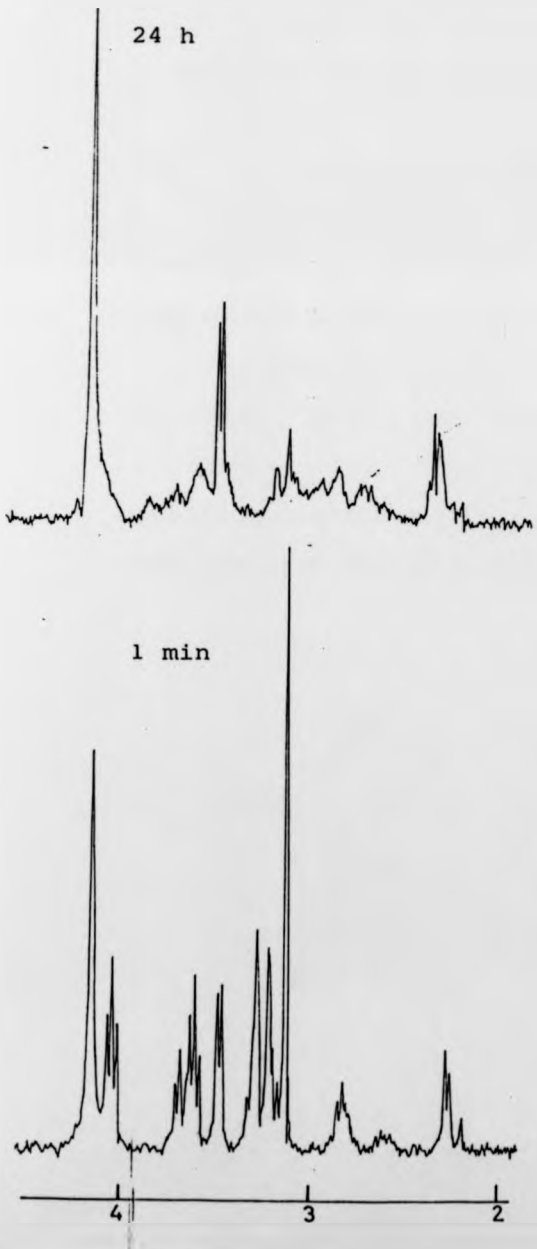
(68)

(c) Reaction with Ammonia (Fig. 4.11)

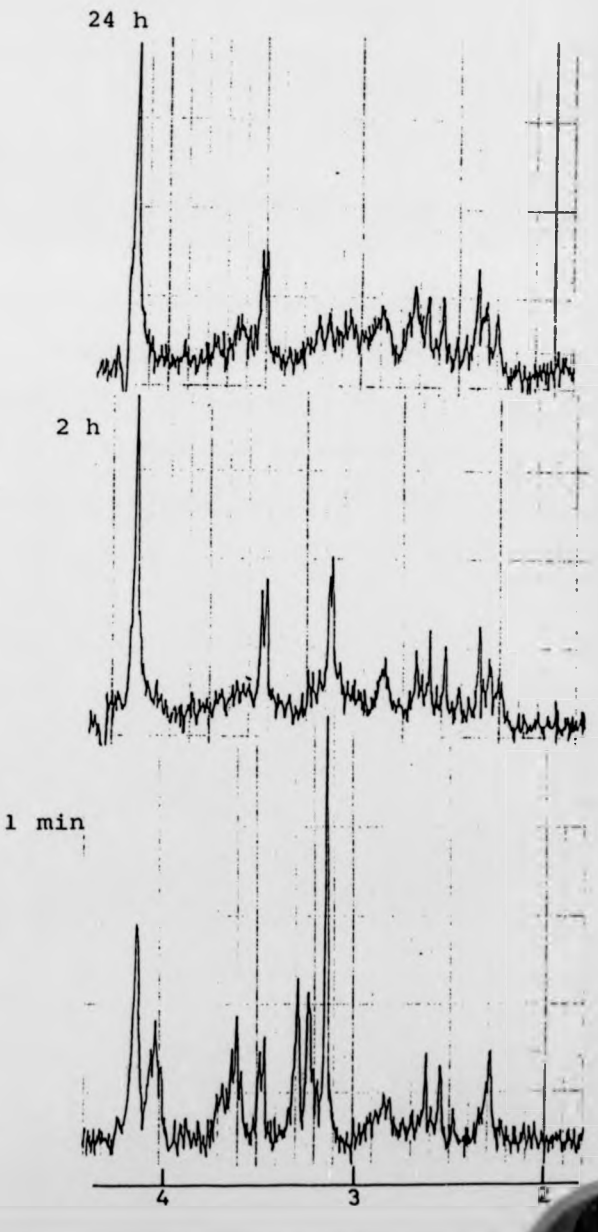
Treatment of (8) in $^{2}H_2O$ with concentrated aqueous ammonia solution produced similar results to (b) above. There was no clear evidence for substituted ammonia products. Repeating the experiment in the presence of buffer gave a similar $^{1}H$ n.m.r. spectrum but with a worse signal-to-noise ratio.

Fig. 4.11 Reaction of N-2-chloroethyl-N-methylaziridinium chloride with ammonia monitored by 220 MHz $^1H$ n.m.r. spectroscopy.

Buffer absent:




In pH 7.2 buffer

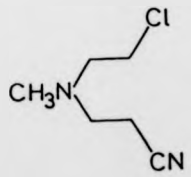


(d) Reaction with $NaN_3$ (Fig. 4.12)

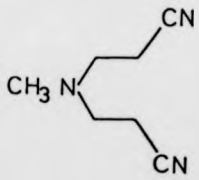
This was a relatively slow reaction with some evidence for the hydrolysis as in (b), but the major feature of the $^1H$ n.m.r. spectrum was a singlet and 2 triplets consistent with the formation of a *bis*-azide, $CH_3N(CH_2CH_2N_3)_2$ (68). In the buffer-mediated reaction slightly more hydrolysis was apparent, but the primary product was the same as that without the buffer.

(e) Reaction with NaCN (Fig. 4.13)

This cleanly gave a single product in a slow reaction which permitted observation of a stepwise substitution proceeding *via* N-2-chloroethyl-N-methyl-2-cyanoethanamine (69) some of which was still present after 20 hours. The product, N-2-cyanoethyl-N-methyl-2-cyanoethanamine, (70), exhibited a complex proton n.m.r. spectrum, of the ABCD type, as discussed above for the product with sodium thiosulphate.




(69)




(70)

Fig. 4.12 Reaction of N-2-chloroethyl-N-methylaziridinium chloride with $NaN_3$ monitored by 220 MHz $^1H$ n.m.r. spectroscopy.

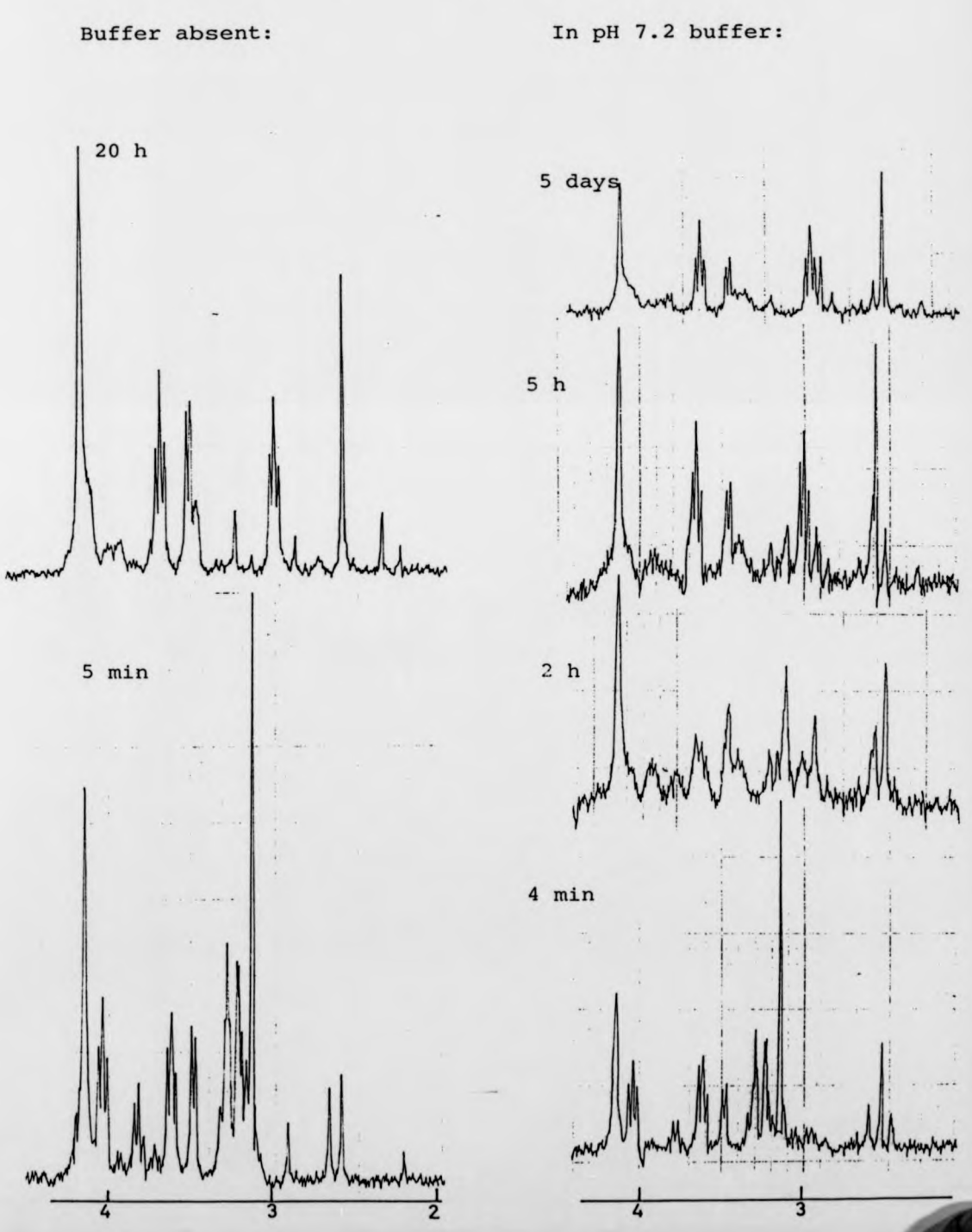
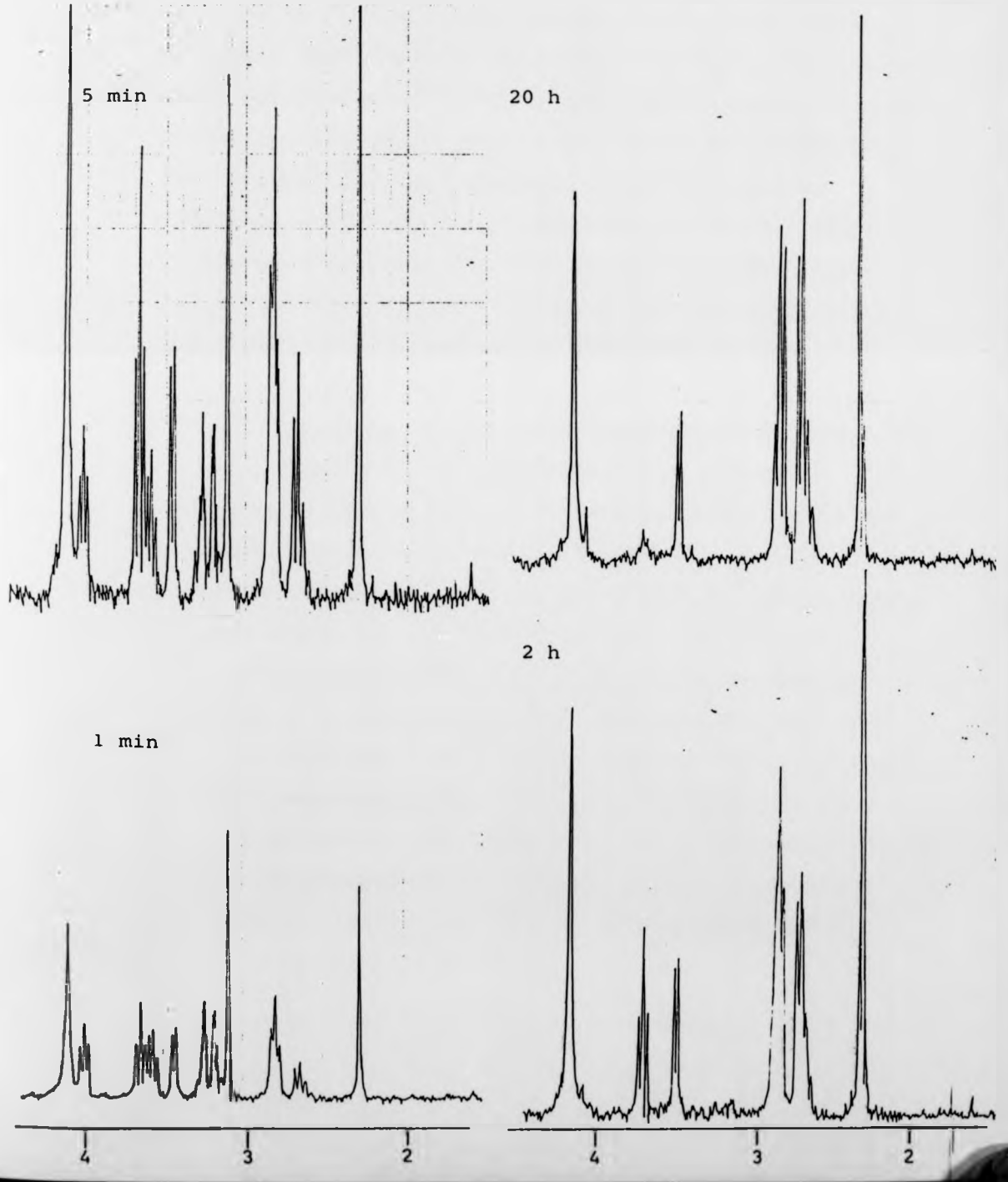


Fig. 4.13 Reaction of N-2-chloroethyl-N-methylaziridinium chloride with NaCN monitored by 220 MHz $^{1}H$ n.m.r. spectroscopy.



(f) Reaction with (*S*)-Methionine (Fig. 4.14 a & b)

A slow reaction was observed which retained a trace of the aziridinium ion (8) even after 2 hours. Analysis by $^1H$ n.m.r. spectroscopy showed that besides resonances from piperazinium dimers, two different N-methyl resonances and one $\overset{+}{S}$-methyl signal were present. This is consistent with the formation of S-alkylated methionines (*cf.* Chapter 5). An intermediate spectrum showing different ratios of these methyl signals and associated methylenes supported this assignment, suggesting that 2/3 of the N-methyl integral arose from monoalkylation, the remainder being due to *bis*-alkylated material.

(g) Reaction with 2-Mercaptoethanol (Fig. 4.15 a & b)

The $^1H$ n.m.r. spectrum of this reaction, performed in the absence of citrate-phosphate buffer and recorded 24 hours from mixing, exhibited 4 different methylene triplets of comparable intensity and 2 N-methyl signals of similar chemical shift ($\delta$ 2.97 and 2.99). The reaction is probably incomplete and these signals represent a mixture of mono- and di-thioethers (71) and (72). In contrast, the buffered reaction clearly passed through a mono-thioether (71) to the di-thioether (72) in a particularly clean reaction. Strict comparison between the two experiments is excluded by a pH difference but a qualitative comparison favours the assignment given.

Fig. 4.14a Reaction of N-2-chloroethyl-N-methylaziridinium chloride with (*S*)-methionine monitored by 220 MHz $^{1}H$ n.m.r. spectroscopy.

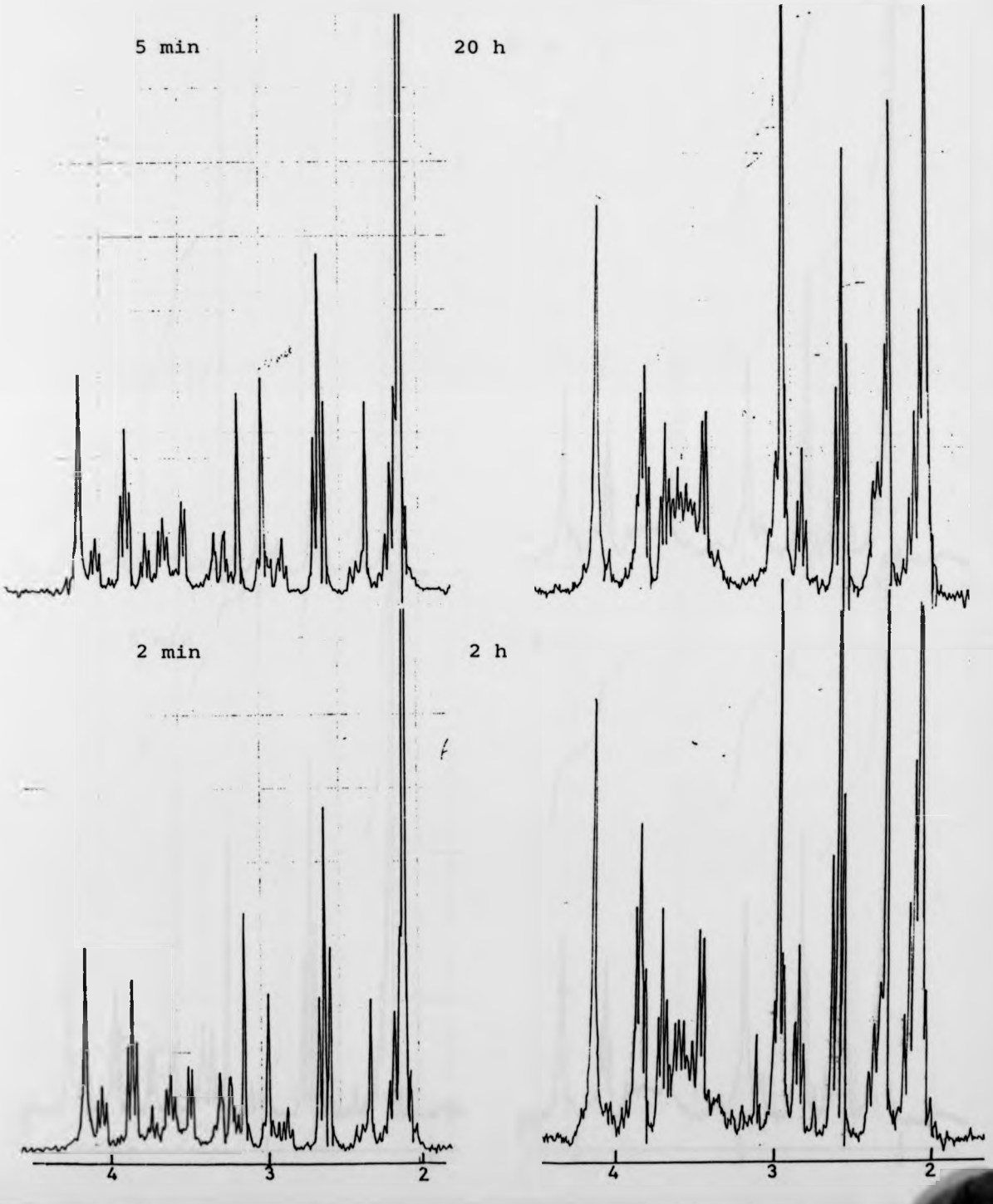


Fig. 4.14b Reactions of N-2-chloroethyl-N-methylaziridinium chloride with *S*-methionine in pH 7.2 buffer monitored by 220 MHz $^{1}H$ n.m.r. spectroscopy.

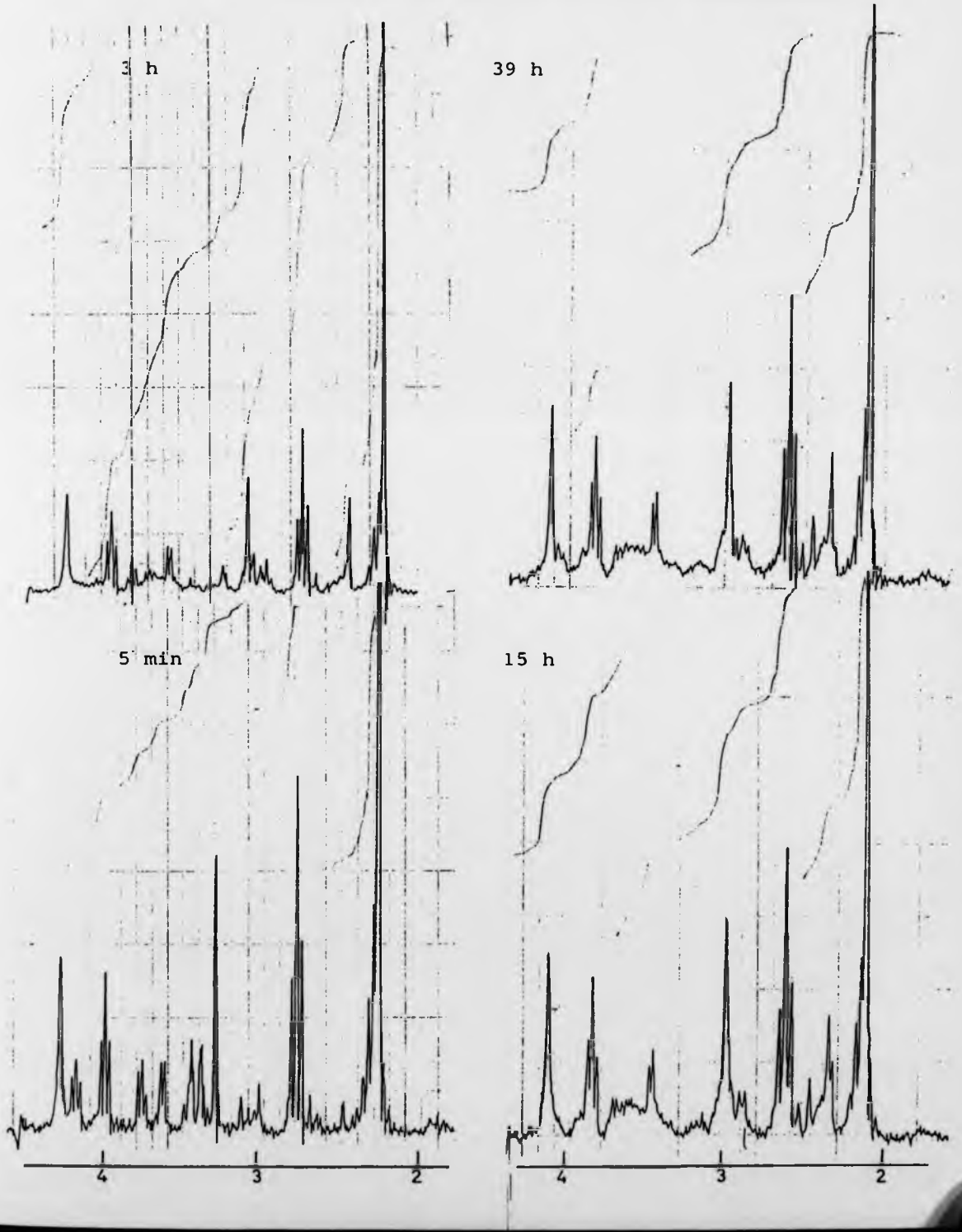
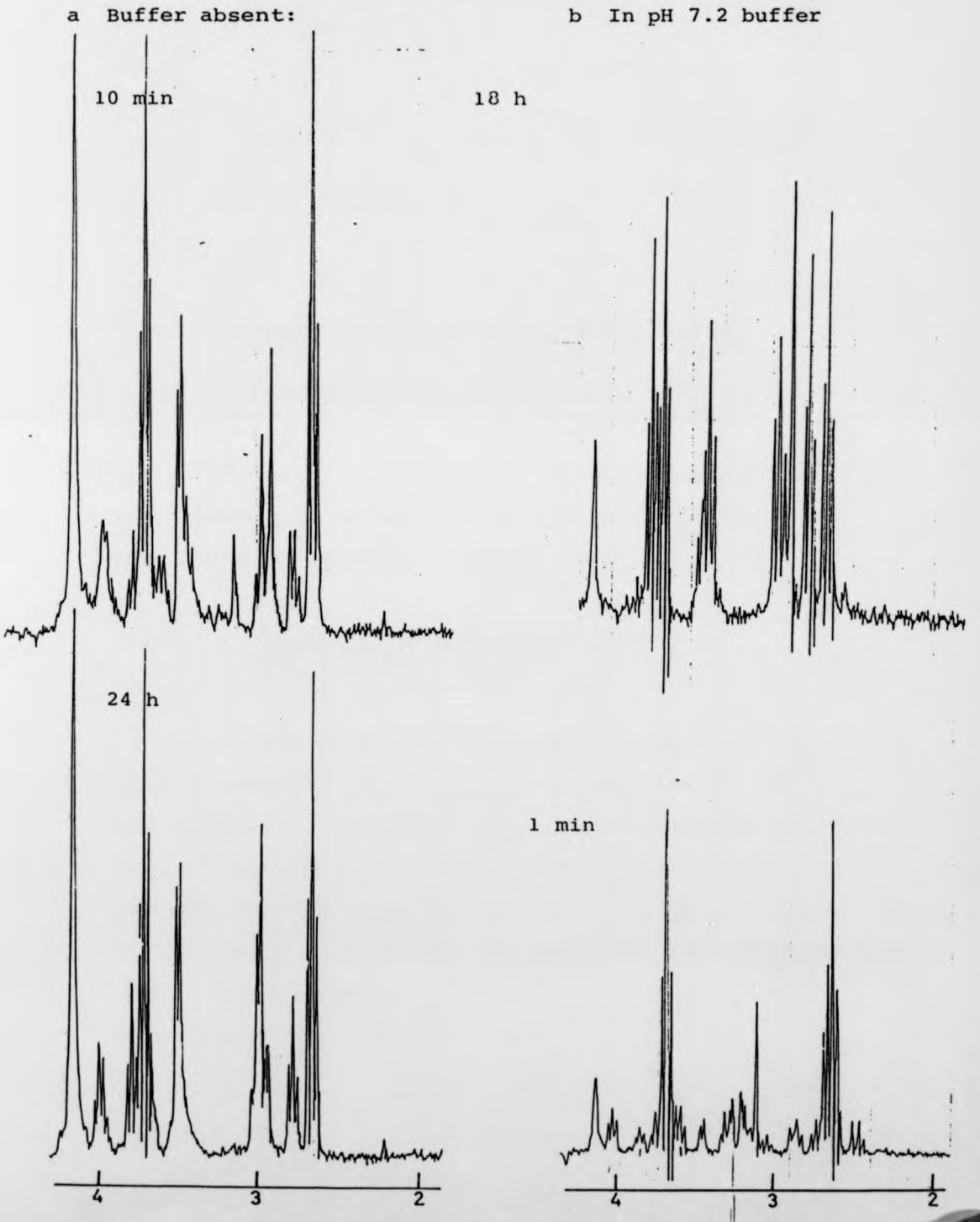
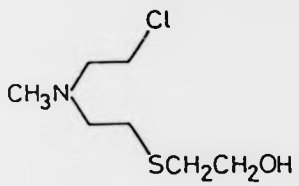
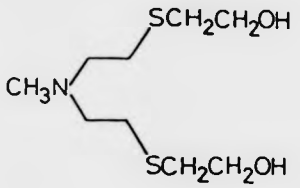


Fig. 4.15 Reaction of N-2-chloroethyl-N-methylaziridinium chloride with 2-mercaptoethanol monitored by 220 MHz $^{1}H$ n.m.r. spectroscopy.






(71)




(72)

(h) Reaction with Guanosine Monophosphate (Fig. 4.16)

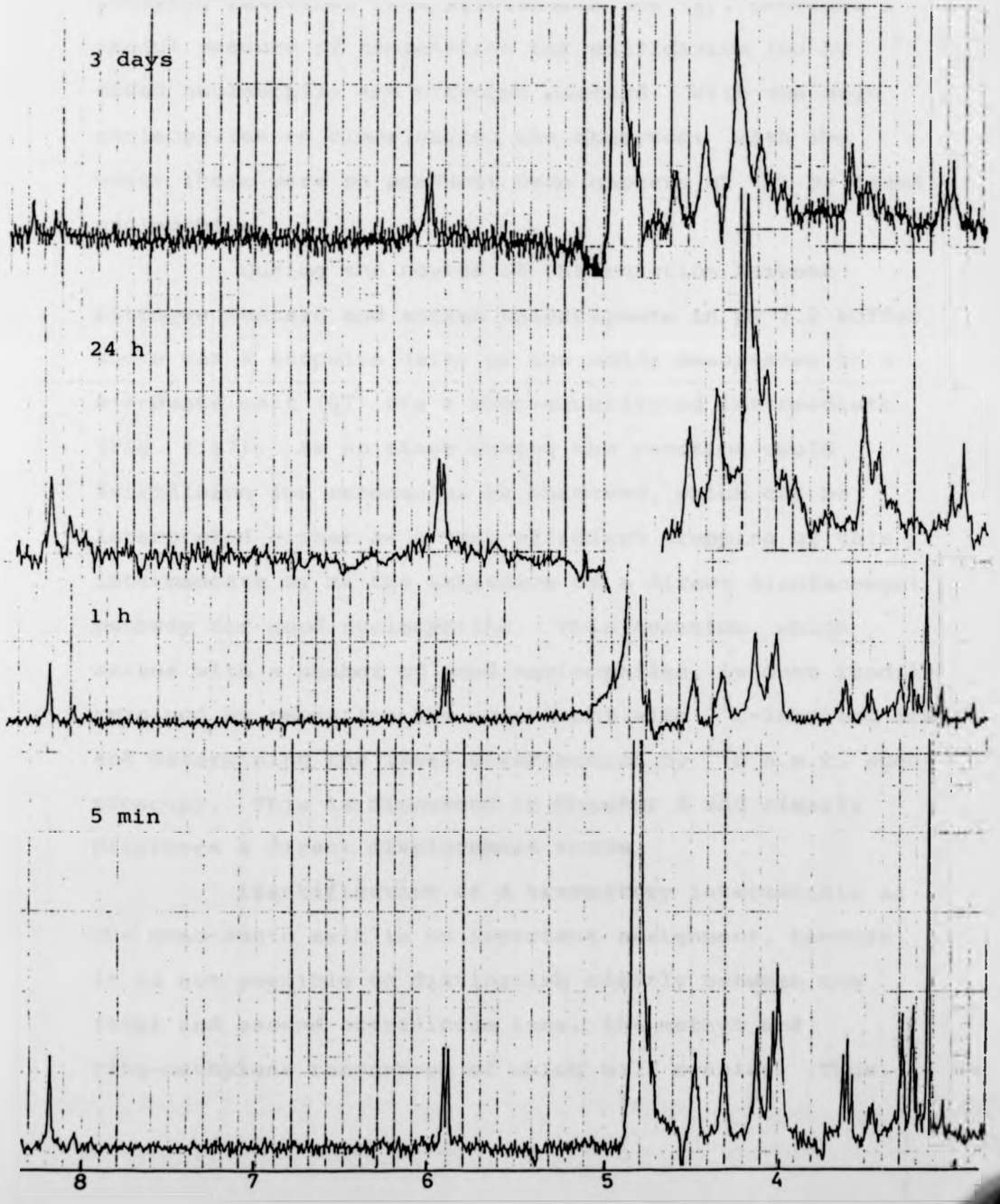
Addition of GMP to a solution of (8) gave no clear reaction save for the conversion of (8) to piperazinium dimer (10). To exclude the possibility of losing information due to proton exchange, the experiment was repeated in water with a similar result.

### 4.3.4 Reactions between Nitrogen Mustard and added Nucleophiles

Because of the problematic conversions of nitrogen mustard indicated above, reactions of the pre-formed aziridinium ion (8) were not further explored. Instead, nucleophiles in pH 7.2 buffer were treated with nitrogen mustard hydrochloride and the subsequent reactions were analysed with the knowledge gained from the preceding work. These reactions fall into three broad groups according to the products:

Group 1: Products derived solely by alkylation of the added nucleophile with negligible dimerisation or hydrolysis.

Fig. 4.16 Reaction of N-2-chloroethyl-N-methylaziridinium chloride with guanosine monophosphate monitored by 220 MHz n.m.r. spectroscopy.



Group 2: Dimerisation or hydrolysis only.

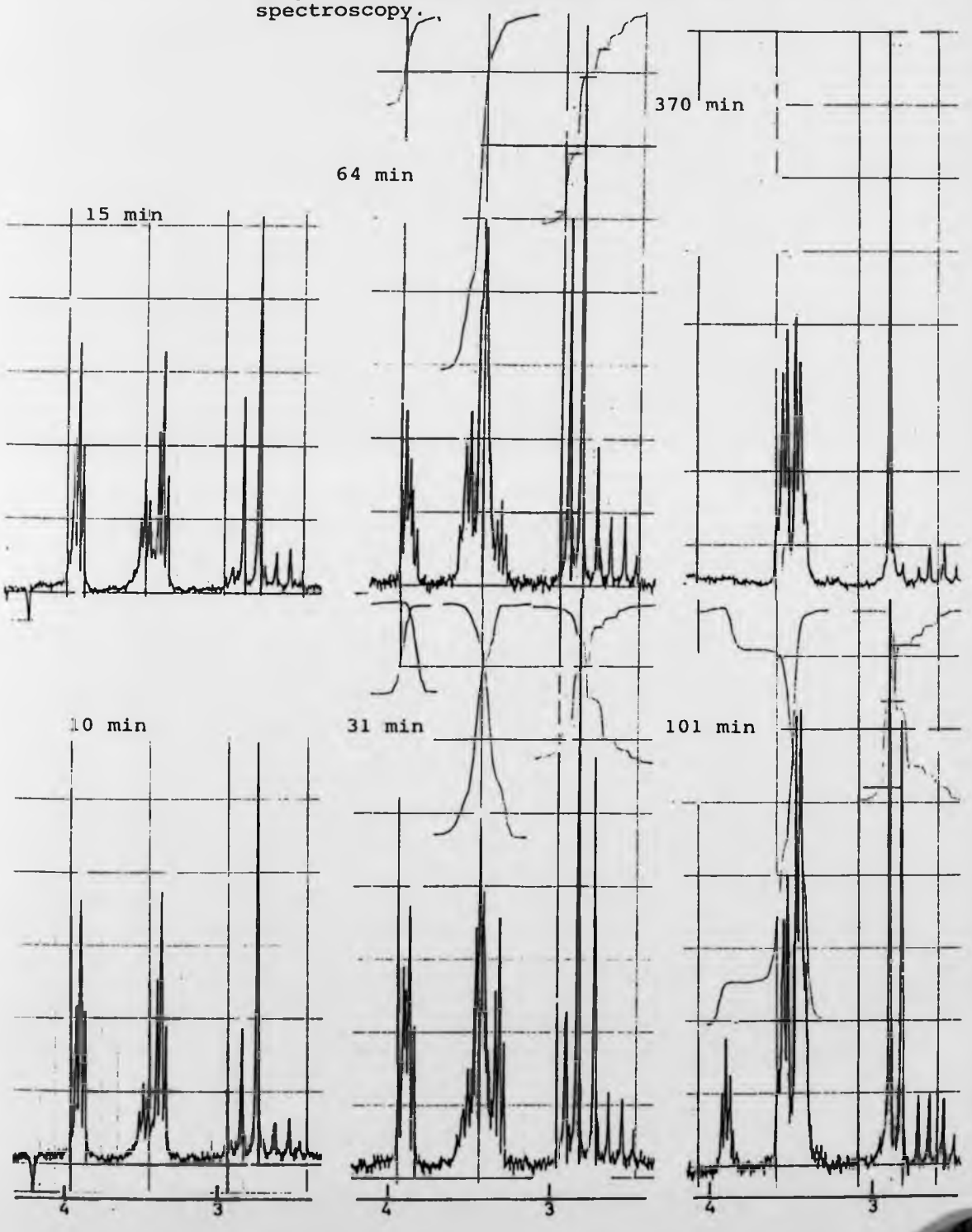
Group 3: A mixture of 1 and 2.

The piperazinium dimers of nitrogen mustard, nuisances in studying reactions from aziridinium ion (8), provided a useful measure of competition for aziridinium ion by added nucleophile and nitrogen mustard. With the best nucleophiles no dimerisation was observed; with the worst there were no products from capture of (8) by added nucleophile.

During the course of the reaction between nitrogen mustard and sodium thiosulphate in pH 7.2 buffer, there was a stepwise decay of the amine resonances to a *bis*-Bunte salt (67) *via* a mono-substituted intermediate (Fig. 4.17). At no stage during the reaction could aziridinium ion resonances be observed, which can be interpreted either as a very efficient trapping of this intermediate or as the existence of a direct displacement pathway for good nucleophiles. This question, which arises with a number of good nucleophiles, is most readily resolved by repeating the experiment with $^{13}C$-labelled drug and determining the label distribution by $^{13}C$ n.m.r. spectroscopy. This is discussed in Chapter 5 and clearly disproves a direct displacement route.

Identification of a transitory intermediate as the mono-Bunte salt is an important assignment, because it is not possible to distinguish clearly between the first and second aziridinium ions, the methyl and ring-methylene resonances of which will overlap. Thus,

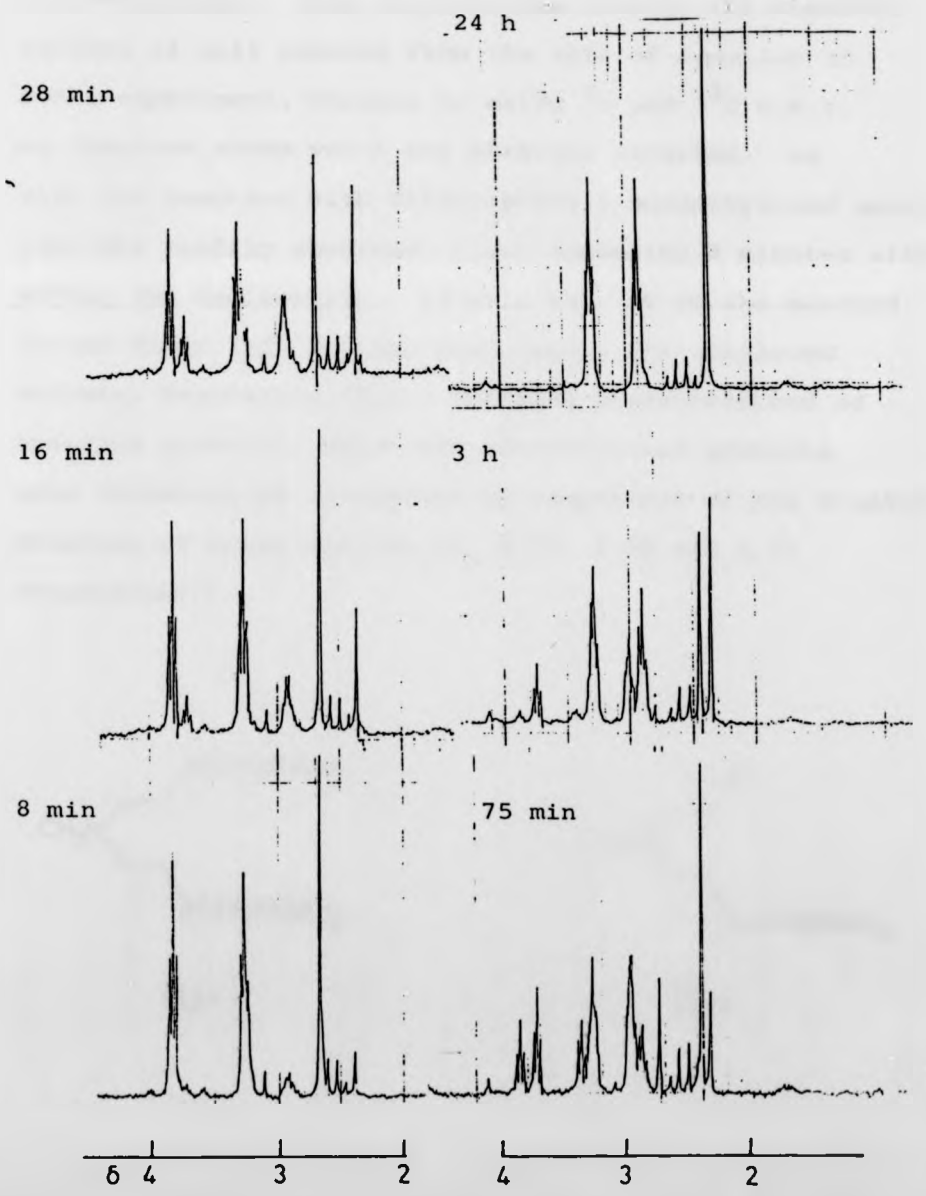
Fig. 4.17 Reaction between nitrogen mustard and $Na_2S_2O_3$ in pH 7.2 buffer monitored by 220 MHz $^1H$ n.m.r. spectroscopy.



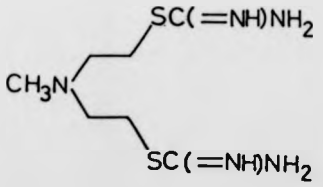
the step-wise nature of the reaction is only apparent from the assignment of three N-methyl singlets to free mustard, half-Bunte salt and Bunte salt. Monitoring the methyl resonances is the most straightforward method for following nitrogen mustard reactions by $^{1}H$ n.m.r. spectroscopy. For nucleophiles which do not give rise to $^{1}H$ n.m.r. spectra. methylenes of the chloroethyl arms of the parent nitrogen mustard or derived first aziridinium ion were sufficiently distinct for clear identification and integration. With thiosulphate as nucleophile, such measurements could be readily made and were in accord with the assignments indicated. Because thiosulphate is such an efficient nucleophile, nitrogen mustard base and hydroxide ion are not capable of competing effectively for aziridinium ions and so this is an especially clean reaction free from dimerisation or hydrolysis products. Therefore, the reaction between nitrogen mustard and thiosulphate is particularly suitable for analysis by $^{1}H$ n.m.r. spectroscopy.

Recent work with phosphoramide mustards dismissed thiourea as an efficient trapping agent for aziridinium ions derived from N,N-*bis*-(2-chloroethyl)phosphorodiamidic acid (6), stating that there was no observable ($^{31}P$ n.m.r. spectroscopy) reaction[36]. However, for nitrogen mustard we have demonstrated a very clean reaction (Fig. 4.18), demonstrating that thiourea is useful for scavenging aziridinium ion (8) as suggested by Kohn[52]. Thus, a cell treated with thiourea before administration of nitrogen mustard would be protected against alkylation. Zon

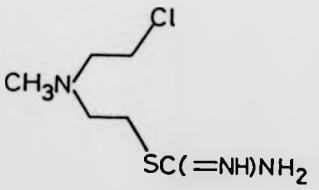
Fig. 4.18 Reaction between nitrogen mustard and thiourea in pH 7.2 buffer monitored by 220 MHz $^{1}H$ n.m.r. spectroscopy.



*et al.* suggested that S-alkylated products formed with (6) should be extremely labile and do not accumulate. However, this so-called labile functionality, $NCH_2CH_2SC(=NH)NH_2$, occurs in the product of nitrogen mustard alkylation (73) and is quite stable. Possibly, the $^{31}P$ resonance of (6) and of the alkylated product are coincident. This is plausible because the observed nucleus is well removed from the site of reaction in Zon's experiment, whereas by using $^{1}H$ and $^{13}C$ n.m.r. we observed atoms which are directly affected. As with the reaction with thiosulphate a monoalkylated species (74) was readily observed, first appearing 8 minutes after adding the nucleophile. After a day, 2% of the mustard formed dimer (10) but the rest gave a *bis*-alkylated thiourea derivative (73). Relative concentrations of starting material, mono- and *bis*-alkylated products were conveniently determined by comparison of the N-methyl singlets of these species ($\delta_H$ 2.73, 2.98 and 2.37 respectively).




(73)




(74)

Fig. 4.19 Reaction between nitrogen mustard and 2-mercaptoethanol in pH 7.2 buffer monitored by 220 MHz $^{1}H$ n.m.r. spectroscopy.

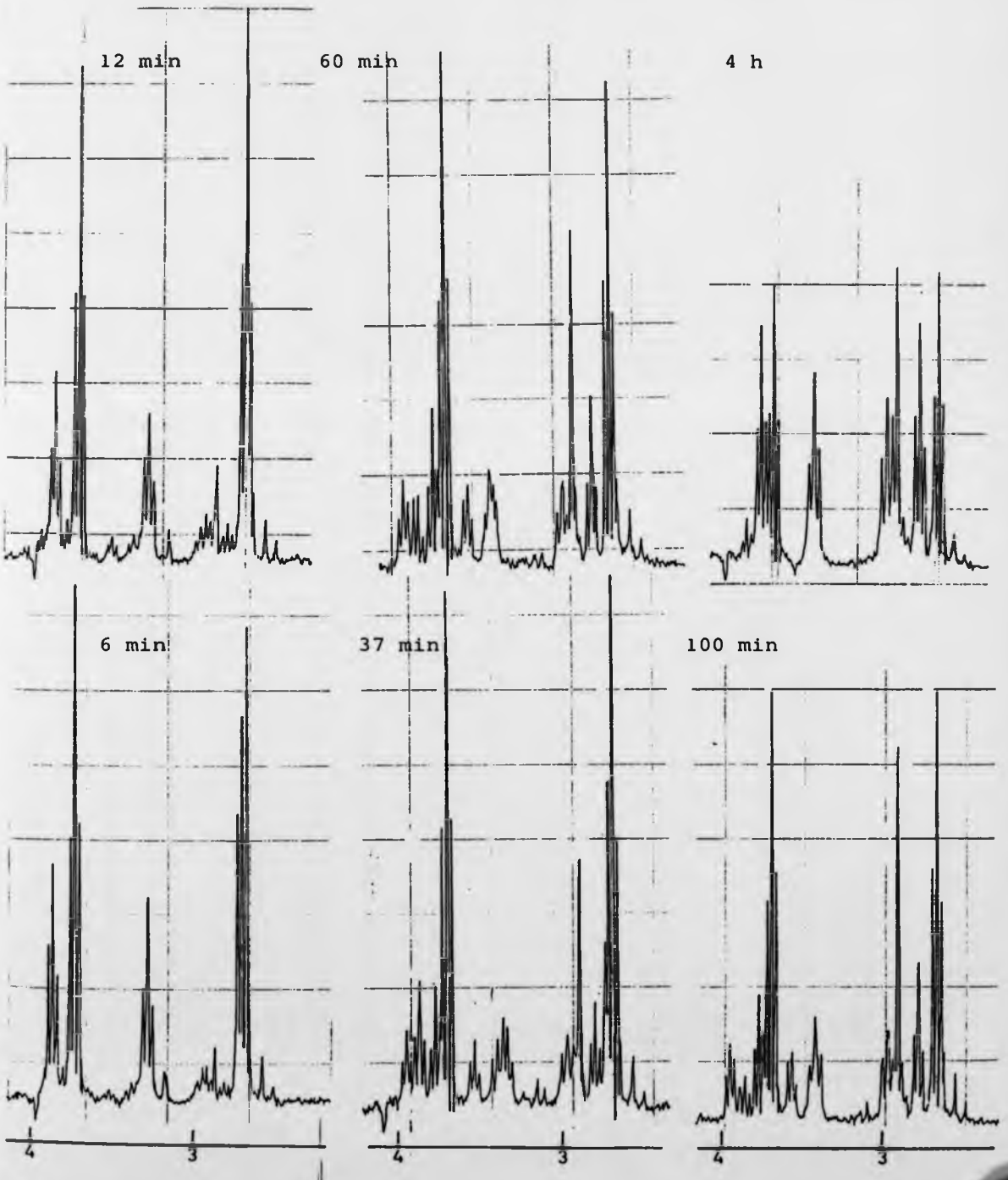


Fig. 4.20 Reaction between nitrogen mustard and (*S*)-methionine in pH 7.2 buffer monitored by 220 MHz $^1$H n.m.r. spectroscopy.

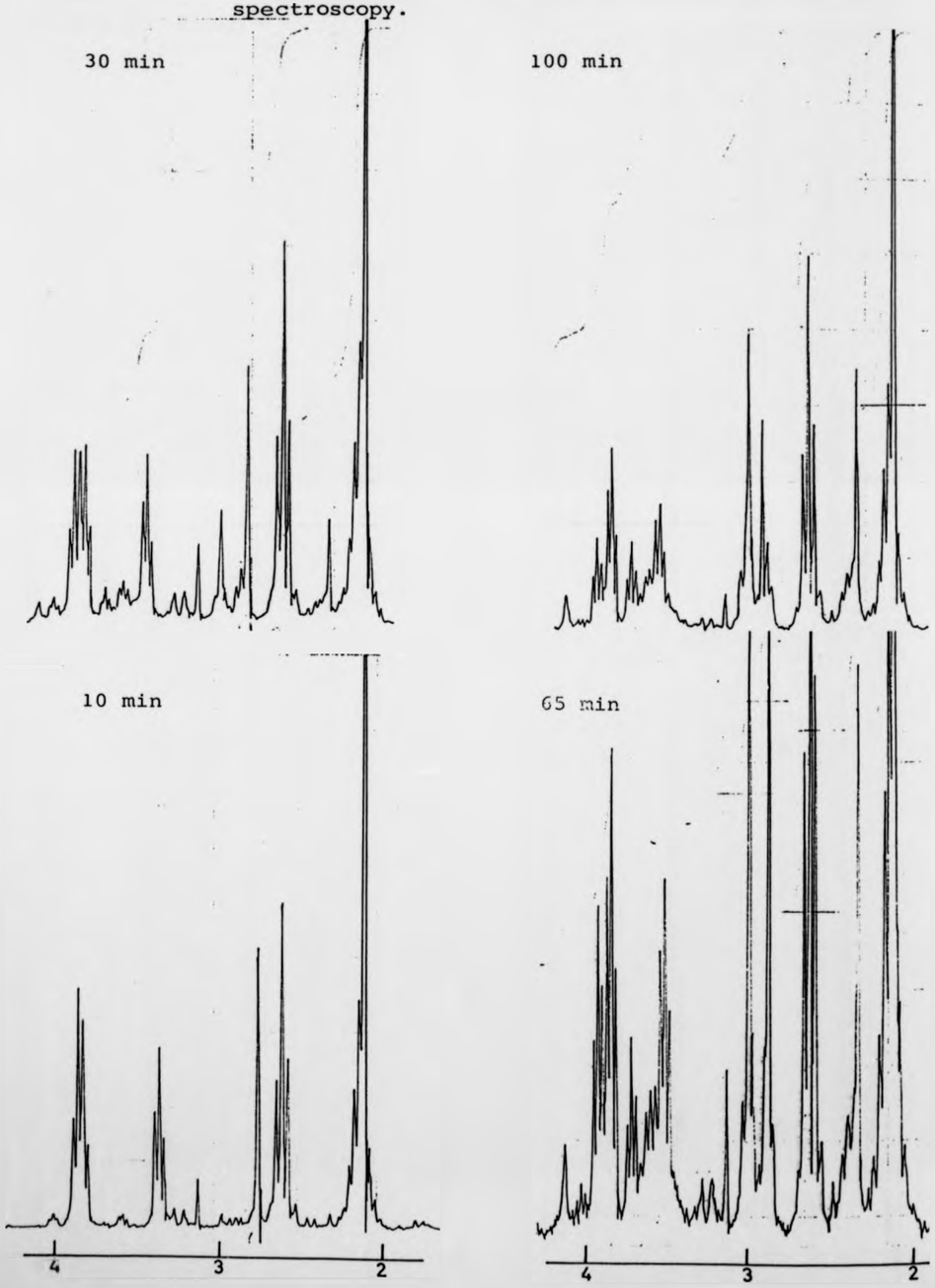



Fig. 4.20 Reaction between nitrogen mustard and (*S*)-methionine in pH 7.2 buffer monitored by 220 MHz $^1$H n.m.r. spectroscopy.

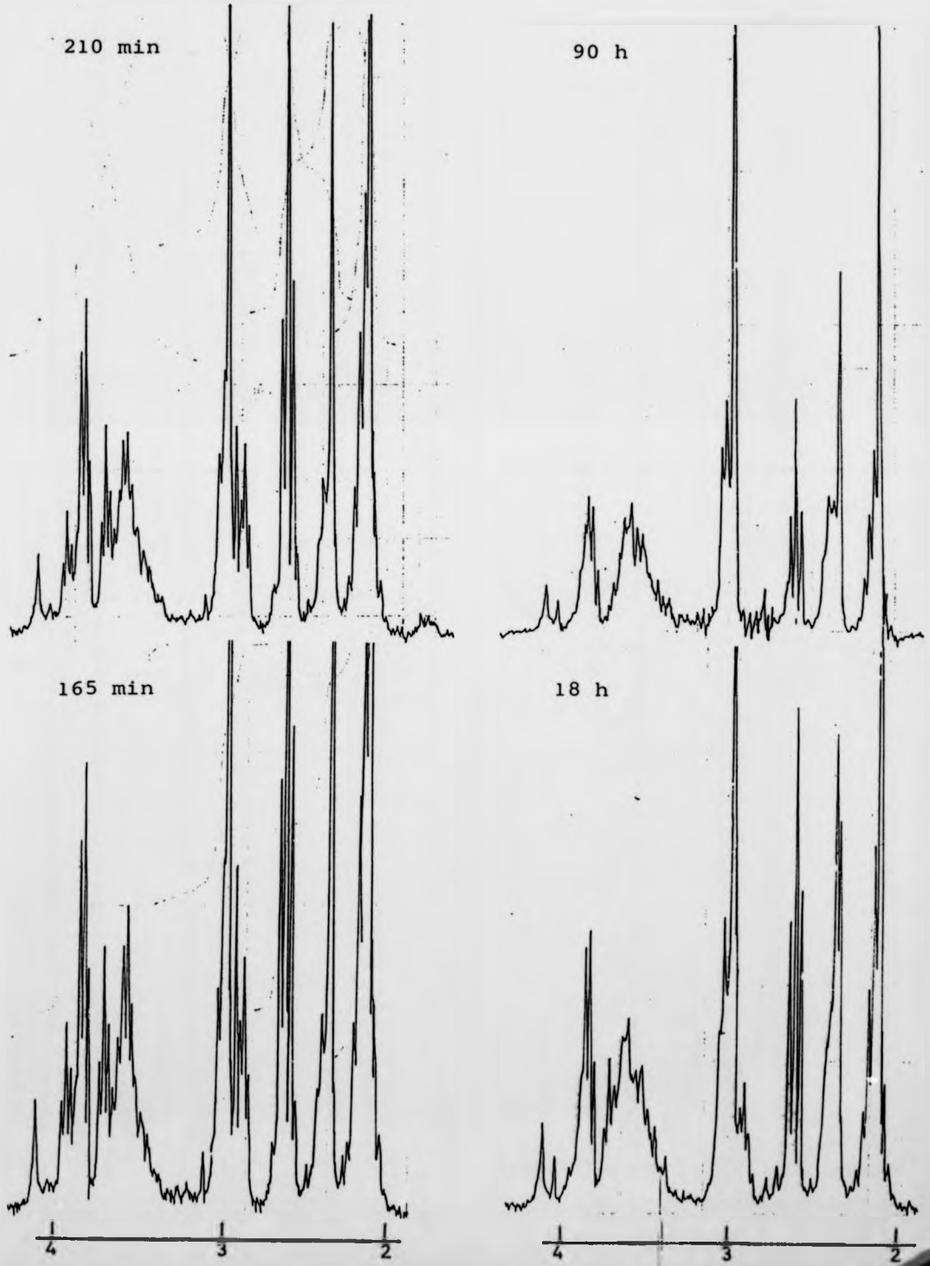


Fig. 4.21 Reaction between nitrogen mustard and N-acetyl-cysteine methyl ester in pH 7.2 buffer by 220 MHz n.m.r. spectroscopy.

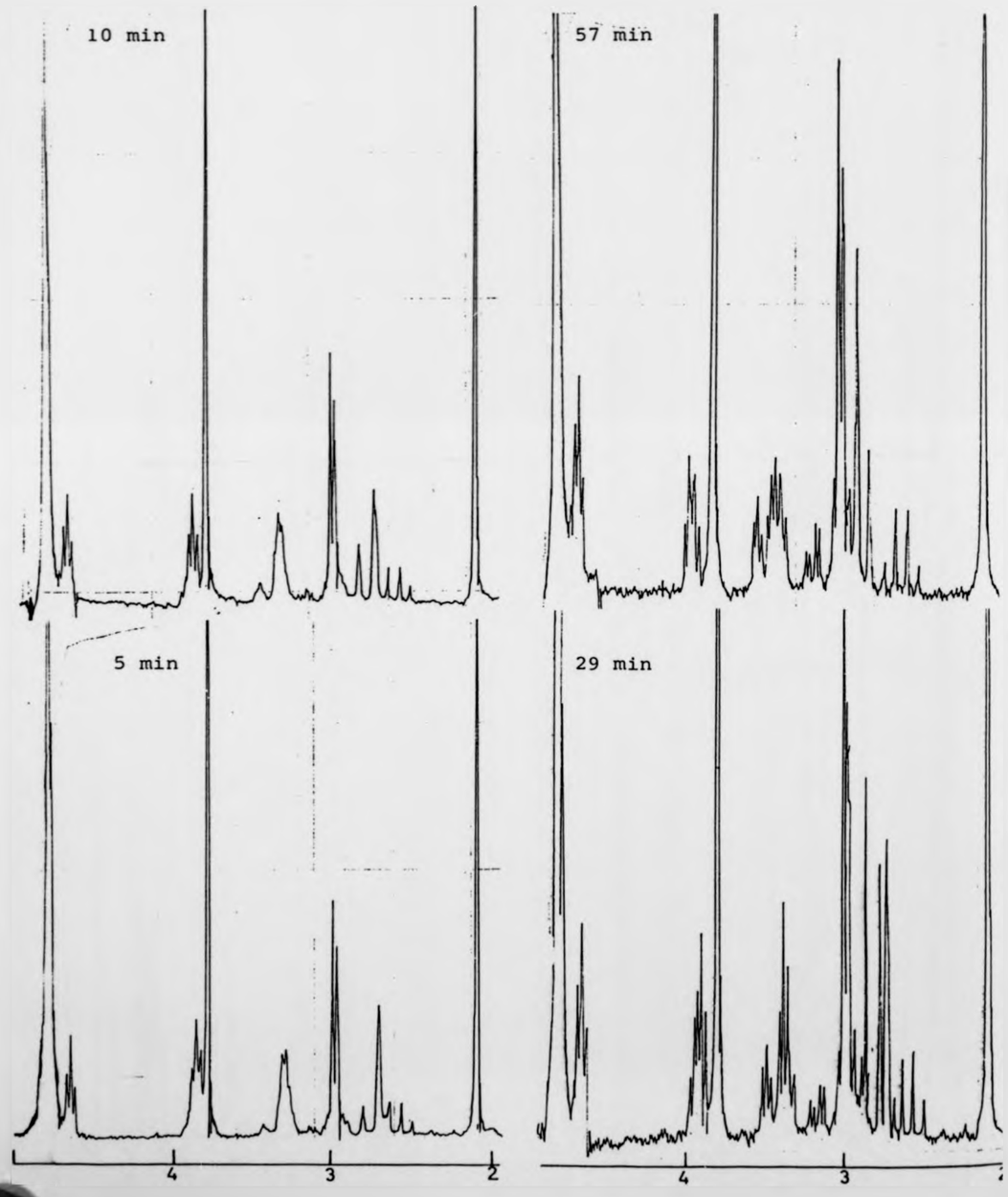
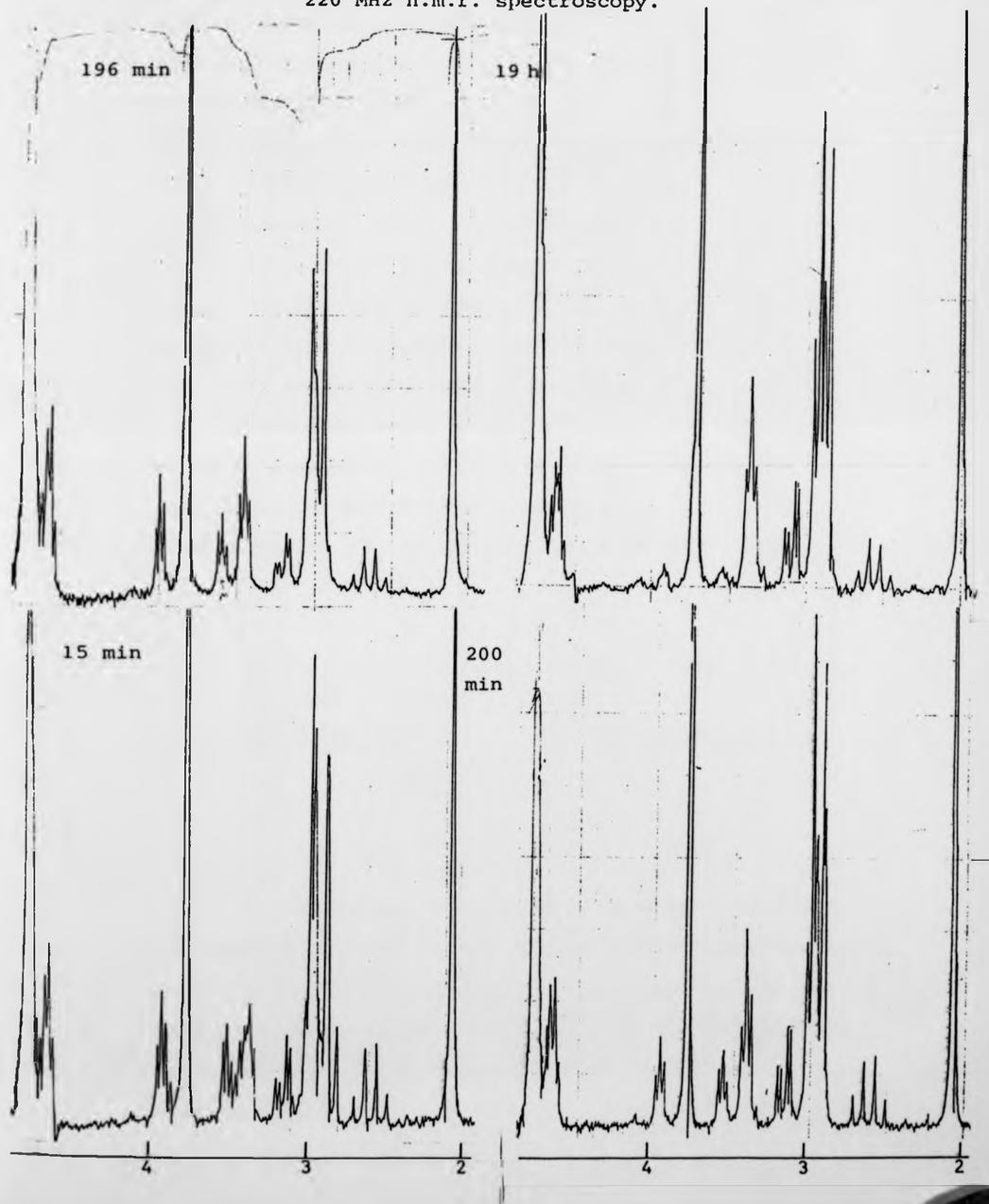
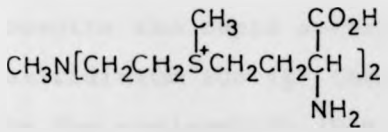


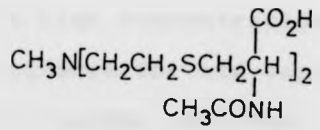

Fig. 4.21 Reaction between nitrogen mustard and N-acetyl-cysteine methyl ester in pH 7.2 buffer by 220 MHz n.m.r. spectroscopy.



Other sulphur nucleophiles studied showed similar behaviour to thiosulphate and thiourea. A stepwise pathway was clearly evident with 2-mercapto-ethanol as nucleophile (Fig. 4.19), which gave the *bis*-alkylated product (72) exclusively in a reaction of similar rate to that with thiourea. With methionine (Fig. 4.20) and N-acetylcysteine methyl ester (Fig. 4.21), there was a slower reaction giving both di-alkylated products (75) and (76) and mono-alkylated materials, which presumably suffered hydrolysis of the remaining chloroethyl arm. The reaction with methionine was particularly slow; one experiment took 7 days to reach an apparently stable condition and significant quantities of piperazinium dimers were formed (approximately 12% of the initial mustard concentration).



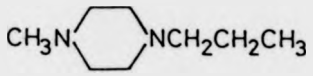
(75)



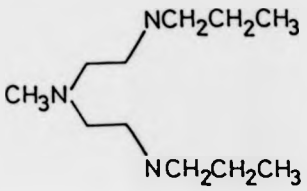
(76)

The cleanest reaction with an amine as added nucleophile occurred after treatment of nitrogen mustard hydrochloride with a solution of the diol (13) in pH 7.2 Sorensen's buffer (Fig. 4.22). Little accumulation of aziridinium intermediates was observed and there was no significant formation of piperazinium dimers. A two step

alkylation to give (66) was seen by observation of the N-methyl signals and noting the relative intensities of quaternary and free N-methyl resonances at $\delta_H$ 3.2 and *ca.* 2.4 respectively. Evidently at a pH where significant hydrolysis of chloroethyl arms occurs, much of the remaining mustard would be consumed in polymerisation reactions.



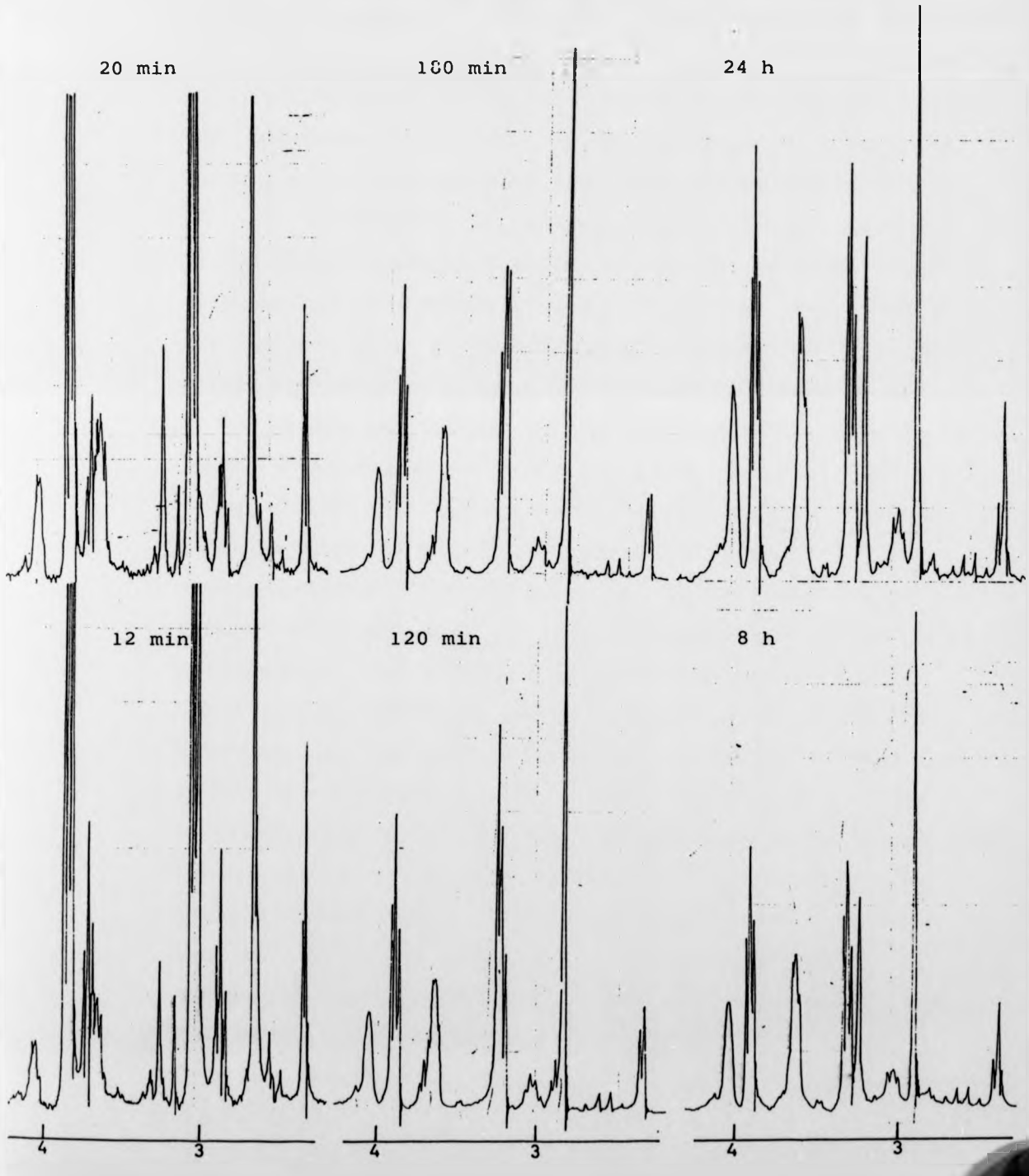

(77)




(78)

Alkylation of *n*-propylamine at pH 7.2 does not yield the anticipated mixture of N-methyl-$N^1$-propylpiperazine (77) and $N^2$-methyl-$N^1$,$N^3$-propyldiethylenetriamine (78). Despite the rapid accumulation of a high concentration of aziridinium ion (8) there was no evidence for its capture by the nucleophile (Fig. 4.23). The buffer had been swamped by an amine which is a strong base (pKa of *n*-propylamine = 9.87) and which was therefore protonated and unavailable for the capture of (8). During four days of observation the $^1$H n.m.r. spectrum of propylamine remained unperturbed, but the aziridinium resonances gave way to signals attributed to a phosphate diester of nitrogen mustard (64) (i.e. the product obtained when nitrogen mustard was exposed to Sorensen's buffer alone).

Fig. 4.22 Reaction between nitrogen mustard and N-2-hydroxyethyl-N-methyl-2-hydroxyethanamine in pH 7.2 buffer monitored by 220 MHz $^{1}H$ n.m.r. spectroscopy.



There was also evidence for the generation of phosphate substituted analogues of piperazinium dimer (10). Thus, *n*-propylamine was not interfering with the reaction between alkylating agent and buffer, save to raise the pH; increased pH serves to speed the cyclisation to aziridinium ion (8). This behaviour occurs whenever the nucleophile is an amine which has a higher pKa than nitrogen mustard (pKa = 6.45).

Golumbic *et al.* measured the nucleophilicity of a nucleophile towards nitrogen mustard by its competition with alanine at a pH of 7.5-8.0. In this pH range most of the alanine (pKa = 9.87) will be protonated. We found very rapid generation of aziridinium species when alanine in pH 7.2 buffer was treated with nitrogen mustard, but these intermediates were long-lived ($t_{\frac{1}{2}}$ ~ 1 hour) and during 4 days there was no change in the alanine $^1H$ n.m.r. spectrum (Fig. 4.24). Again, the products derived from nitrogen mustard were those seen in buffered reactions without added nucleophiles. These results differ from those of Golumbic *et al.*, because these workers did not buffer their system. Instead, nitrogen mustard was neutralised with NaOH and then four mol. equiv. of alanine and four mol. equiv. of $NaHCO_3$ were added. The consumption of 0.75 mmol of alanine per mmol of mustard was reported. Apparently until the $NaHCO_3$ was consumed the medium was basic and the amino group of alanine was available to capture the aziridinium ion (8). Between 1 and 2 mols of alanine per mol of mustard should be consumed depending on the degree of alkylation. The failure in the experiment of Golumbic *et al.* to consume all the mustard may be due to a decrease

Fig. 4.23 Reaction between nitrogen mustard and *n*-propylamine in pH 7.2 buffer monitored by 220 MHz $^1$H n.m.r. spectroscopy.

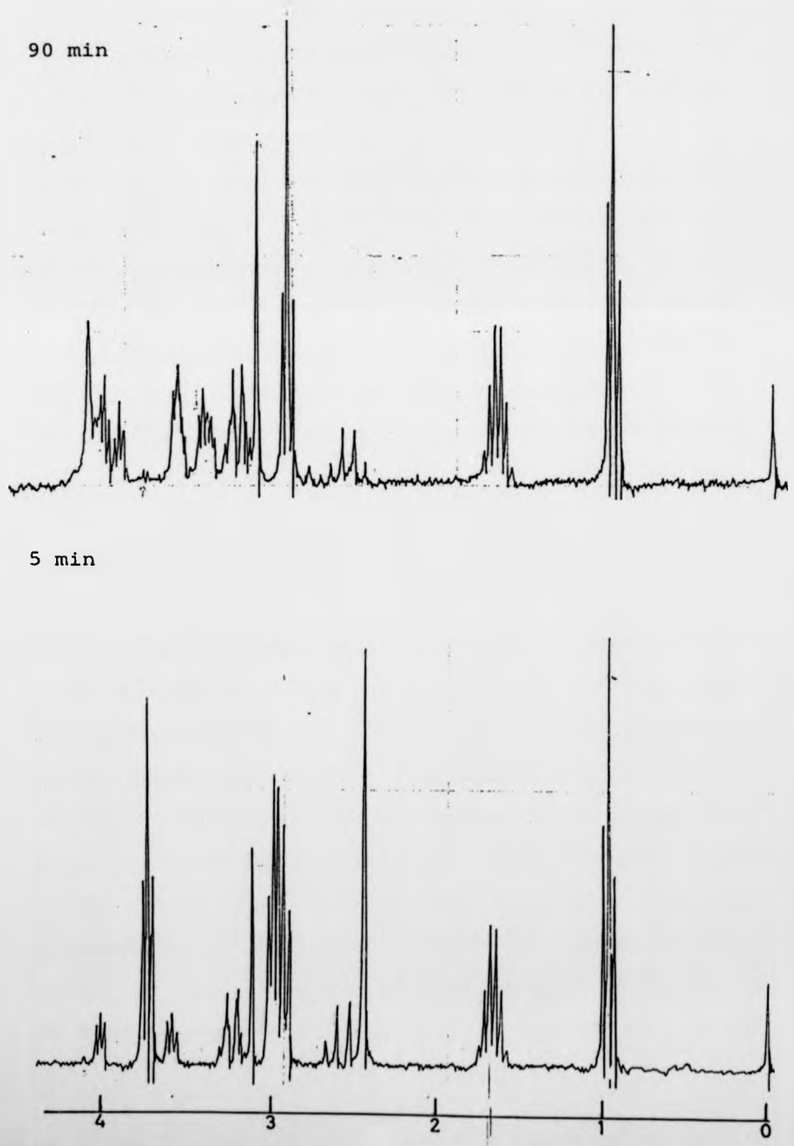



Fig. 4.23 Reaction between nitrogen mustard and *n*-propylamine in pH 7.2 buffer monitored by 220 MHz $^1$H n.m.r. spectroscopy.

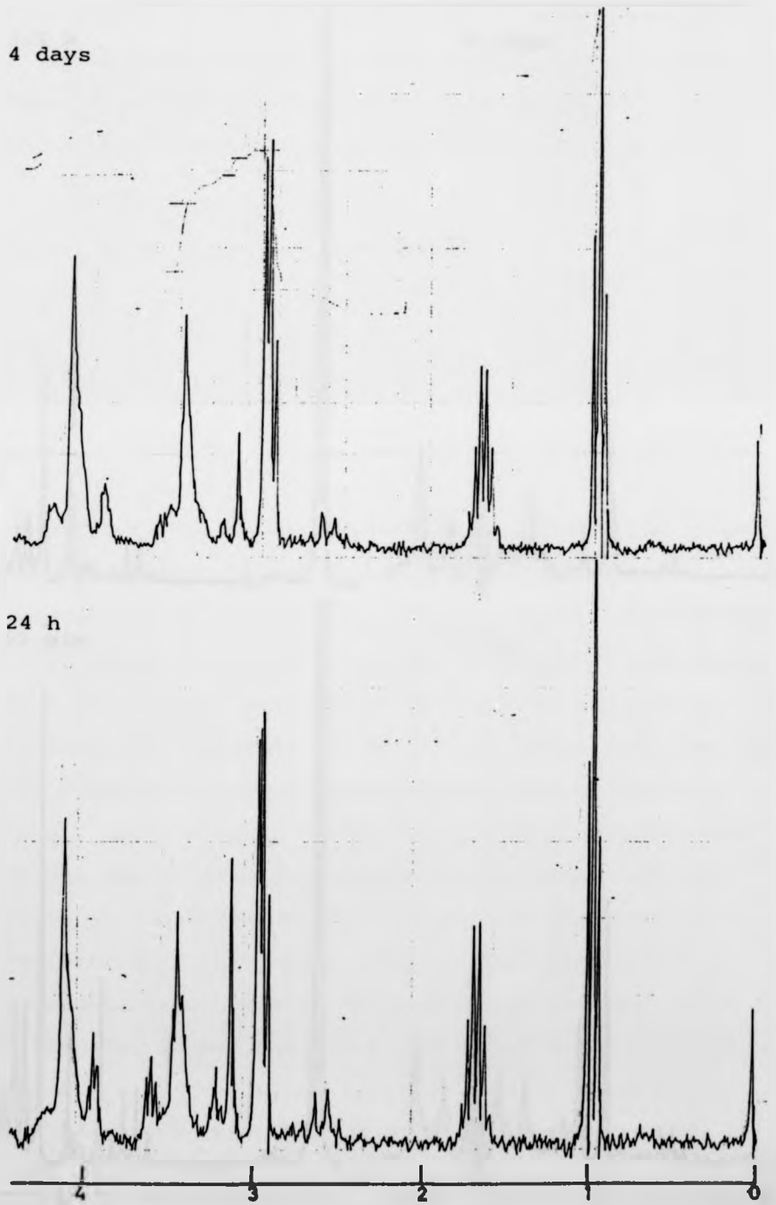
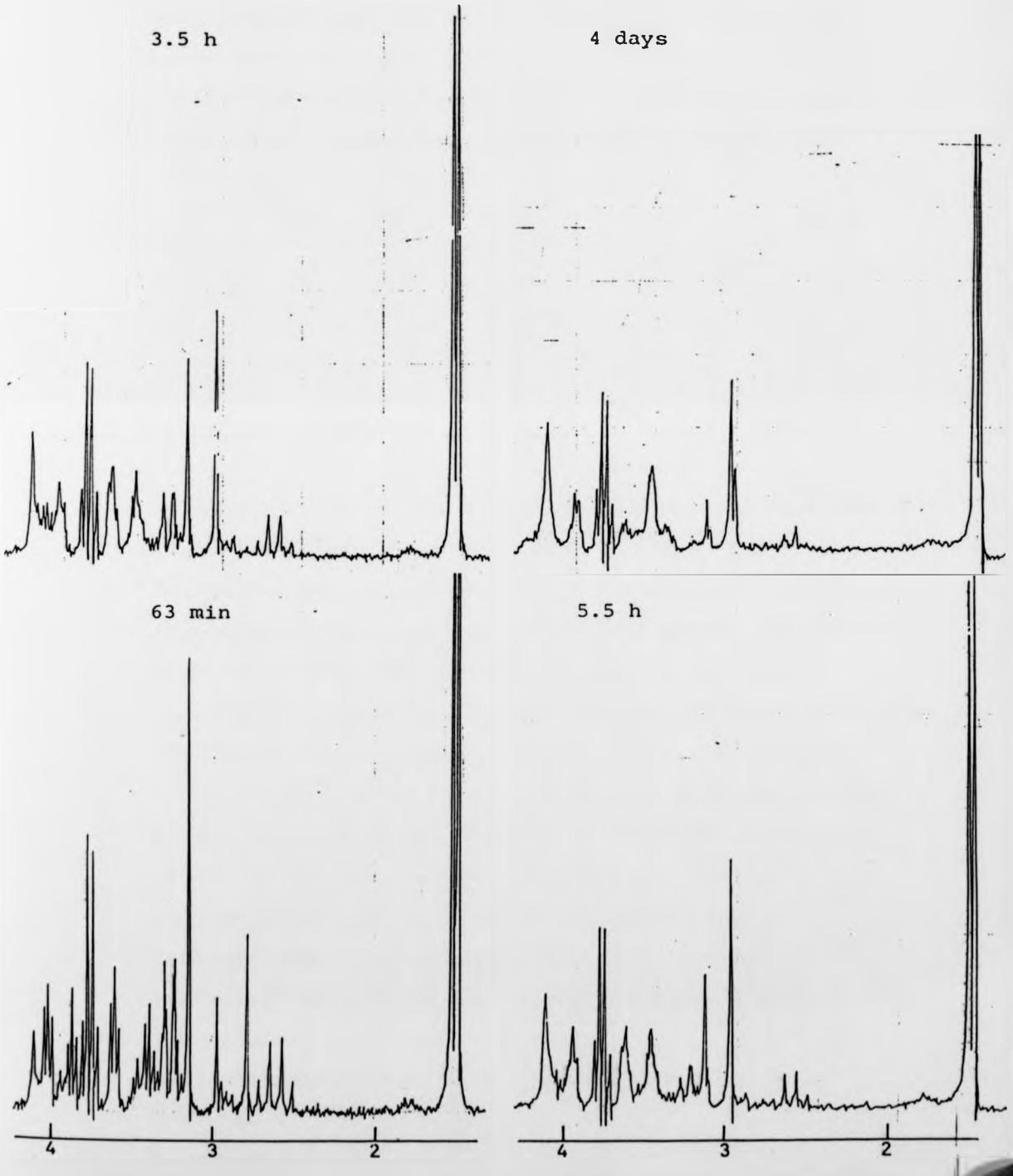
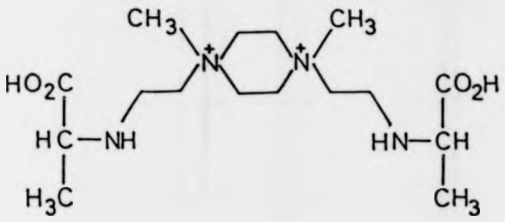


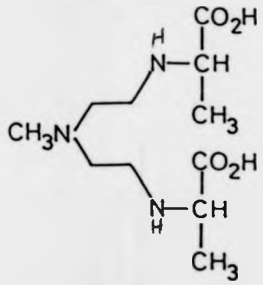
Fig. 4.24 Reaction between nitrogen mustard and alanine in pH 7.2 buffer monitored by 220 MHz $^{1}H$ n.m.r. spectroscopy.



in pH as the reaction proceeds. They presented data indicating that 92% of the chloroethyl groups lost chloride and that only 3% were capable of reacting with thiosulphate after 24 hours, which indicates substantial hydrolysis. The end product of this process was suggested to be the $N,N^1$-methyl-$N,N^1$-ethylalanine piperazinium dication (79).



(79)



(80)

Di-substitution of chloride with alanine to give $N^2$-methyl-$N^1,N^3$-(2-methylglycine)diethylenetriamine (80) or intramolecular cyclisation to give N-methyl-$N^1$-(2-methylglycine)piperazine (81) were not considered. By analogy with our results when excess of ammonia was used as nucleophile, (Chapter 5), these are the products to expect. The comparison between our work and that of Golumbic *et al.* shows a sharp difference in the conditions used. By the use of dilute solutions of reactants and a pH of *ca.* 7, we intended that our results could be indicative of what might occur in physiological circumstances. Because amino nitrogen was usually protonated in our reactions this provides a better model system than that of Golumbic *et al.*, except in circumstances where the pH is significantly higher than *ca.* 7.

Fig. 4.25 Reaction between nitrogen mustard and N-acetyl-histidine in pH 7.2 buffer monitored by 220 MHz $^{1}H$ n.m.r. spectroscopy.

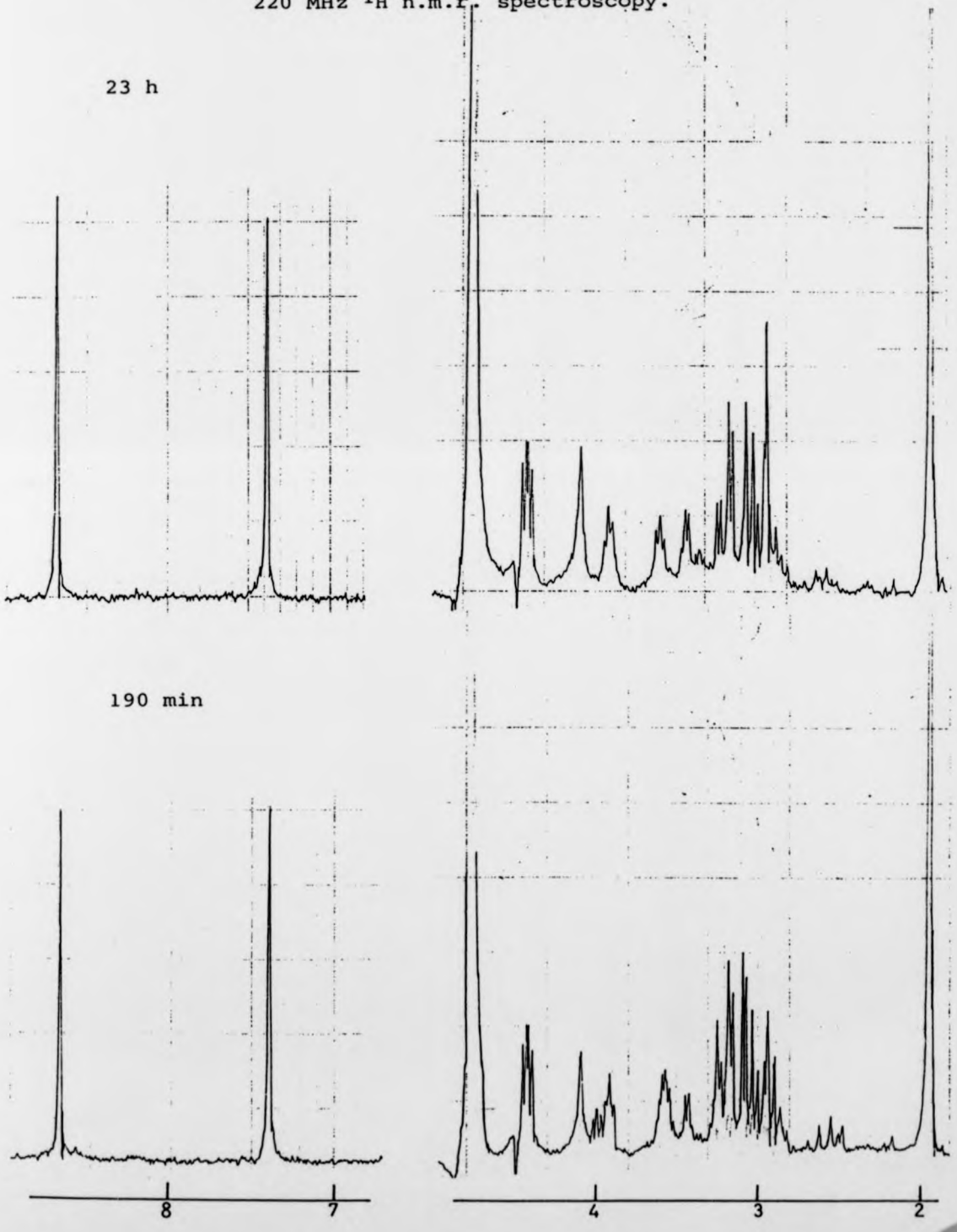
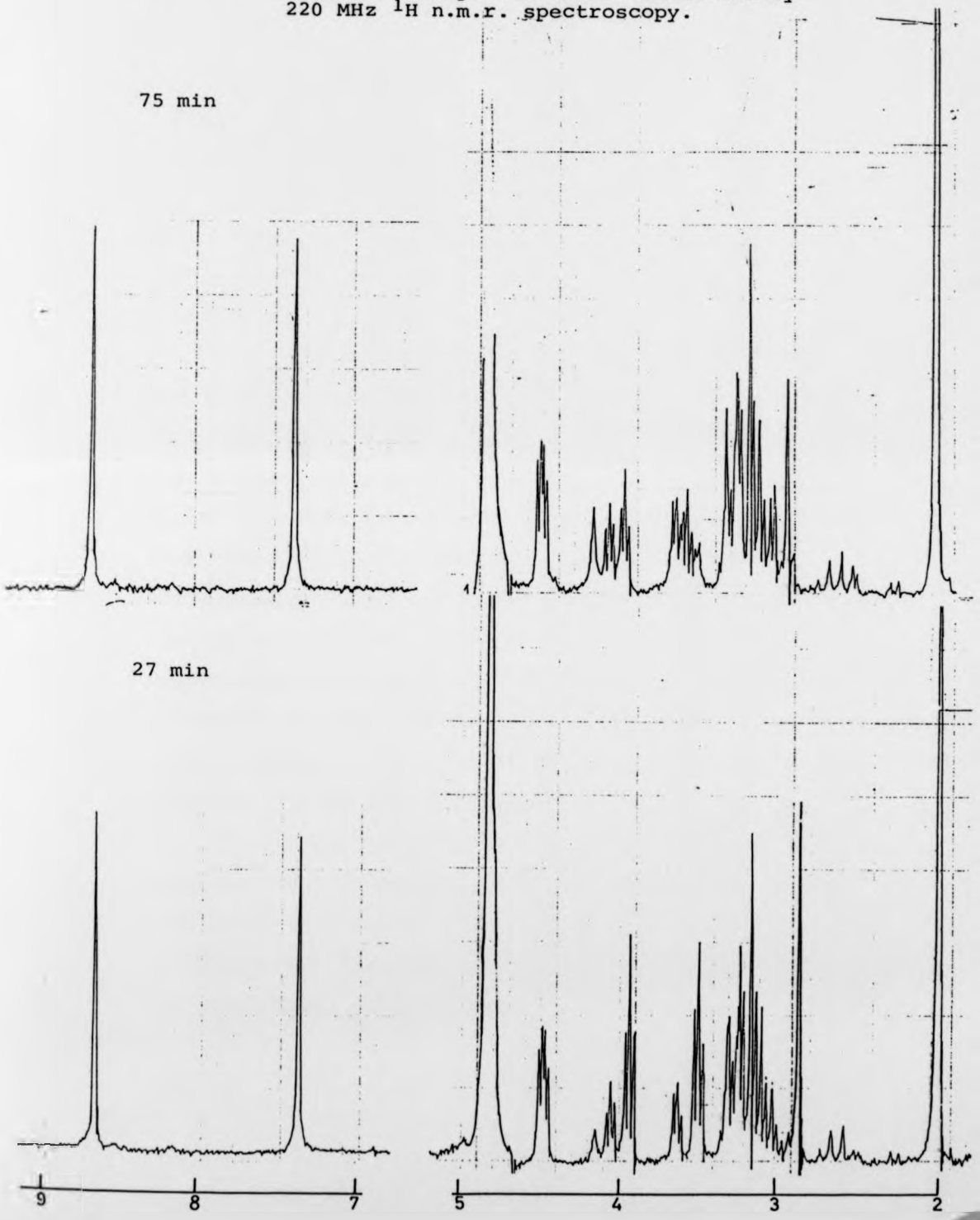
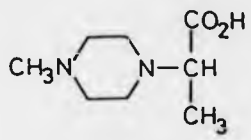
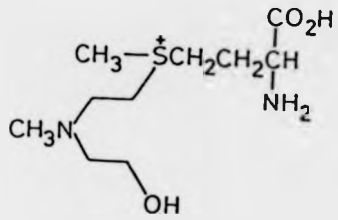


Fig. 4.25 Reaction between nitrogen mustard and N-acetyl-histidine in pH 7.2 buffer monitored by 220 MHz $^{1}H$ n.m.r. spectroscopy.





(81)

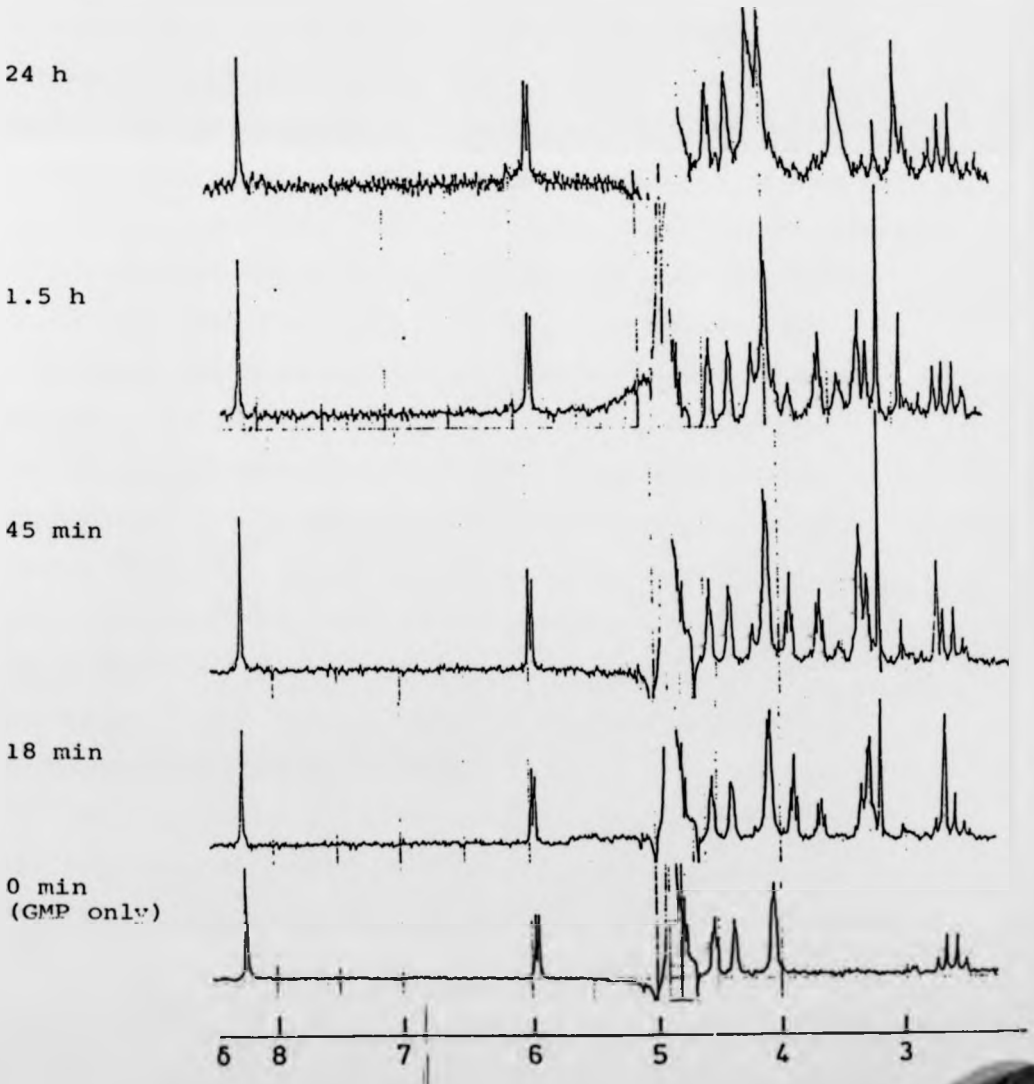


(82)

Lack of reactivity towards aziridinium ion (8) was also shown by the imidazole function of N-acetyl-*S*-histidine (Fig. 4.25). This is surprising because the pKa of this function (5.97) is below that of nitrogen mustard itself, i.e. if the drug is capable of cyclising then the imidazole nitrogens must be unprotonated. Despite generating a high concentration of aziridinium ion (8) the $^{1}H$ n.m.r. spectrum of the amino acid is unperturbed save for a slight pH shift. Alkylation of an imidazole nitrogen should affect the shifts of the ring protons adjacent to it. However, these remain as sharp singlets throughout the generation and decay of (8).

Reaction of nitrogen mustard with guanosine monophosphate is widely cited as a model for alkylation of guanosine in nucleic acids. Once again, we were unable to detect any reaction of this potential nucleophile under our conditions (Fig. 4.26)

Fig. 4.26 Reaction between nitrogen mustard and guanosine monophosphate in pH 7.2 buffer monitored by 220 MHz $^{1}H$ n.m.r. spectroscopy.



## 4.4 EXPERIMENTAL

### 4.4.1 Generation of N-2-Chloroethyl-N-methylaziridine

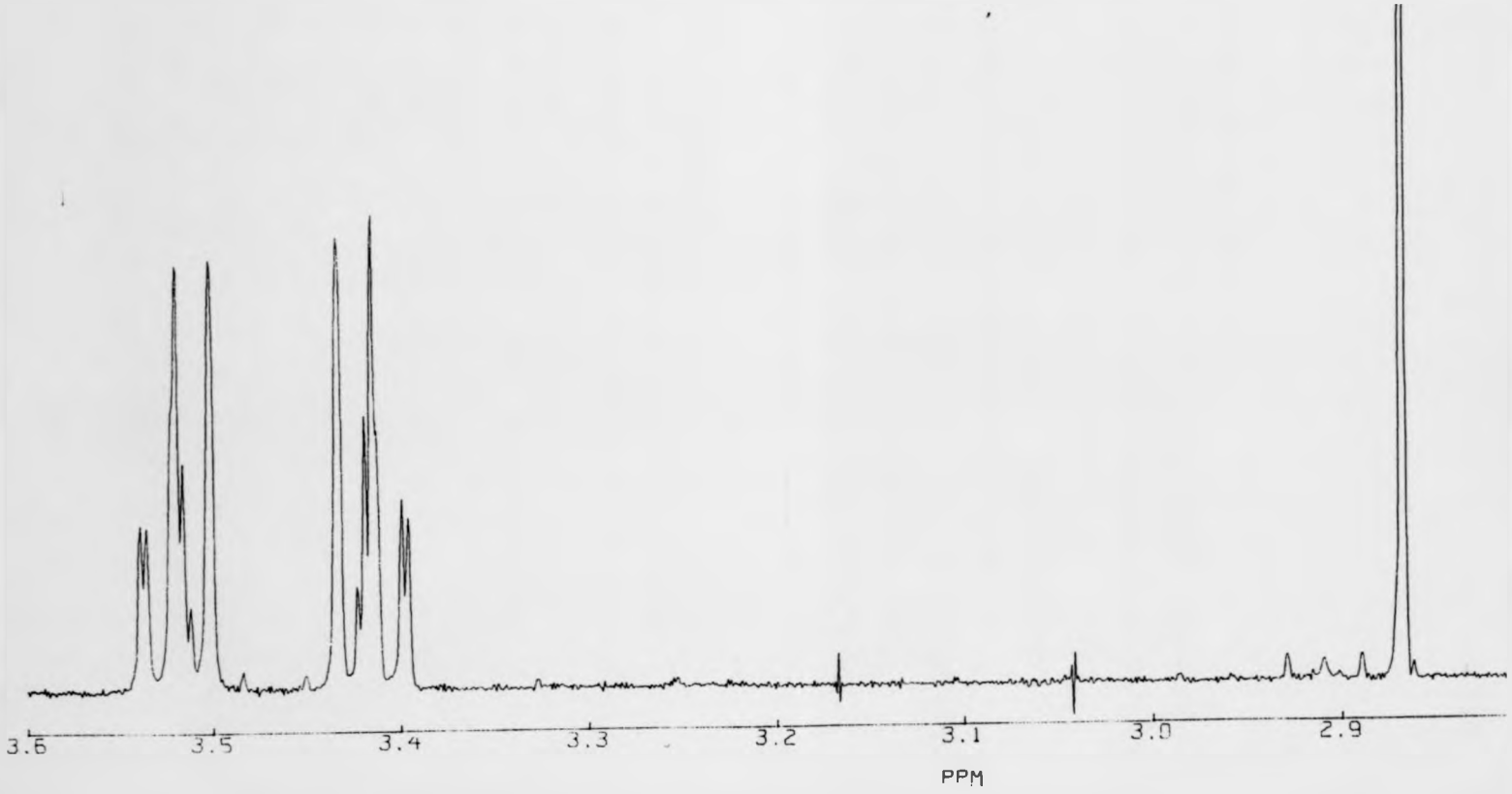
$^2H_2O$ (0.5 $cm^3$) was added to a vial containing nitrogen mustard hydrochloride (10 mg) which rapidly dissolved. Treatment with a 1.7 M solution of $Na_2CO_3$ in $^2H_2O$ (31 µl, 1 mol. equiv.) gave a briefly opaque solution as the free base was liberated. Monitoring by $^1H$ n.m.r. spectroscopy showed the title compound to be present 2 minutes from mixing, $\delta_H$ ($^2H_2O$): 3.16, (s, $\overset{+}{N}Me$), 3.32 (m, ring-$CH_2$), 3.64 (t, $CH_2\overset{+}{N}$), 4.08 (t, $CH_2Cl$), growing to a maximum concentration of 80-90% of starting mustard after *ca.* 2 hours. N,N'-2-chloroethyl-N,N'-methylpiperazinium dichloride (10) was detectable 20 minutes after neutralisation and increased while uncyclised nitrogen mustard was still present. After reaching its maximum concentration, aziridinium ion (8) is slowly converted to piperazinium dimer which conversion is virtually complete in 48 hours. At this time a $^1H$ n.m.r. spectrum shows traces of $MeN(CH_2CH_2OH)_2$ (13) and the linear dimer derived thereof, $HOCH_2CH_2NMe.CH_2CH_2\overset{+}{N}Me.(CH_2CH_2OH)_2$ (65), $\delta_H$ ($^2H_2O$) (13): 2.40 (s, $CH_3N$), 2.76 (t, 2 x $CH_2N$), 3.74 (t, 2 x $CH_2OH$). A solution of nitrogen mustard hydrochloride (10 mg) in 2 M $Na_2CO_3$ (0.5 $cm^3$) showed only $MeN(CH_2CH_2OH)_2$ by $^1H$ n.m.r. analysis. The reaction was fast and no intermediate species were identified. Warming solutions of (8) to 30 $^oC$ for 1 hour gave only piperazinium dimer,

no aziridinium species remaining at the end of this time. This behaviour caused serious problems when attempting to record decoupled $^{13}C$ n.m.r. spectra. The $^{1}H$ n.m.r. spectrum of (10) was remarkably uninformative: $\delta_H$ 3.46 (s, $CH_3\overset{+}{N}$, 3 H), 3.48 (s, $CH_3\overset{+}{N}$, 3 H), 4.15 (broad singlet, $CH_2$, 16 H). The 2 methyl resonances arise from *cis*- and *trans*-isomers. Confirmation of this assignment was provided by synthesis. Nitrogen mustard hydrochloride (0.25 g) was dissolved in water (2 $cm^3$) and neutralised with 2 M NaOH (0.65 $cm^3$). The free base was twice extracted with chloroform (2 x 3 $cm^3$), evaporated and taken up in methanol (5 $cm^3$). After 40 hours a fine white deposit of *cis*-N,N'-2-chloroethyl-N,N'-methyl piperazine was recovered by filtration. Analysis by $^{1}H$ n.m.r. confirmed the above assignment: $\delta_H$ ($^{2}H_2O$): 3.46 (s, $CH_3\overset{+}{N}$ *cis*), 4.16 (broad singlet, $CH_2$). The presence of quaternary nitrogen makes the methylene protons diastereotopic but no couplings may be observed even at 400 MHz.

### 4.4.2 Reactions between N-2-Chloroethyl-N-methyl-aziridine and Nucleophiles in the Absence of Buffer

In all cases, nitrogen mustard hydrochloride (10 mg) in $^{2}H_2O$ (0.5 $cm^3$) was treated with 1.7 M $Na_2CO_3$ in $^{2}H_2O$ (31 µl, 1 mol. equiv.) and allowed to stand at room temperature (18-20 $^{o}C$) for 2 hours when $^{1}H$ n.m.r. analysis showed at least 80% conversion to the title compound. The nucleophile, 2.5 mol. equivs. in 0.5 $cm^3$ $^{2}H_2O$ was added and $^{1}H$ n.m.r. spectra were recorded at

Fig. 4.27



*ca.* 1 minute, 5 minutes, 1 hour, 2 hours and 24 hours from mixing.

(a) Sodium Thiosulphate

Treatment with 21 mg of $Na_2S_2O_3$ shows predominantly mono-Bunte salt after 5 minutes, $MeN(CH_2CH_2Cl)(CH_2CH_2S_2O_3)$: $\delta_H$ ($^2H_2O$): 2.47 (s, $CH_3N$), 2.08 (m, 2 x $CH_2$-N), 3.27 (t, $CH_2S_2O_3$), 3.76 (t, $CH_2Cl$). The 24 hours spectrum showed only piperazine dimer and *bis*-Bunte salt, $MeN(CH_2CH_2S_2O_3)_2$: δ 2.47 (s, $CH_3N$), 3.18 (m, 2 x $CH_2CH_2S_2O_3$). The latter resonance is due to the ABCD spin system (Fig. 4.27) mentioned above; this sort of coupling is frequently useful for identifying mustard derivatives.

(b) Sodium Hydroxide

Treatment with 0.52 $cm^3$ of a $^2H_2O$ solution containing 10 $mg/cm^3$ of NaOH gave after 24 hours a 2:1 mixture of (13) and (65). $MeN(CH_2CH_2OH)_2$: δ 2.27 (s, $CH_3N$), 2.60 (t, 2 x $CH_2N$), 3.70 (t, 2 x $CH_2OH$). $MeN(CH_2CH_2OH)CH_2CH_2\overset{+}{N}Me(CH_2CH_2OH)_2$: δ 2.33 (s, $CH_3N$), 2.98 (m, 2 x $CH_2N$), 3.21 (s, $Me\overset{+}{N}$), 3.60 (m, 3 x $CH_2$-$\overset{+}{N}$), 4.02 (m, 3 x $CH_2OH$).

(c) Ammonia

Treatment with 9 µl of 880 ammonia solution gave a very poor 24 hours $^1H$ n.m.r. spectrum broadly similar to that obtained with hydroxide ion.

(d) Sodium Azide

Treatment with 0.5 $cm^3$ of a solution of 25 mg of $NaN_3$ in 1.5 $cm^3$ $^2H_2O$ gave rise to a clean 24 hours spectrum consistent with $MeN(CH_2CH_2N_3)$: $\delta_H$ 2.56 (s, $CH_3N$), 2.98 (t, 2 x $CH_2N$), 3.65 (t, 2 x $CH_2N_3$). Addition of $MeN(CH_2CH_2OH)_2$ did not enhance these signals. Resonances similar to those of the 24 hour spectrum in (b) above were also present at *ca.* 15% of the mustard intensity indicating significant hydrolysis.

(e) Sodium Cyanide

Treatment with 0.5 $cm^3$ of a solution of 20 mg NaCN in 1.5 $cm^3$ $^2H_2O$ showed after 2 hours 2 methyl resonances separated by 4 Hz and 3 methylene resonances. At 24 hours the spectrum is consistent with a *bis*-nitrile only. $MeN(CH_2CH_2CN)_2$: $\delta_H$ 2.32 (s, $CH_3N$), 2.75 (m, 2 x $CH_2CH_2$, ABCD spin system). The earlier spectrum probably represents a mixture of this compound and a mono-substituted product displaying a triplet at $\delta$ 3.71 for the remaining $CH_2$-Cl.

(f) *S*-Methionine

Treatment with 19 mg of methionine gave a system which displays a sharp $\overset{+}{S}Me$ resonance of alkylated methionine in the first $^1H$ n.m.r. spectrum (1 minute) but traces of the $\overset{+}{N}$ methyl signal persist after 2 hours. The 24 hour spectrum is assigned to a 3:1 mixture of $MeN(CH_2CH_2\overset{+}{S}Me.CH_2CH_2CHCO_2HNH_2)_2$ (75) and $MeN(CH_2CH_2OH)$-$(CH_2CH_2\overset{+}{S}Me.CH_2CH_2CHCO_2HNH_2)$ (82). These are assigned

as (75): $\delta_H$($^2H_2O$) 2.37 (s, $CH_3N$), 2.38 (m, 2 x $C\underline{H}_2CH$), 3.01 (s, 2 x $\overset{+}{S}Me$) 3.05 (m, 2 x $CH_2N$), 3.62 (m, 2 x $C\underline{H}_2$-$\overset{+}{S}C\underline{H}_2$) 3.74 (m, 2 x CH), and (81): $\delta$ 2.34 (s, $CH_3N$), 2.38 (m, $C\underline{H}_2CH$), 2.89 (t, $HOCH_2C\underline{H}_2$), 3.01 (s, $\overset{+}{S}Me$), 3.05 (m, $SCH_2C\underline{H}_2N$), 3.62 (m, $C\underline{H}_2$-S-$C\underline{H}_2$) and $C\underline{H}_2OH$), 3.74 (m, CH).

(g) Thioethanol

Treatment with 10 mg of $HSCH_2CH_2OH$ gave primarily $MeN(CH_2CH_2SCH_2CH_2OH)_2$: $\delta$ 2.78 (t, 2 x $HOCH_2C\underline{H}_2S$), 2.93 (m, 2 x $NC\underline{H}_2CH_2S$), 2.97 (s, $CH_3N$), 3.47 (m, 2 x $NCH_2C\underline{H}_2S$), 3.76 (t, 2 x $C\underline{H}_2OH$). Partially reacted material is indicated by an N-Me singlet at $\delta$ 2.99 and a multiplet at $\delta$ 3.97.

(h) Guanosine Monophosphate, Disodium Salt

Treatment with GMP gave only piperazinium dimer (10): $\delta_H$ ($^2H_2O$) 3.46, 3.48 (s, 2 x $\overset{+}{N}CH_3$), 4.15 (broad s, 8 x $CH_2$).

## 4.4.3 Reactions between N-2-Chloroethyl-N-methylaziridinium Chloride and added Nucleophile in Sorensen's Buffer

In each experiment, nitrogen mustard hydrochloride (10 mg) in 0.5 $cm^3$ $^2H_2O$ was treated with 1.7 M $Na_2CO_3$ in $^2H_2O$ (31 µl, 1 mol. equiv.) and left for 2 hours at room temperature when $^1H$ n.m.r. analysis showed high concentrations of (8). The nucleophile, 2.5 mol. equiv. was added as a solution in 0.5 $cm^3$ of pH 7.2 Sorensen's buffer in $^2H_2O$. Following thorough mixing, the reaction

was monitored by $^{1}H$ n.m.r. spectroscopy. All data refers to the $^{1}H$ n.m.r. spectrum recorded 24 hours from mixing unless otherwise indicated.

(a) Sodium Thiosulphate

Addition of $Na_2S_2O_3$ (21 mg) initiates a fast reaction giving only the *bis*-Bunte salt, $MeN(CH_2CH_2S_2O_3)_2$: $\delta$ ($^{2}H_2O$): 2.59 (s, $CH_3N$), 3.33 (m, 2 x $CH_2CH_2$, an ABCD spin system).

(b) Ammonia

Treatment with .880 aqueous ammonia solution in buffer gives a very poor 24 hour spectrum in which only the piperazinium dimer (10) may clearly be identified. Aziridinium species were still present after 2 hours.

(c) Sodium Azide

Addition of 0.5 $cm^3$ of a solution containing 25 mg of $NaN_3$ in 1.5 $cm^3$ of $^{2}H_2O$ slowly gives $MeN(CH_2CH_2N_3)_2$ (68): $\delta_H$ ($^{2}H_2O$): 2.57 (s, N-$CH_3$), 3.0 (t, N-$CH_2$), 3.67 (t, $CH_2$-$N_3$) which is the only product after 24 hours. Aziridinium species are clearly present after 2 hours and may persist up to 5 hours from mixing. Comparing the 2 hours and 5 hours spectra shows a singlet at $\delta$ 2.60 losing intensity to a similar resonance at $\delta$ 2.55, suggesting that the former is an N-Me resonance associated with a mono-substitution product.

(d) *S*-Methionine

Addition of a solution of *S*-methionine (19 mg in 1.5 $cm^3$ $^2H_2O$) initiates a slow, stepwise reaction. An N-Me singlet at δ 2.34 appears in the 5 minute spectrum; after 3 hours it has increased and displays a downfield shoulder which becomes a strong singlet at δ 2.37 as the upfield signal decays. This is supported by the steady increase in the $\overset{+}{S}Me$ signal at δ 3.0. The disubstituted product is characterised: δ 2.37 (s, $N-CH_3$), 2.4 (m, 2 x $N-CH_2$), 3.00 (s, 2 x $\overset{+}{S}CH_3$), and (under) (m, 2 x $CH-C\underline{H}_2$), 3.65 (v broad m, 2 x CH and 2 x $CHCH_2C\underline{H}_2$), 3.93 (m, 2 x $NCH_2C\underline{H}_2$). Much of the distortion is due to the survival of an unidentified mono-substituted species.

(e) Thioethanol

Addition of this nucleophile (10 mg) gives a remarkably clean, well resolved 24 hour spectrum suggesting di-substitution only, $MeN(CH_2CH_2SCH_2CH_2OH)_2$: $\delta_H$ ($^2H_2O$) 2.79 (t, 2 x $HOCH_2C\underline{H}_2$), 2.93 (S, $NCH_3$), 3.0 (t, 2 x $N-CH_2$), 3.44 (t, 2 x $NCH_2C\underline{H}_2$) 3.80 (t, 2 x $CH_2OH$). Earlier spectra, e.g. 3 hours from mixing, clearly demonstrate stepwise substitution, with the following transitory peaks for (71): $\delta_H$ 2.85 (s, $CH_3N$), 3.36 (t, $CH_2-N$), 3.55 (t, $CH_2-Cl$). Assignments are made on the assumption that resonances from the substituted arm of $ClCH_2CH_2NMe.CH_2CH_2SCH_2CH_2OH$ are co-incident with those of the di-substituted material. No aziridinium or mustard species are present at this stage (3 hours) but the final spectrum shows none of the half-reacted material, therefore, the aziridinium

ion derived therefrom cannot accumulate and is rapidly captured by thioethanol.

### 4.4.4 Reactions between Nitrogen Mustard Hydrochloride and added Nucleophile in Sorensen's Buffer

An excess (2.5 mol. equivalents) of the nucleophile was dissolved in 0.5 $cm^3$ of pH 7.2 citrate-phosphate buffer and the $^1H$ n.m.r. spectrum was recorded. This served both to provide a reference spectrum of the nucleophile (assuming it gave one) and additionally to allow the spectrometer to be adjusted for the sample. Although such tuning cannot, except by luck, be optimal for the reaction mixture, in practice only minor further adjustments were required. On removing the n.m.r. tube (5 mm precision glass) from the probe, the contents were added to a sample vial into which *ca.* 10 mg of nitrogen mustard hydrochloride had been weighed. Addition of the buffer solution immediately gave rise to an opaque solution of the nitrogen mustard base which was transferred back to the n.m.r. tube, vigorously shaken to lose its opacity and replaced in the instrument. A spectrum was recorded directly, while adjusting the fine tuning of the spectrometer. By this time the solution appeared homogenous but broad mustard peaks were often observed in the initial spectrum, presumably due to incomplete dissolution because subsequent spectra were resolved without significant re-tuning. Timing of the interval between recording spectra depended on the apparent velocity of the reaction, faster reactions

demanding shorter intervals. Spectra were usually recorded at 4-5 minute intervals for the first 20 minutes which meant that the sample remained in the probe during this time. Subsequent spectra were recorded at up to 30 minute intervals until 2 hours had elapsed and then at 1 or 2 hourly intervals as the situation dictated. Additional spectra of the "completed" reaction were recorded after 24 hours and sometimes up to a week from starting. The recorded timing intervals are the delays between commencing recording one spectrum and reaching an identical place on the next. Most of the spectra were recorded over 5 → 0 p.p.m. at a scan speed equivalent to 2½ minutes for the journey and then integrated over relevant parts of the same region at a speed equivalent to 1½ minutes for the full 5 p.p.m. Thus, 4-5 minutes represent the minimum practical time interval between sequential integrated spectra occupying the whole 5 p.p.m. Reduction of the interval is achieved either by omitting to integrate a spectrum or by scanning manually, but the instrument was returned to its normal scan-speed for recording peaks to avoid variations in shift due to the lag in pen response.

(a) Sodium Thiosulphate as Nucleophile

Treatment with $Na_2S_2O_3$ (21 mg) and following the methyl resonances shows that the mustard ($\delta_H$ 2.76-2.71) is fully consumed between spectra recorded at 64 and 107 minutes. The mono-Bunte salt ($\delta_H$ NMe 2.82-2.84) is present from 2 minutes; the *bis*-Bunte salt ($\delta_H$ NMe 2.89)

appears after 15 minutes. The concentration of the 2 Bunte salts is about equal after 12 minutes giving way to a 6 hour spectrum which displays resonances for unperturbed buffer and di-substituted material only. No dimeric species or hydrolysis products were detectable $CH_3N(CH_2CH_2S_2O_3)_2$: $\delta_H$ ($^2H_2O$) 2.88 (s, $CH_3N$), 3.47 (m, 2 x $CH_2CH_2$ ABCD spin system), $ClCH_2CH_2NMeCH_2CH_2S_2O_3$: $\delta$ 2.82 (s, N-$CH_3$), 3.33 (t, 2 x $CH_2$-N), 3.46 (t, $CH_2S_2O_3$), 3.89 (t, $CH_2Cl$). The pH of this system drops from 7.2 to 6.9 during the 6 hours required for the reaction to reach completion.

(b) Thiourea

The methyl groups of a mono-substituted product ($\delta$ 2.42) and aziridinium ion are discernable 8 minutes after adding the alkylating agent to 9.9 mg of this nucleophile. Concentrations of mustard and mono-alkylated product are equal 28 minutes from the addition allowing characterisation of $ClCH_2CH_2NMeCH_2CH_2SC(NH_2)_2$: $\delta_H$ ($^2H_2O$) 2.42 (s, N-$CH_3$), 2.98 (m, 2 x $NCH_2$), 3.29 (m, $CH_2$-S), 3.74 (m, $CH_2Cl$). Nearly all the starting material has been consumed by 175 minutes when mono- and di-substituted mustards were present in equal amounts. The final spectrum (24 hours) shows less than 2% conversion to dimer, the dominant feature being $MeN(CH_2CH_2SC(NH_2)_2)_2$: $\delta_H$ ($^2H_2O$) 2.37 (s, N-$CH_3$), 2.9 (t, 2 x $CH_2N$), 3.31 (t, 2 x S-$CH_2$). Addition of $MeN(CH_2CH_2OH)_2$ does not enhance any of these resonances.

(c) Thioethanol

Addition of nitrogen mustard hydrochloride to thioethanol (10 mg) in our buffer system gave a stepwise substitution which allowed only brief observation of aziridinium intermediates and did not give rise to any piperazine dimer (10) producing only $MeN(CH_2CH_2SCH_2CH_2OH)_2$: $\delta_H(^2H_2O)$ 2.78 (t, 2 x $HOCH_2C\underline{H}_2$), 2.91 (s, $NCH_3$), 2.98 (t, 2 x $N-CH_2$), 3.43 (t, 2 x $NCH_2C\underline{H}_2$), 3.76 (t, 2 x $CH_2OH$). The methyl signal of this material is evident 6 minutes from mixing; after 12 minutes the methylene signals may also be distinguished. Transitory resonances, slightly de-shielded from mustard chloroethyl arms, are consistent with a monosubsituted intermediate, $MeN(CH_2CH_2Cl)(CH_2CH_2SCH_2CH_2OH)$: $\delta_H(^2H_2O)$ 2.78 (t, $HOCH_2C\underline{H}_2$), 2.91 (s, $N-CH_3$), 2.98 (t, $SCH_2C\underline{H}_2N$), 3.36 (t, $ClCH_2C\underline{H}_2N$, 3.43 (t, $NCH_2C\underline{H}_2S$), 3.76 (t, $HOCH_2$), 3.93 (t, $Cl-CH_2$). The concentration of this species never exceeds that of the fully reacted material, remaining at *ca.* 1/3 that of the di-substituted product until all the mustard is consumed (2 hours). Between 2 and 4 hours from mixing the mono-alkyl material gives way completely to the *bis*-alkylated product, supporting the assignment.

(d) N-Acetyl Cysteine Methyl Ester

This protected amino acid (19 mg), the gift of Mr. M. K. Ellis, when treated with nitrogen mustard hydrochloride in the standard system showed simultaneous production of 1:1 and 2:1 adducts without any accumulation of aziridinium intermediates. There is considerable

peak overlap with unreacted nucleophile, the α-H, ester and acetyl signals being insufficiently shifted on alkylation to distinguish them from those of excess nucleophile. The alkylated species are distinguished by the doublets of the β-methylene which are clearly discernable 5-10 minutes from mixing. As resonances assigned to the chloroethyl arm and $CH_2CH$ of the mono-substituted mustard decrease, the corresponding resonances in the di-substituted mustard increase proportionately. The fully reacted material is characterised, $MeN(CH_2CH_2SCH_2CH\text{-}NHAc.CO_2Me)_2$: $\delta_H$ ($^2H_2O$) 2.07 (s, 2 x $CH_3CO$), 2.91 (s, N-$CH_3$), 2.98 (m, 2 x N-$CH_2$), 3.13 (d, J = 5 Hz, 2 x $C\underline{H}_2$-CH), 3.39 (t, 2 x $CH_2C\underline{H}_2S$), 3.78 (s, 2 x $CO_2Me$), 4.66 (m, 2 x CH). Mono-substituted material is characterised: $\delta_H$ ($^2H_2O$) 2.07 (s, $CH_3CO$), 2.94 (m, $CH_3N$ and $SCH_2C\underline{H}_2$), 3.19 (d, $C\underline{H}_2CH$), 3.39 (t, $CH_2C\underline{H}_2S$), 3.60 (t, $ClCH_2C\underline{H}_2$), 3.78 (s, $CO_2Me$), 3.96 (t, $CH_2Cl$). Hydroxy-mono-substituted mustard is discounted by decay of the δ 3.60 triplet from *ca.* 2 hours onwards. After 24 hours the solution was neutralised with $NaHCO_3$ and extracted into $C^2HCl_3$. Unreacted and cross-linked cysteine residues are extracted in preference to the mono-alkyl species. Serious peak overlap still occurs but the N-Me and both α-H signals may be distinguished allowing the integral to assist fully in assigning remaining peaks. A relative integral value of 14.5 for the $CH_3$-N of cross-linked material is consistent with a value of 10 for the resonance assigned to the 2 α protons. Thus, one hydrogen of the dialkylated product has an integral

value of approximately 5. The remaining α hydrogen resonance suggests a value of 11 for each hydrogen of the unreacted amino acid. The slightly split N-acetyl and methyl ester resonances are apportioned in a 10:11 ratio and the predicted integral (3 x 10 + 3 x 11 = 63) agrees with the observed value of 62. Integral values of the 2 remaining multiplets agree with the assignment 4 x $CH_2$-S (integral = 4 x 10 *cf.* observed value of 40) of the alkylated compound for the upfield one and 2 x N-$CH_2$ + S-$CH_2$ of unreacted amino acid (integral = 2 x 10 + 2 x 11 = 42, *cf.* 45) for the downfield multiplet. Thus, $CH_3N(CH_2CH_2SCH_2CHNH_2CO_2H)_2$ is characterised: $\delta_H(C^2HCl_3)$ 2.05 (s, 2 x $CH_3CO$), 2.24 (s, N-$CH_3$), 2.60 (m, 2 x N-$CH_2$), 3.0 (m, 4 x $SCH_2$), 3.77 (s, 2 x $CH_3$-O), 4.84 (t, 2 x $NH_2$-C<u>H</u>). The corresponding aqueous portion from the neutralised reaction mixture was treated with 1 drop of HCl to prevent ester hydrolysis and the $^1H$ n.m.r. recorded. Such an ionic sample gave a poor spectrum which suggested $HOCH_2CH_2NMe(CH_2CH_2SCH_2CH.CO_2HNH_2)$: $\delta_H$ ($^2H_2O$) 1.96 (s, N-Ac), 2.93 (s, N-$CH_3$), 3.0 (m, 2 x $CH_2$-N and $CH_2C\underline{H}_2S$), 3.13 (d, CH-$NH_2$).

(e) <u>*S*-Methionine</u>

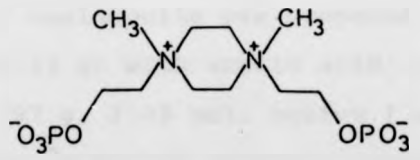
Treatment of *S*-methionine (19 mg) with nitrogen mustard as per general method showed a stepwise alkylation along with some dimerisation of nitrogen mustard. The reaction is complicated by accumulation of N-2-chloroethyl-N-methylaziridine which accounts for 20% of the total mustard content after 30 minutes. A

singlet at δ 2.98 was detected 8 minutes from mixing and grown rapidly, accompanied by another singlet at δ 2.33. These are respectively assigned to the $\overset{+}{S}Me$ and NMe of the 1:1 mustard:methionine product. An upfield shoulder on the NMe signal develops slowly to after *ca.* 18 hours. At 90 hours only 1 NMe resonance is evident and 2 methylene triplets present at 18 hours have also been consumed, behaviour which suggests a mono-substituted product slowly undergoing a second alkylation. This is well supported by integration. The 90 hours spectrum characterises the fully reacted product, $CH_3N(CH_2CH_2\overset{+}{S}Me.CH_2CH_2CH.NH_2.CO_2H)_2$: δ ($^2H_2O$) 2.36 (s, N-$CH_3$), 2.40 (m, 2 x N-$CH_2$), 2.98 (s, 2 x $\overset{+}{S}$-$CH_3$), 3.04 (m, 2 x CH-$C\underline{H}_2$), 3.6 (broad m, 2 x CH and $CHCH_2\underline{CH}_2$), 3.87 (m, 2 x $NCH_2C\underline{H}_2$). The mono-substituted product may be characterised, $ClCH_2CH_2NMe.CH_2CH_2\overset{+}{S}MeCH_2CH_2CH.NH_2.CO_2H$: $\delta_H$ ($^2H_2O$) 2.35 (s, N-$CH_3$), 2.38 (m, 2 x N-$C\underline{H}_2CH_2$-S), 2.91 (t, $C\underline{H}_2CH_2Cl$), 2.99 (s, $\overset{+}{S}$-Me), 3.04 (m, CH-$C\underline{H}_2$), 3.60 (broad m, CH and $NCH_2C\underline{H}_2\overset{+}{S}$), 3.73 (t, Cl-$C\underline{H}_2$), 3.84 (m, $CHCH_2C\underline{H}_2$). Persistence of reactive chloroethyl groups is associated with a drop in pH to 6.6 and possible change in pKa of the mustard nitrogen on mono-alkylation.

(f) *n*-Propylamine

During 48 hours, following treatment of this nucleophile (8 mg) with nitrogen mustard hydrochloride as per general method, there was no measurable perturbation of the amine $^1H$ n.m.r. spectrum. A very rapid generation of aziridinium ion (8) was observed, mustard resonances

being absent 40 minutes from mixing. Traces of aziridinium signals persist after 48 hours. The major product is similar to piperazinium dimer (10) together with the phosphate diester suggested earlier as the product of mustard and buffer only. This latter species is identifiable after 1 hour when the aziridinium resonances are perturbed: the N-Me is split, the chloroethyl methylenes lose resolution and the ring methylenes lose intensity. A day after mixing the minor aziridinium N-Me resonance accounted for 25% of the total aziridinium N-Me integral, phosphate diester is decreased and resonances overlapping piperazinium dimer signals are growing in intensity. Four days from mixing most of the phosphate diester was consumed and the aziridinium N-Me depleted. Both the piperazinium dimer signals are enhanced but have lost resolution. New methylene signals have appeared on either side of the dimer methylene signal. We postulate reaction of aziridinium species with phosphate diester to give a piperazinium species with N-ethyl phosphate substituents (83). Ring methylenes and methyl groups of such a species should be similar to dimer derived from




(83)

nitrogen mustard but methylenes of the external ethyl arms might differ from their chloroethyl analogues. Thus, we suggest: $\delta_H$ ($^2H_2O$) 3.47 (broad, $\overset{+}{N}-CH_3$), 3.91 (broad, N-$CH_2CH_2OP$), 4.13 (broad, $\overset{+}{N}CH_2$ ring), 4.23 (broad, $CH_2OP$). The ratio of total methylene integral to total methyl signal supports this assignment.

(g) *R,S*-Alanine

With this nucleophile (11.8 mg) similar behaviour to the propylamine reaction is observed, with the alanine $^1H$ n.m.r. signals remaining unperturbed during four days of observation. Nitrogen mustard has disappeared after 1 hour giving way to aziridinium ion (8) which in turn is consumed 5½ hours from mixing. At this point phosphate diester and derived piperazinium dimer predominate. There was little change in these signals during the remaining period of observation, presumably further reaction was prevented by protonation of the diester.

(h) N-Acetyl-*S*-histidine

This nucleophile was prepared by treating *S*-histidine (0.43 g) with acetic acid (5 $cm^3$) and acetic anhydride (0.297 g, 1.05 mol. equivs.) in a flask in an oil bath at 120 $^oC$ for 40 seconds, protected throughout with a $CaCl_2$ drying tube. The mixture was cooled rapidly, evaporated and re-evaporated from water whereupon a white crystalline deposit of N-acetyl-*S*-histidine was recovered[105] (0.38 g, 70%), m.p. 167-169 $^oC$, lit. m.p. =

169 $^{o}$C, $[\alpha]_D$ = +42$^{o}$, C = .05, lit. $[\alpha]_D$ = +44, pure by $^{1}$H n.m.r. spectroscopy, single spot by t.l.c.

Reaction with this nucleophile (25 mg) under standard conditions consumed the starting mustard by 1 hour. The concentration of aziridinium ion decreased rapidly to give after 24 hours a reaction mixture containing piperazinium dimer (10) and phosphate diester in a 1:3 ratio. There was no indication of dimer derived from the diester.

(i) Guanosine Monophosphate, Di-sodium Salt

Treatment of 50 mg of GMP under standard conditions consumed all starting mustard 1½ hours from mixing. After 3 hours there was a 1:1 concentration of phosphate diester and aziridinium ion which gave way to phosphate diester only after 4 days.

This experiment was repeated, attempting to dissolve 50 mg of nitrogen mustard hydrochloride neutralised with $NaHCO_3$ in 0.5 cm$^3$ of buffer containing 250 mg GMP after the method of Brookes and Lawley[44]. The drug did not dissolve properly and the opaque mixture had the texture of silicone grease. Small portions were removed with a microspatula and diluted with $^{2}H_2O$ at 2 and 24 hours. Both spectra showed that the mustard was unreacted.

(j) N-2-(Hydroxyethyl)-N-methylethanamine

Treatment with 15.5 mg of $MeN(CH_2CH_2OH)_2$ gave only (66): $\delta_H$ ($^{2}H_2O$) 2.34 (s, $CH_3N$), 3.02 (t, 2 x $CH_2N$),

3.21 (s, 2 x $CH_3\overset{+}{N}$), 3.67 (broad s, 6 x $CH_2\overset{+}{N}$), 4.05 (broad s, 8 x $CH_2OH$).

# CHAPTER 5

# $^{13}C$ N.M.R. SPECTROSCOPIC STUDIES OF THE REACTIONS OF NITROGEN MUSTARD

## 5.1 INTRODUCTION

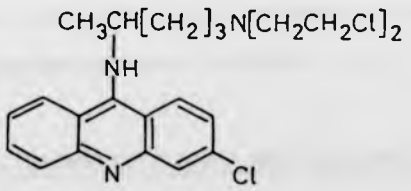
The identification of sites of alkylation in proteins and nucleic acids exposed to reactive chemical reagents has been achieved by the use of radiolabelling techniques (see Chapters 1 and 3). Indications of likely sites for attack can also be obtained from model studies (see Chapters 1 and 4). The use of an alkylating agent specifically labelled with a stable isotope possessing favourable properties for n.m.r. spectroscopy (e.g. $^{13}C$, $^{15}N$) offers the possibility of determining sites of alkylation by direct observation of a reaction in an n.m.r. tube. By the use of nitrogen mustard selectively enriched at C-2 and C-2' with $^{13}C$, in principle, the nature of a site of alkylation could be determined from the chemical shift of the resonance corresponding to a product of alkylation. Furthermore, the intermediacy of an aziridinium ion in this process of alkylation would be evident from the appearance of two resonances of approximately equal intensity, one corresponding to a labelled carbon directly bonded to the nucleophilic site and the other corresponding to a labelled carbon one carbon removed from this site.

The technique of n.m.r. spectroscopy has recently

been exploited by Chang[106] in studies of alkylations of nucleic acids and their components by [$^{13}$C-Me]-methyl methanesulphonate. Thus, products of the methylation of DNA with methyl methanesulphonate were identified by comparison of the $^{13}$C n.m.r. spectra of several methylated nucleotides and nucleosides with the $^{13}$C n.m.r. spectrum of the alkylated DNA. Four products were detected: 3-methyldeoxycytidine, 7-methyldeoxyguanosine, 1-methyl-deoxyadenosine and a phosphomethyl ester. Comparison of the spectrum of native DNA with that of DNA alkylated with unenriched methyl methanesulphonate was not helpful. When the alkylating agent was enriched to 90 atom % $^{13}$C, the products of alkylation of DNA were readily detected with a shorter accumulation time than necessary for natural abundance material. Moreover, alkylation of DNA from a different source showed a different product distribution. Degradative studies would not have permitted such a ready comparison. Other analytical methods may have destroyed or failed to detect the phosphate ester; it has been shown that 1-methyldeoxyadenosine is not conventionally observed intact, but usually rearranges to the 6-aminopyrimidine[103]. Similar work with RNA allowed six methylated products to be determined: 7-methylguanosine, 1-methyladenosine, 3-methylcytidine, 1-methylguanosine, 3-methyluridine and methylphosphodiester. The latter arose from extensive reaction with the phosphate buffer which lowered the solution pH and may have deactivated nucleophilic centres by protonation. Co-alkylation with [$^{14}$C-Me]-methyl methanesulphonate was used to assess

the overall extent of reaction illustrating a complementary application of radiolabelling.

Although it is widely accepted that nitrogen mustards attack N-7 of guanine residues, it would be valuable to be able to confirm this by direct observation of a reaction. Furthermore, minor sites of alkylation (e.g. O-6), not amenable to quantitative assessment by existing techniques, might be identified. There is considerable uncertainty concerning sites of alkylation of proteins by nitrogen mustards. Thus, a recent study[107] of the alkylation of the nicotinic acetylcholine receptor of *Torpedo californica* by quinacrine mustard (84) considered all possible nucleophilic sites as targets, but was unable to specify the actual site alkylated, although some evidence was provided to exclude a thiol grouping. The problem of identifying the target for nitrogen mustard in hemoglobin has been discussed in Chapter 1, along with other studies of protein alkylation.




(84)

The viability of direct observation of alkylation by a nitrogen mustard selectively labelled with a stable isotope needs to be tested by studying reactions with simple nucleophiles, which may be suitable models for nucleophilic sites in biological macromolecules. This chapter describes a study of reactions of [2,2'-$^{13}C_2$]-N-(2-chloroethyl)-N-methyl-2-chloroethanamine with ammonia, thiosulphate, cyanide, methanethiolate and thiourea. It is shown that intermediate aziridinium ions participate in all of these reactions. Of particular importance is the discovery of two competing pathways of decomposition of an intermediate (produced by capture of an initial aziridinium ion) in the reaction between nitrogen mustard and ammonia, only one of which proceeds to a second aziridinium ion.

An alternative strategy to that described, in which the alkylating agent is labelled, is to label selectively its nucleophilic substrate. This approach has been tested by investigating the reaction between unlabelled nitrogen mustard and [$^{13}CH_3$]-methionine. We have found that sulphonium salts are rapidly formed in this reaction and that they undergo slow decomposition to further products (see Section 5.5).

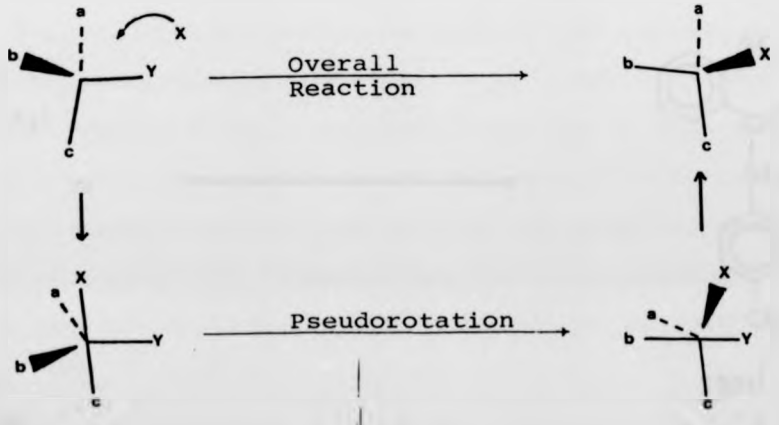
## 5.2 $S_N2$ SUBSTITUTIONS AND BALDWIN'S RULES

The classical pathway for $S_N2$ substitution at carbon involves Walden inversion *via* a pentacoordinated intermediate or transition state. Following the discovery

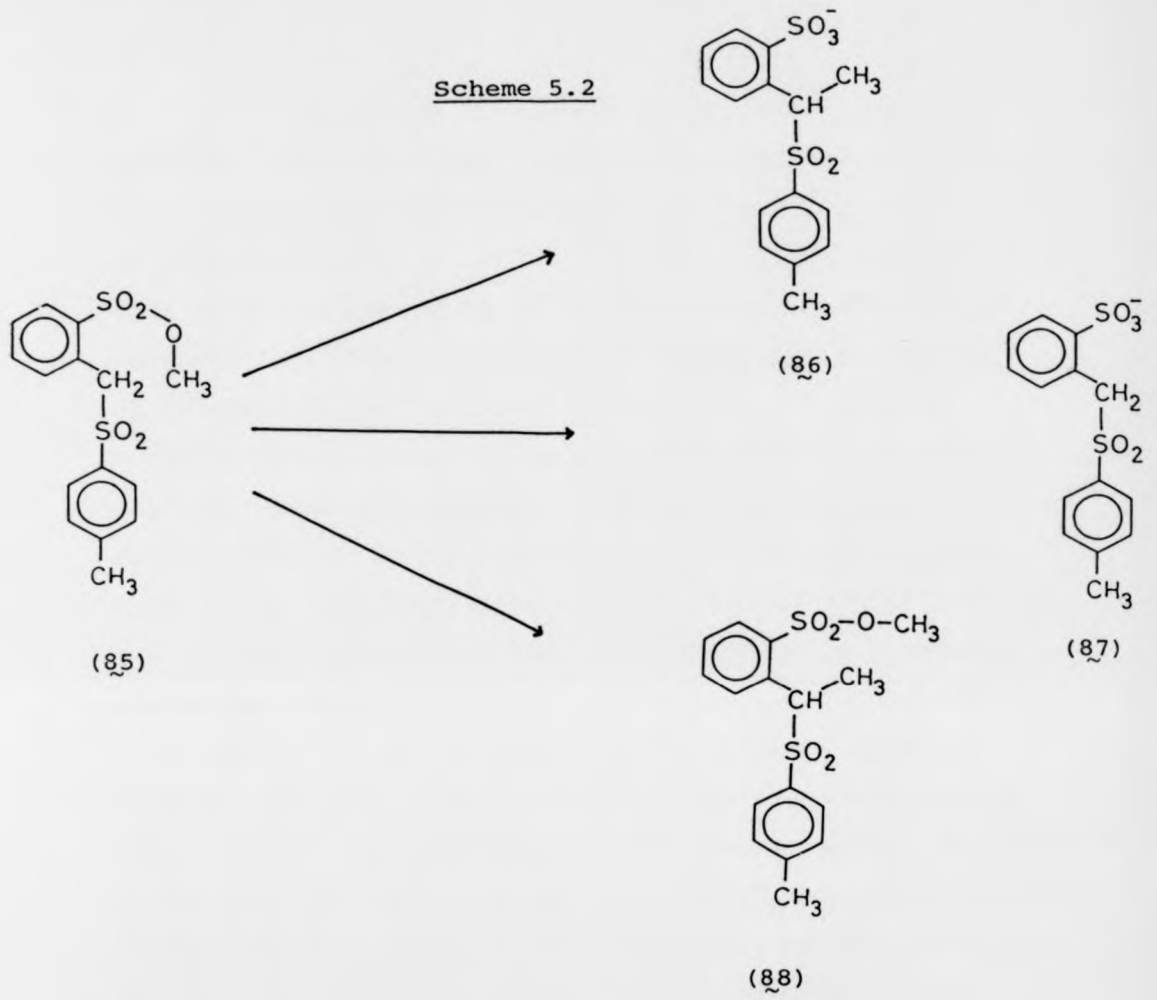
of the occurrence of pseudorotation in pentacoordinated phosphorous systems, which can cause isomerisations of these compounds, the possibility of such processes with carbon was considered. If the $S_N2$ substitution proceeds *via* a pentacoordinated carbon species of finite lifetime, then the consequence of pseudorotation within this species, would be to offer substitution at carbon with retention, provided that the initial nucleophilic attack occurs along the axis of a bond other than that connected to the leaving group (*cf.* Scheme 5.1).

Eschenmoser devised an experiment[37] to test this premise, in which transfer of a methyl group was examined in a system restricted to intramolecular attack along the axis of a C-H bond, i.e. not along the axis of the leaving group (Scheme 5.2). This mechanism is described as an "endocyclic" intramolecular substitution reaction, whereas attack along the axis of the leaving group bond to carbon is described as an "exocyclic" intermolecular substitution.

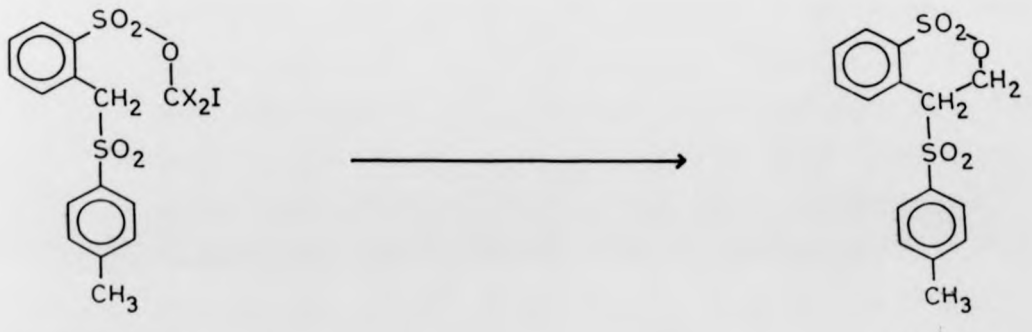
Scheme 5.1



Scheme 5.2




Scheme 5.3

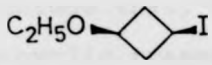


Analysis of the reaction fifteen minutes after treating (85) with 1 mol. equiv. of NaH in dioxan, showed a mixture of the anticipated product (86) (23%), a de-methylated material (87) (38%) and a di-methyl species (88) (36%). Cross-reaction of (85) with its deuteriated analogue (85a) using a variety of bases and solvents for an extended period gave over 90% of (86). However, analysis by n.m.r. spectroscopy showed that approximately half the product contained two $C^2H_3$ units, a quarter had one and a quarter contained no deuterium. This demonstrated that the product (86) arose from an intermolecular pathway: the authors reasoned that de-protonation of (85) formed a carbanion which abstracted a methyl group from the sulphate ester moiety of another molecule to yield the dimethyl species (88). On further reaction, the sulphonate methyl ester of (88) was transferred to the anion of (87) thereby giving the high yield of (86) isolated after longer reaction times. The experiment with deuterated material showed that the toluenyl methyl group was not transferred and the ratio of deuterium in the product represented a statistical distribution of the sulphonate methyl groups. When the methyl ester was replaced with an iodomethyl function, a different carbon-leaving group axis was introduced such that intramolecular reaction is an exocyclic process. Thus, base treatment of an equimolar mixture of this material (89) and its pentadeuteriated analogue (89a) followed by n.m.r. spectroscopic analysis of the products showed retention of the original deuterium distribution, confirming an intramolecular reaction. This route was presumed

to include the cyclic intermediate or transition state (90) shown in Scheme 5.3.

In systems smaller than the 6-membered ring used by Eschenmoser it was supposed that the effect of incorporating two of the bonds of the carbon undergoing nucleophilic attack into the ring, might force the nucleophile to approach along the axis of a bond other than that bearing the leaving group. Experimental evidence for this view was advanced by Ugi *et al.*[108]. Reaction of *cis*-3-ethoxycyclobutyl brosylate (91) with iodide under $S_N2$ conditions allowed the isolation of *cis*-3-ethoxycyclobutyl iodide (92) in high yield. Similarly, sequential reaction of (91) with bromide and then iodide under the same conditions also gave (92). This was interpreted as an $S_N2$ reaction proceeding with retention of configuration. The postulated pentacoordinate transition state was presumed to be long-lived and to undergo pseudorotation because of energetic factors arising from the strain induced by the incorporation of two ligands into the ring.

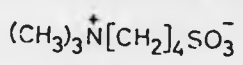

(91)



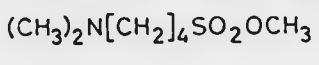

(92)

This theory was dispelled by Mislow *et al.*[109] who showed that the *cis*-product from this reaction was the result of two $S_N2$ displacements proceeding with inversion. G.l.c. analysis of the reaction mixture over a period of time showed initial production of the *trans*-isomer, which was later converted to the *cis*-form in accordance with the known epimerisation of halocyclobutanes by halide ion. A second claim[110] for an $S_N2$ reaction proceeding with retention of configuration in a cyclopropane system was also shown to be the product of a two-stage process[111].

However, on proceeding to larger ring sizes with greater flexibility, some workers[112] reasoned that an endocyclic ring closure should be able to attain the required geometry for backside $S_N2$ attack. Formation of the betaine (93) from an equimolar mixture of methyl 4-(dimethylamino)butanesulphonate (94) and the deuteriated analogue (94a) showed the reaction to be entirely intermolecular with a 1:1:1:1 distribution of the nona-, hexa-, tri- and undeuteriated betaine. An intramolecular reaction would proceed *via* an eight-membered cyclic transition state which appeared feasible from a molecular model. The authors conclude that, because the maximum concentration of the cyclic transition state was 1/50th of that reported for the analogous exocyclic lactonisation of 8-bromooctanoate, the requirement for endocyclic attack provided substantial additional strain on the system other than that expected for formation of an eight-membered ring.



(93)



(94)

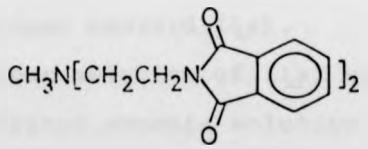
The results of these studies and other experimental observations were summarised by Baldwin in a set of rules for ring closure[38]. The theoretical basis of the rules is the geometric one discussed above. By specifying the size of the ring to be formed, the geometry of the carbon atom undergoing attack and the relationship of the breaking bond to the smallest ring formed, the rules classify ring closure as either favoured or disfavoured. Thus, only the relative facility of ring-making reactions is specified. Due to the larger atomic radii and longer bond lengths of atoms from the second, or greater, row of the periodic table the rules are restricted to first row elements. Baldwin *et al.* have demonstrated that a cyclisation disfavoured with first row elements is possible when sulphur is introduced into the system, but no exceptions with first row elements have been discovered.

Isotope Effects in Displacement Reactions

A majority of work on isotope effects has been concerned with isotopes of hydrogen. Heavy atom work with $^{13}C$ or $^{14}C$ is comparatively scarce, but interesting correlations between the magnitude of the isotope effect and the reaction type have emerged[113]. $S_N1$ reactions involve additional bond formation to the developing charge centres in the activated complex compared to the reactants, (i.e. solvation and charge stabilisation by hyperconjugation), which off-sets the bond rupture of the leaving group. Bond formation contributions of this type are absent in $S_N2$ reactions. The additional bonding appears as a negative function in the calculation of isotope effects. Thus, $S_N2$ reactions such as the displacement reactions of $^{14}C$-methyliodide with pyridine give $k_{12}/k_{14} = 1.142 \pm 0.009$ whereas $S_N1$ reactions such as ethanolysis of 1-bromo-1-phenylethane give $k_{12}/k_{13} = 1.0065 \pm 0.0006$. Therefore, isotope effects at a central atom are large for $S_N2$ reactions and small for $S_N1$ reactions. Ionisation of triphenylmethyl chloride gives an ion which is resonance stabilised with respect to the reactant: the bond formation contribution is so great that an inverse isotope effect is observed[114].

## 5.3 REACTION OF NITROGEN MUSTARD WITH AMMONIA

Remarkably there is no report in the literature concerning the reaction between nitrogen mustard and ammonia, although the possibility of reaction was considered in the context of the alkylation of hexamethylenetetramine by nitrogen mustard. From the reaction of (1) with excess of ammonia we have identified the products as 2-amino-N-(2-aminoethyl)-N-methylethanamine (95) [60%] and N-methylpiperazine (96) [40%]. When a limited amount of ammonia was reacted with (1), additional products were formed as described in Chapter 4. The amines (95) and (96) were separated by ion exchange chromatography and characterised by the $^{1}H$ and $^{13}C$ n.m.r. spectral properties. Authentic N-methylpiperazine was commercially available. A reference sample of amine (95) was synthesised by a published procedure by reacting diol (13) with phthalimide and hydrolysing the intermediate *bis*-phthalimide (97) with hydrochloric acid. Further characterisation of the products from reacting (1) with ammonia was achieved by the preparation of N-phenylamino-thiocarbonyl derivatives.

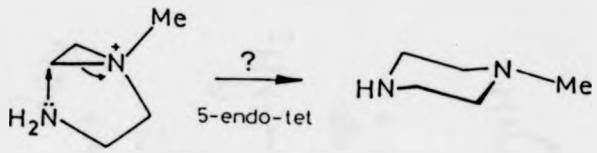


(97)

The possible routes of formation of amines (95) and (96) are shown in Scheme 5.4, ignoring pathways in which chloride is directly displaced by ammonia. Amine (95) arises *via* two intermediate aziridinium ions. These ions are connected by the "one-armed" intermediate (98), a "half-reacted" mustard. There are two routes for the formation of N-methylpiperazine from intermediate (98). In pathway A cyclisation to aziridinium ion (99) occurs. This can either be captured by intermolecular reaction with ammonia to give amine (95) or could proceed to (96) by intramolecular attack of its amino function on one of the carbon atoms of the aziridinium ring. The intramolecular pathway is an endocyclic $S_N2$ (or 5-endo-tet) reaction, which is expected to be disfavoured compared to the exocyclic (3-exo-tet) cyclisation giving (95). However, it is interesting to consider whether a favourable ground state interaction in the intermediate (99) could lead to insertion of the amino nitrogen into a $C\text{-}N^+$ bond of the aziridinium ring (*cf.* Scheme 5.5). In pathway B, intramolecular attack by the amino nitrogen of (98) on the $CH_2Cl$ carbon leads directly to N-methylpiperazine. Pathways A and B can be distinguished by analysis of the distribution of $^{13}C$ in N-methylpiperazine derived from $^{13}C$-labelled nitrogen mustard (1a).

An aqueous solution of (1a) was added to an excess of concentrated ammonia solution. The reaction was allowed to proceed for two hours and then evaporated to dryness. Previous $^{13}C$ n.m.r. examination of the products *in situ* had shown no loss of product due to

Scheme 5.5




this procedure, but a considerable improvement in the signal-to-noise ratio was achieved by the removal of ammonia. The presence of amines (95) and (96) was indicated by $^{1}H$ and $^{13}C$ n.m.r. spectra of the solid residue in $^{2}HCl$ (Fig. 5.1). The sample was applied to an ion exchange column (C.G.120) and the product separated by elution with 3 M HCl. Amines (95) and (96) were isolated as the major products, but a trace of piperazinium dimer (10) was also detected. The NOE suppressed $^{13}C\{^{1}H\}$ n.m.r. spectrum of (95) (Fig. 5.2) showed two enriched peaks at $\delta$ 34.61 and 53.69. Similarly, the spectrum of N-methylpiperazine (Fig. 5.3) showed enriched peaks at $\delta$ 41.59 and 50.82. For the linear amine (95) the ratio of C-1(C-1') to C-2(C-2') was 1.14 $\pm$ 0.05:1. The same ratio C-1(C-1') to C-2(C-2') for N-methylpiperazine (96) was 2.04 $\pm$ 0.12 :1. The former ratio represents an isotope effect in opening the aziridinium ring with ammonia as nucleophile. A lower

Scheme 5.4 Reaction of nitrogen mustard with ammonia

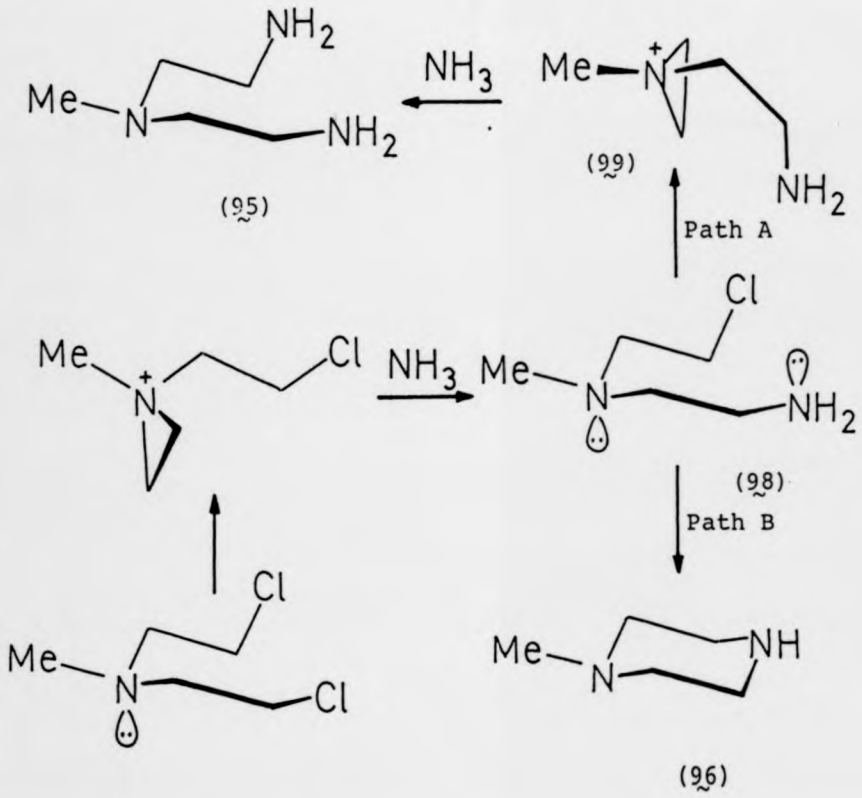
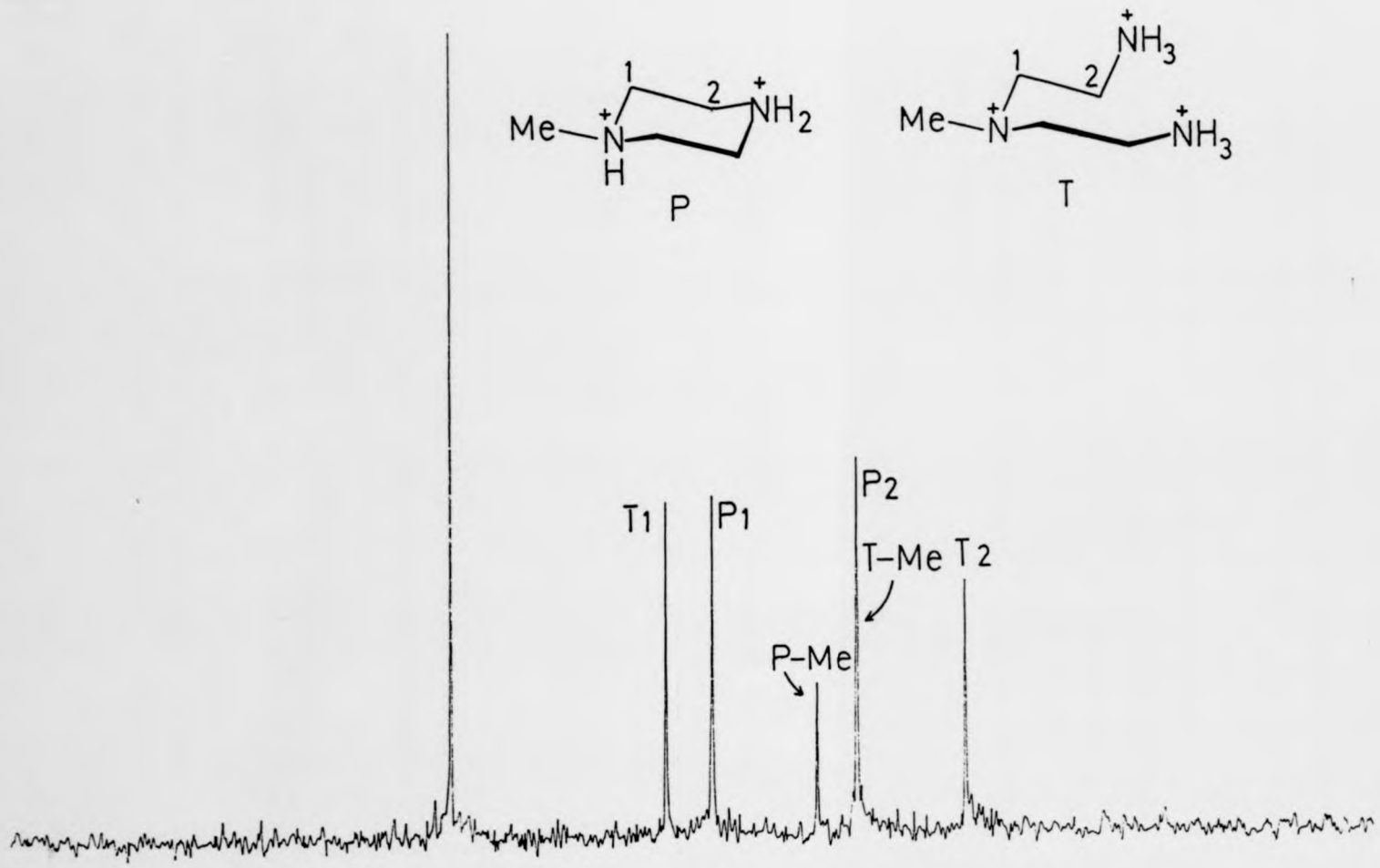


Fig. 5.1 $^{13}C\{^{1}H\}$ n.m.r. spectrum of the products of the reaction between nitrogen mustard and ammonia in $^{2}HCl$.



zero point energy for $^{13}C$ than for $^{12}C$ makes the $\overset{+}{N}$ - $^{13}C$ bond stronger than the $\overset{+}{N}$-$^{12}C$ bond, thus giving a product with higher $^{13}C$ enrichment at C-1. Using this isotope effect, the theoretical $^{13}C$ distribution ratio for (96) can be calculated assuming ring closure by direct displacement of chlorine from the half-reacted mustard intermediate (98). Thus, taking natural abundance as 1.1% $^{13}C$ and the enrichment at C-2 is known to be 10.8% $^{13}C$, the integral ratio of the $^{13}C$ n.m.r. spectrum can be calculated.

$$\text{C-1 + C-1'} = \frac{(1.1 + 10.8)}{2} \times 1.14 + 1.1$$

$$= 7.88$$

$$\text{C-2 + C-2'} = \frac{(1.1 + 10.8)}{2} \times \frac{1}{1.14} + 10.8$$

$$= 16.02$$

$$\frac{\text{C-2 + C-2'}}{\text{C-1 + C-1'}} = \frac{16.02}{7.88}$$

$$= 2.03$$

The calculated $^{13}C$ distribution ratio of 2.03 is in excellent agreement with the experimentally observed ratio of 2.04 $\pm$ .12.

The observation that $^{13}C$ is approximately equally distributed between C-1(C-1') and C-2(C-2') of triamine (95) is not *per se* proof that this compound is formed *via* aziridinium ions (8) and (99). It is conceivable that (1a) equilibrates with the isomeric

Fig. 5.2 $^{13}C\{^{1}H\}$n.O.e. suppressed n.m.r. spectrum of 2-amino-N-(2-aminoethyl)-N-methylethanamine from the reaction of nitrogen mustard (1a) with ammonia.

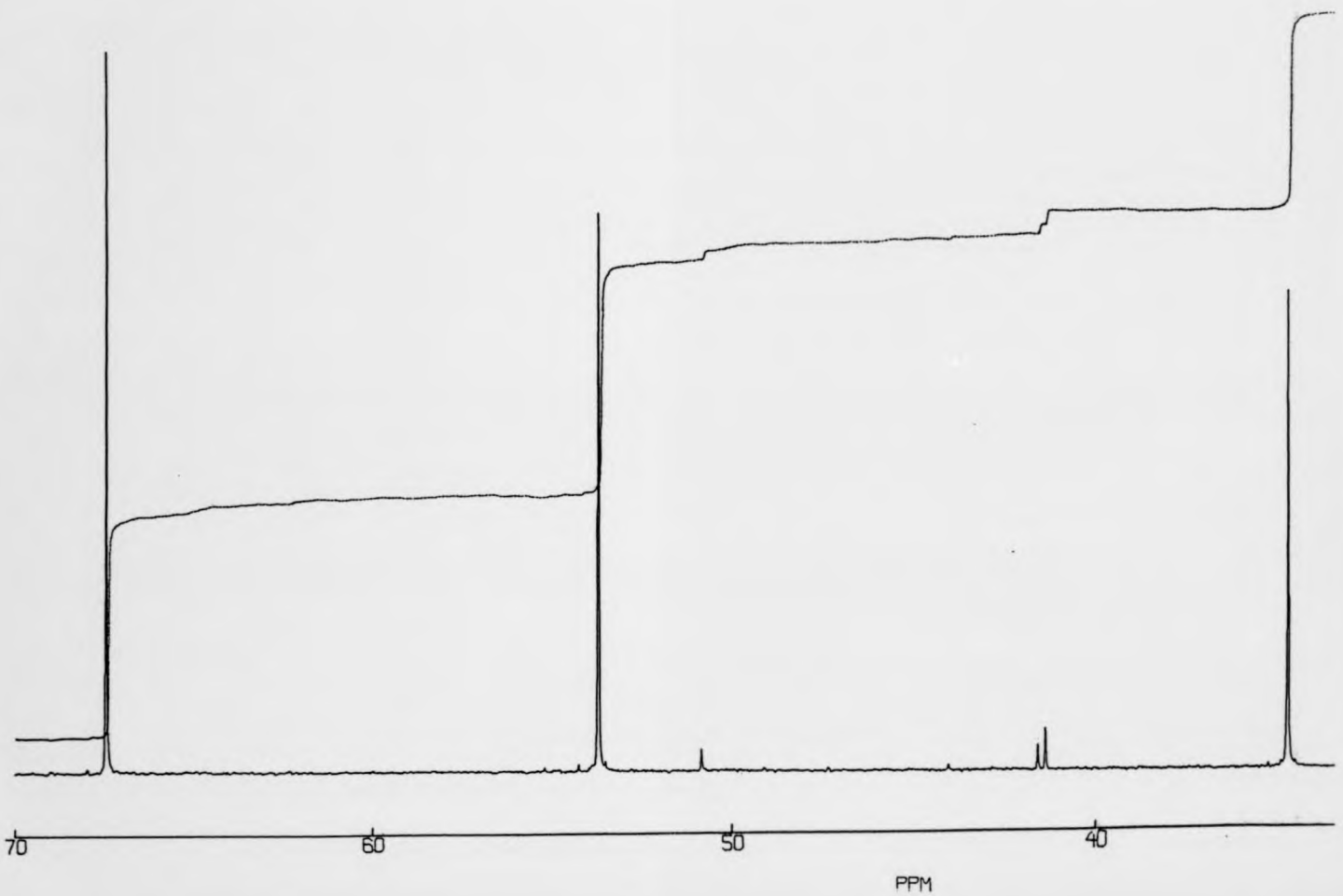
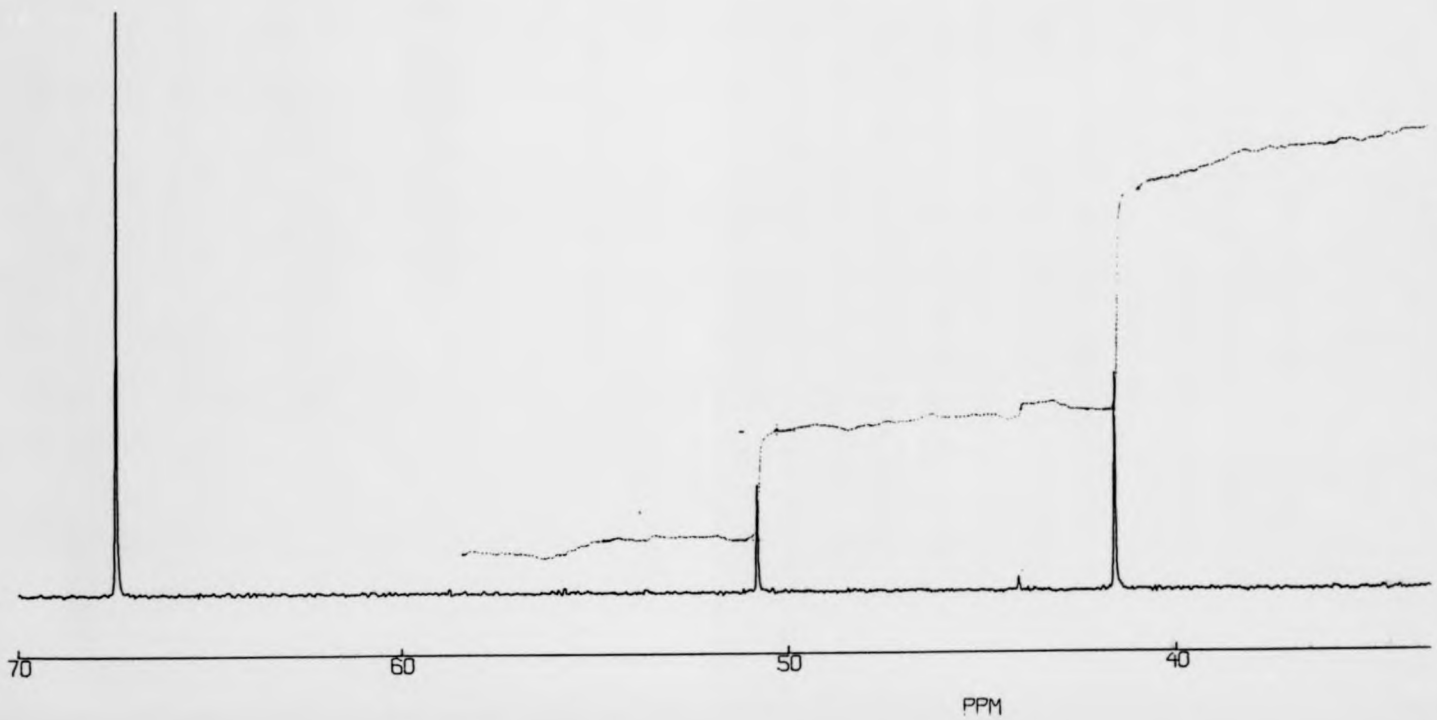


Fig.5.3 $^{13}C\{^{1}H\}$ n.O.e. suppressed n.m.r. spectrum of N-methylpiperazine from the reaction of nitrogen mustard (1a) with ammonia.



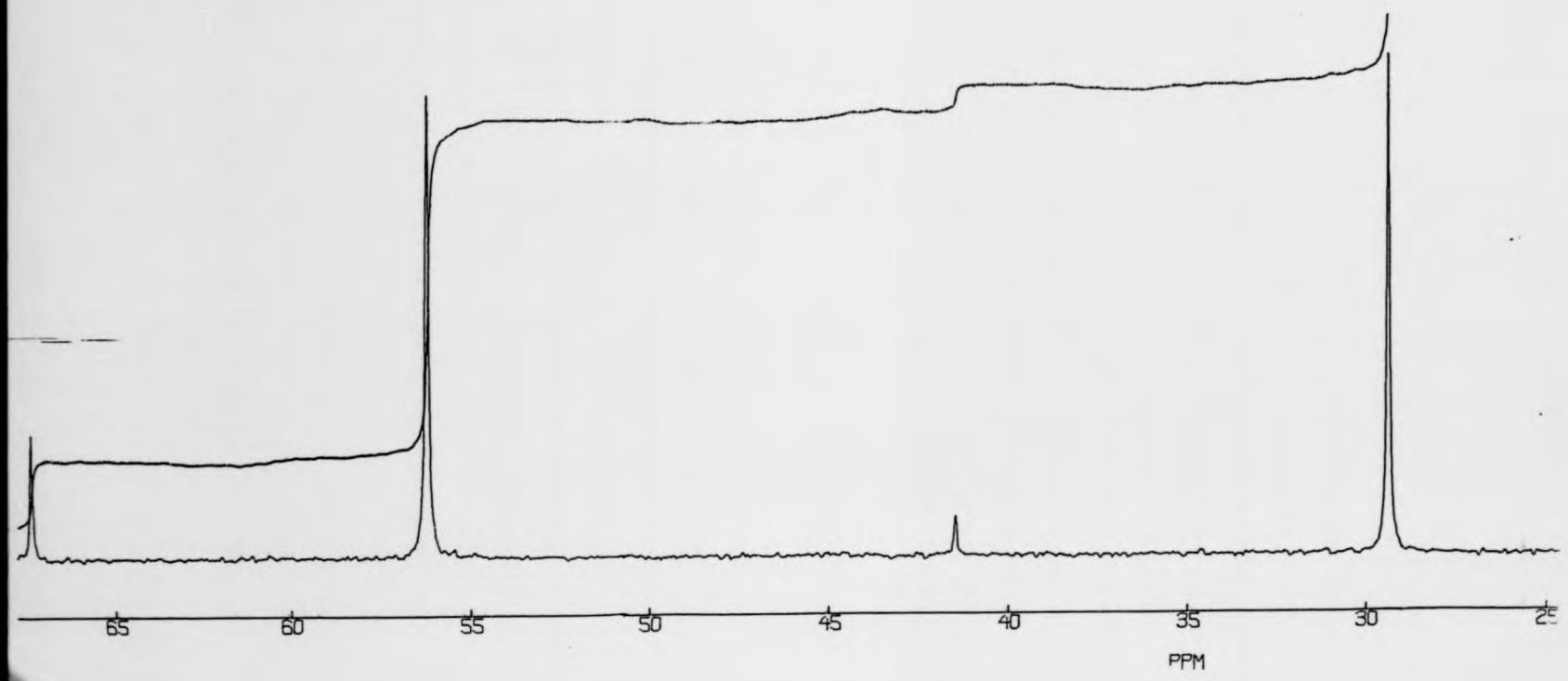
labelled nitrogen mustard (1b) and (1c) *via* (8). The mustards then react with ammonia by direct displacement. Two items of evidence exclude this possibility, besides its intrinsic lack of plausibility. The ratio of $^{13}C$ at C-1(C-1') and C-2(C-2') of (9) was not altered in an experiment to which NaCl was added (total $[Cl^-]$ at the start of the experiment increased from 0.1 M to 0.6 M). This shows that the capture of (8) by chloride to give (1a)/(1b) does not effectively compete with its capture by ammonia. Of decisive relevance is the fact that the two ethylene fragments of N-methylpiperazine are differently labelled with $^{13}C$. If (1a) had equilibrated with (1b) and (1c) prior to reaction with ammonia, this would have given N-methylpiperazine with very similar labelling of each of its ethylene units, irrespective of its mechanism of formation.

## 5.4 REACTION OF NITROGEN MUSTARD WITH SIMPLE NUCLEOPHILES

### 5.4.1 Reaction of [2,2'-$^{13}C$]-2-Chloro-N-(2-chloroethyl)-N-methylethanamine with Sodium Thiosulphate

Sodium thiosulphate solution is the recommended reagent for neutralising nitrogen mustard solutions and has been adopted for rinsing accidential skin contamination. The kinetics of the reaction of nitrogen mustard with thiosulphate are well established and reflect the high nucleophilicity of this anion.

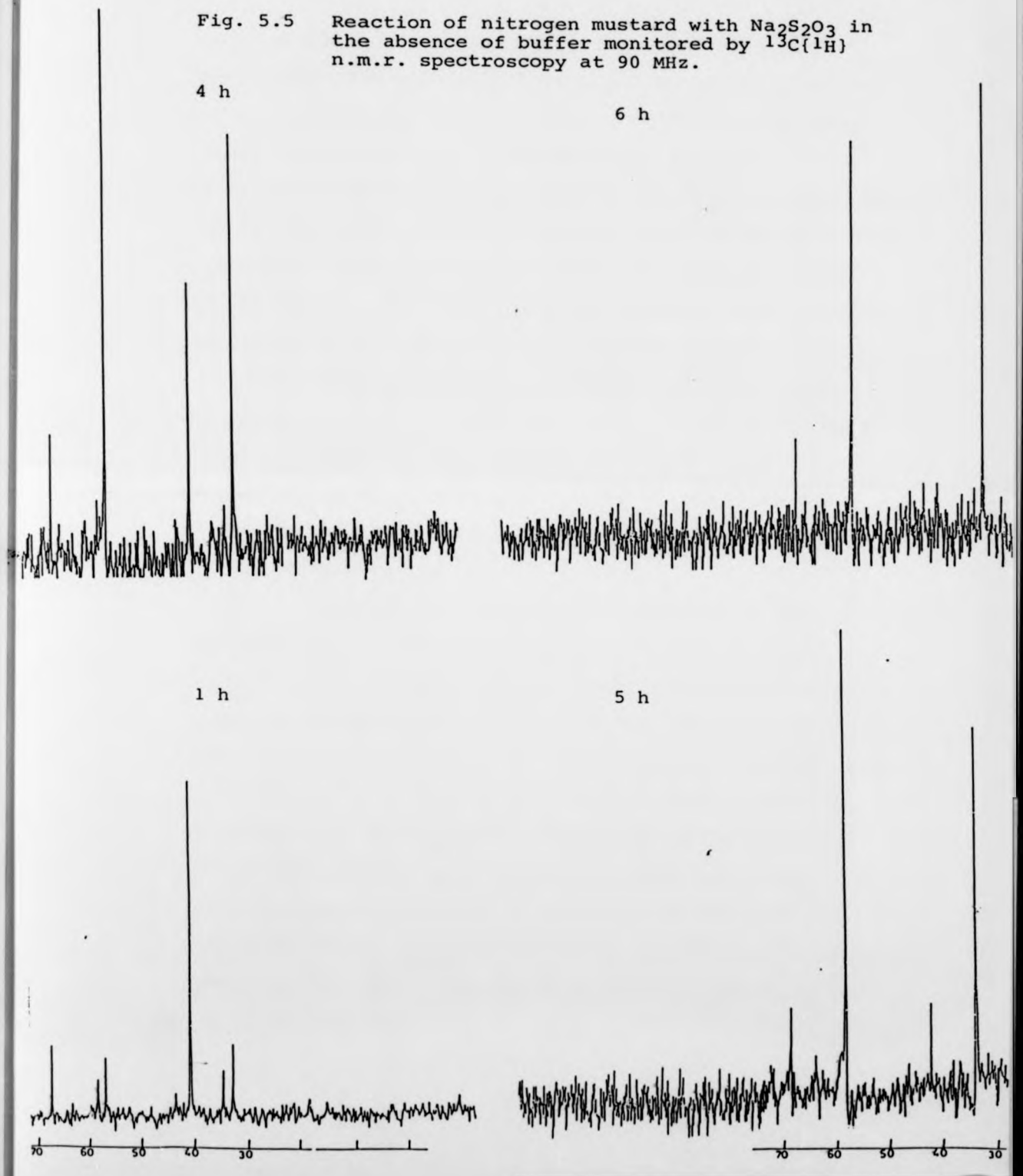
Fig. 5.4 $^{13}C\{^{1}H\}$ n.O.e. suppressed n.m.r. spectrum of the product of the reaction between nitrogen mustard (1a) with $Na_2S_2O_3$.



With such an efficient nucleophile it was of interest to observe any variation in the C-1(C-1')/C-2(C-2') labelling ratio of the product *bis*-Bunte salt. The protocol employed was identical with that described in the previous chapter for $^{1}H$ n.m.r. spectroscopic studies, i.e. (1a). HCl was added to an excess of $Na_2S_2O_3$ in pH 7.2 citrate-phosphate buffer. The subsequent reaction was monitored by $^{1}H$ n.m.r. spectroscopy and when completed the sample was analysed by high field $^{13}C\{^{1}H\}$ n.m.r. spectroscopy. At this stage (24 hours from mixing) the spectrum (Fig. 5.4) was consistent with *bis*-Bunte salt (67) as the sole product. The ratio of C-1(C-1')/C-2(C-2'), measured *in situ* was 1.09 $\pm$ .03 which compares favourably with a ratio of 1.14 $\pm$ .05 obtained for the formation of amine (95).

The un-buffered reaction between nitrogen mustard and $Na_2S_2O_3$ is slower than that at pH 7.2 and has a convenient half-life for continuous monitoring by $^{13}C$ n.m.r. spectroscopy. Addition of nitrogen mustard to a solution of $Na_2S_2O_3$ in $^{2}H_2O$ containing an internal standard (dioxan) followed by spectral accumulation interrupted at *ca.* 1 hour intervals gave a series of $^{13}C\{^{1}H\}$ spectra showing the change from nitrogen mustard to *bis*-Bunte salt (Fig. 5.5). The N-$CH_2$ of nitrogen mustard can be distinguished from the corresponding methylene resonance of the product; after the first hour the mustard N-$CH_2$ was *ca.* 10% of the intensity of the $CH_2$-Cl fragment confirming that the $^{13}C$ label is retained in the 2,2'-positions of the mustard. At no stage during the reactions

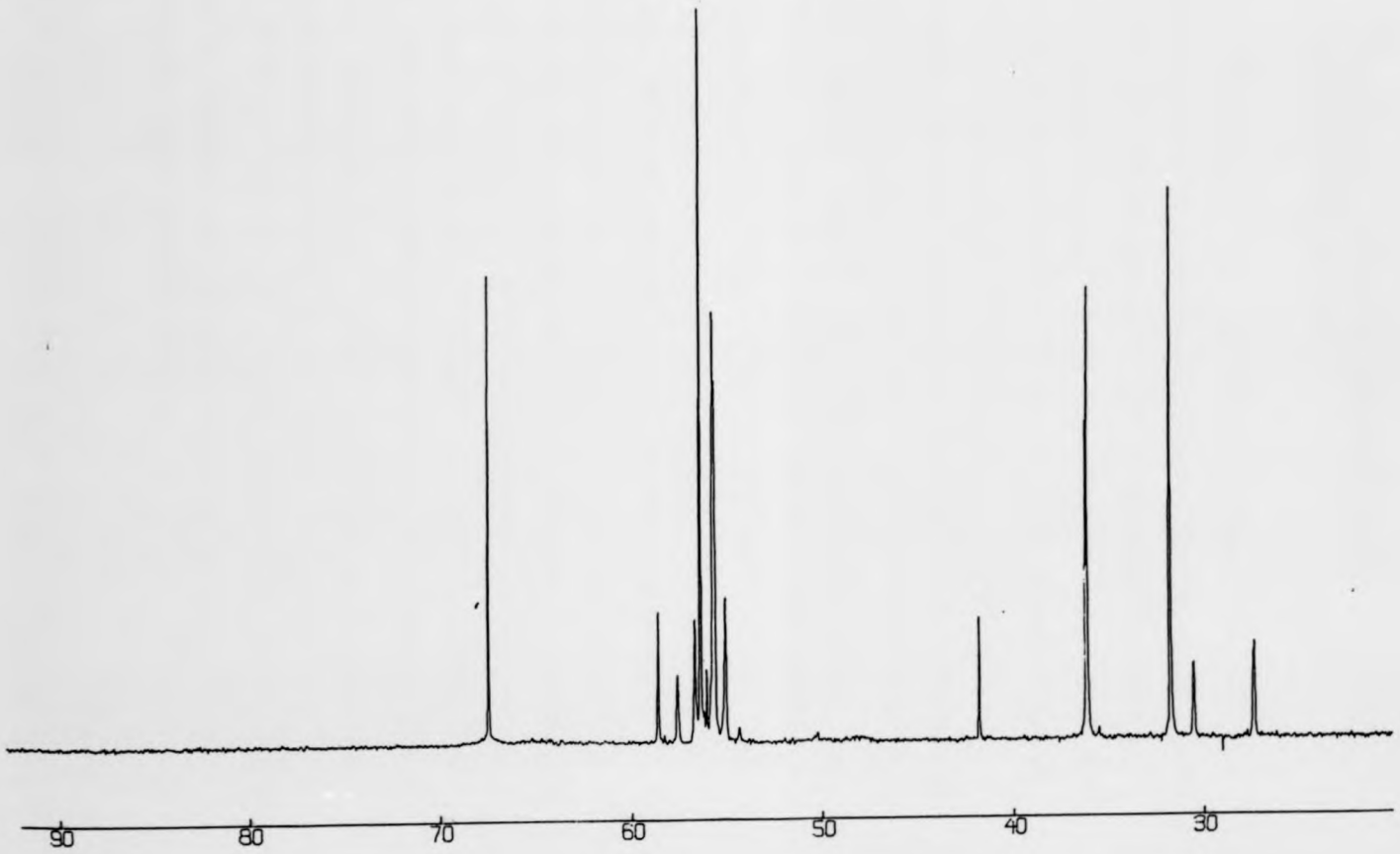
Fig. 5.5 Reaction of nitrogen mustard with $Na_2S_2O_3$ in the absence of buffer monitored by $^{13}C\{^1H\}$ n.m.r. spectroscopy at 90 MHz.



did the mustard N-$CH_2$ resonance show any indication of enrichment demonstrating that there is no equilibration of label between C-1(C-1') and C-2(C-2') such as could arise from reversible aziridinium ion formation. Subsequent spectra displayed peaks for enriched resonances only, that of the $CH_2Cl$ methylenes decaying steadily with a half-life of *ca.* 1 hour, in agreement with published measurements. The first spectrum includes an unassigned signal at δ 31.7 which does not appear in later spectra. The only transitory species expected are aziridinium intermediates and a half-Bunte salt. Observations with $^1H$ n.m.r. spectroscopy suggest that there is no accumulation of aziridinium ions in the presence of thiosulphate. Thus, the δ 31.7 resonance must arise from the half-Bunte salt.

Addition of $Na_2S_2O_3$ to a solution of the aziridinium ion (8a) generated by treating labelled mustard (1a) with base gave an identical product to that obtained without pre-treatment of the mustard (Fig. 5.6). The ratio of $^{13}C$ at C-1(C-1') and C-2(C-2') was measured as 1.07 ± .05 *in situ*, and is in good agreement with that obtained when starting with (1a), confirming that (8a) is on the reaction coordinate. Numerous resonances not attributable to the Bunte salt were also observed and are assigned to piperazinium dimers and hydrolysis products which nearly always accompany aziridinium ion generation.

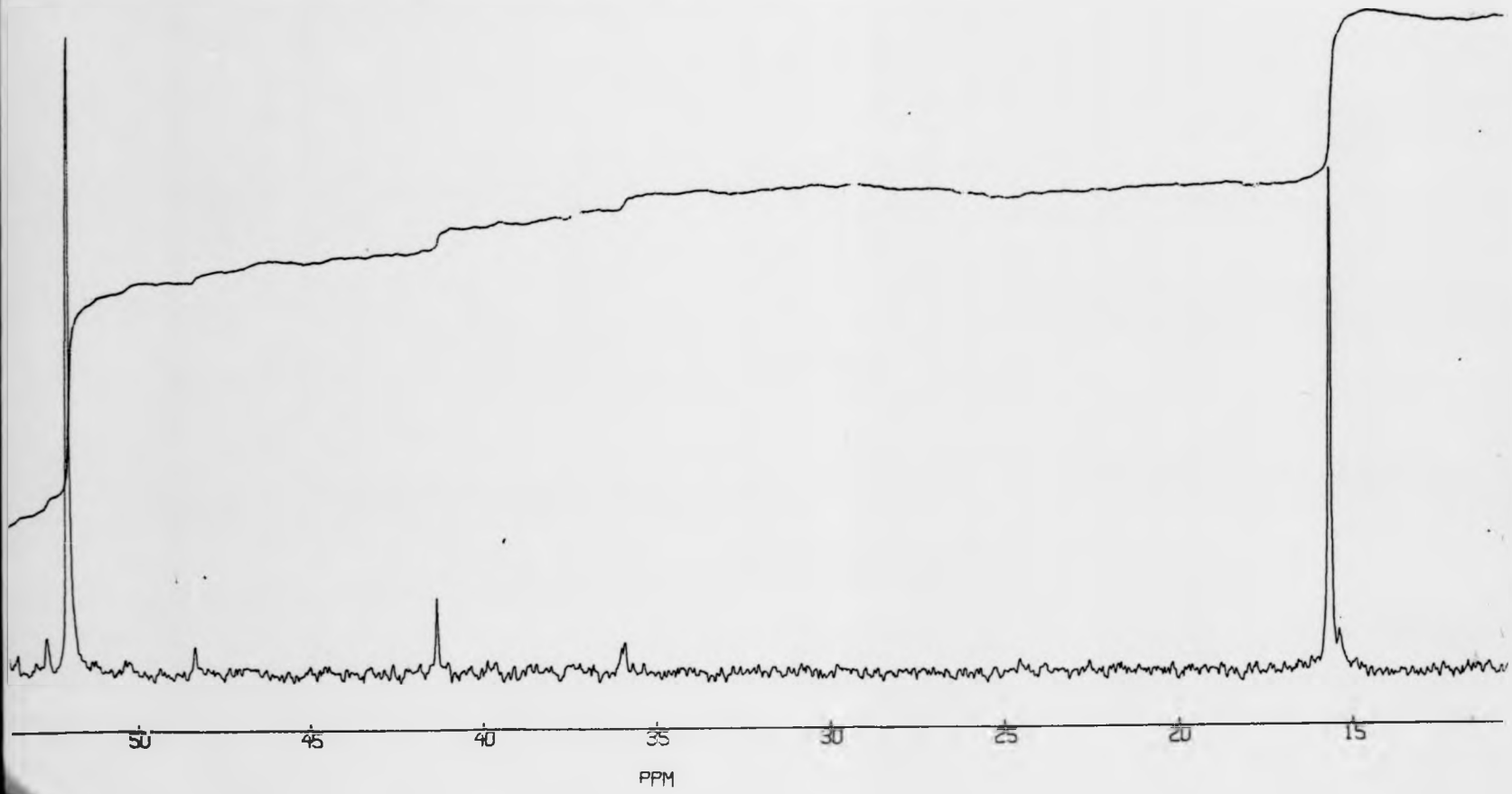
Fig. 5.6 $^{13}C$ $\{^{1}H\}$ n.O.e. suppressed n.m.r. spectrum of the products of the reaction between aziridinium ion (8a) and $Na_2S_2O_3$.



### 5.4.2 Reaction of [2,2'-$^{13}$C]-2-Chloro-N-(2-chloroethyl)-N-methylethanamine with Sodium Cyanide

Measurement of the isotope effect observed in the capture of an aziridinium ion by a carbon nucleophile is of interest for comparison with that determined for other nucleophiles. Recording the $^{13}C\{^{1}H\}$ n.m.r. spectrum 24 hours from mixing the reagents in $^{2}H_2O$ showed 3 peaks (Fig. 5.7) attributable to 2-cyano-N-(2-cyanoethyl)-N-methylethanamine (69) with a C-1(C-1')/C-2(C-2') integral ratio of 1.12 ± 0.05. Within the limits of experimental error this is the same as that determined for other nucleophiles. Interestingly, there were seven minor peaks present in addition to those assigned to (69) and the piperazinium dimer. On re-recording the spectrum seven days from mixing, two of these resonances had disappeared indicating that they may have belonged to a mono substituted intermediate, an assignment supported by $^{1}H$ n.m.r. evidence. A slow reaction such as this would permit some hydrolysis. Accordingly the 5 unidentified signals are tentatively assigned to mustard hydrolysis products, although their rapid appearance argues against this possibility. There is no measurable difference in the C-1(C-1')/C-2(C-2') ratio and none of the unidentified signals is sufficiently intense to suggest a significant contribution from labelled carbon.

Fig. 5.7 $^{13}C\ \{^{1}H\}$ n.O.e. suppressed n.m.r. spectrum of the product of the reaction between mustard (1a) and NaCN.



### 5.4.3 Reaction of [2,2'-$^{13}$C]-2-Chloro-N-(2-chloroethyl)-N-methylethanamine with Methanethiol

A solution of (1a) in water was treated with $Na_2CO_3$ and the solution saturated with methanethiol. When the reaction was over, extraction with $CH_2Cl_2$ gave N-2-(methylthio)ethyl-N-methyl-2-(methylthio)ethanamine (100) which was then analysed by $^{13}C\{^1H\}$ n.m.r. spectroscopy. This showed (Fig. 5.8) two intense peaks in the ratio 1.2:1 ± 0.1 which is consistent with the C-1(C-1')/C-2(C-2') ratio observed with other nucleophiles. Reference to the literature indicated that the dithioether (100) was a new compound. Therefore it was fully characterised (in unlabelled form) by elemental analysis, mass spectroscopy and $^1H$ n.m.r. spectroscopy.

$$CH_3N[CH_2CH_2SCH_3]_2$$

(100)

The C-1/C-2 isotope distribution ratios discussed above are summarised in Table 5.1. They range between 1.09 ± 0.03 and 1.2 ± 0.1, well within the theoretical maximum of 1.25 (see ref. 113, table 4.1) and in sufficiently good agreement to suggest a common mechanism. The error limits were estimated from the

Fig. 5.8 $^{13}C$ $\{^{1}H\}$ n.O.e. suppressed n.m.r. spectrum of the product of the reaction between (1a) and methanethiol.

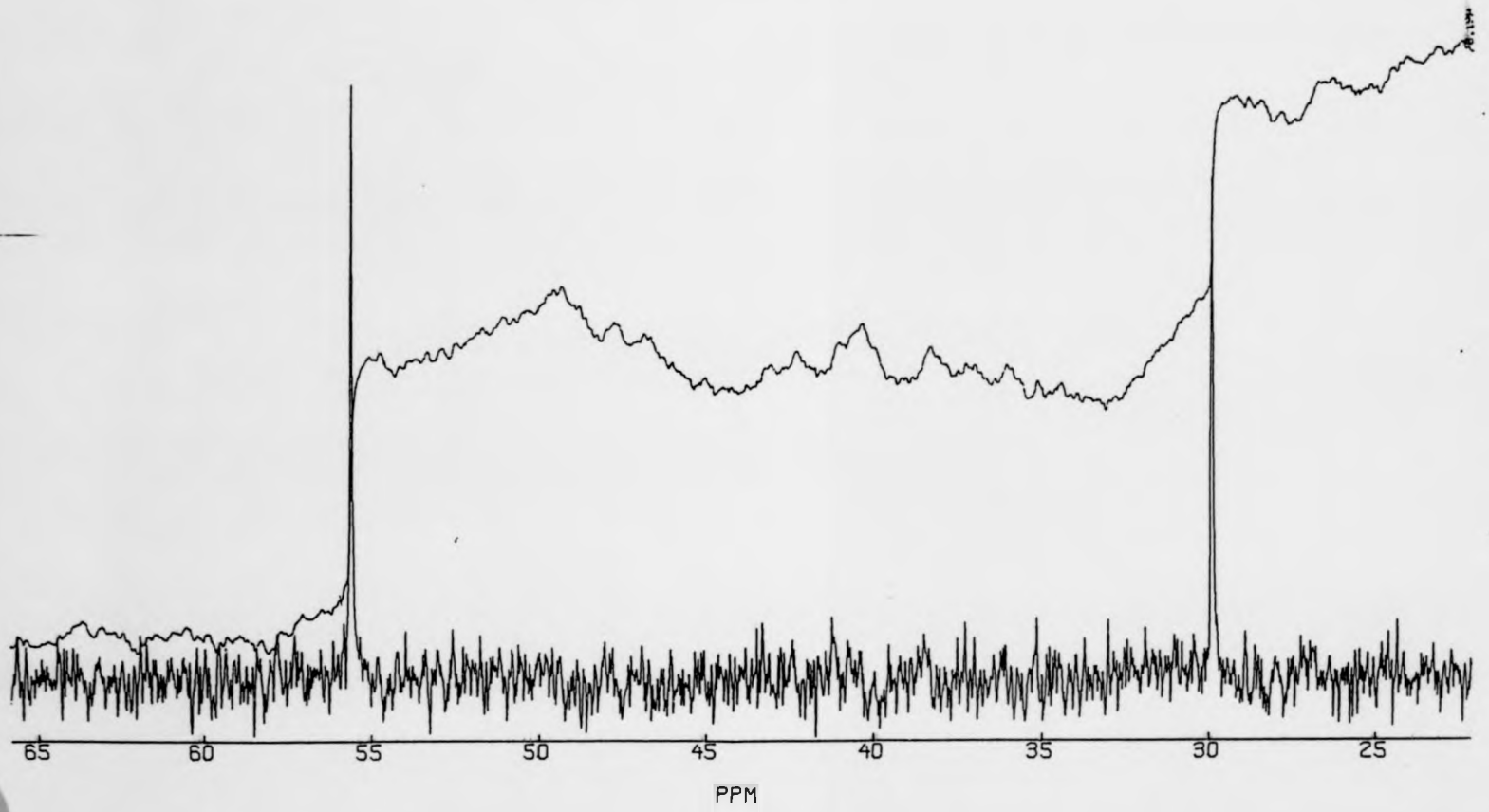
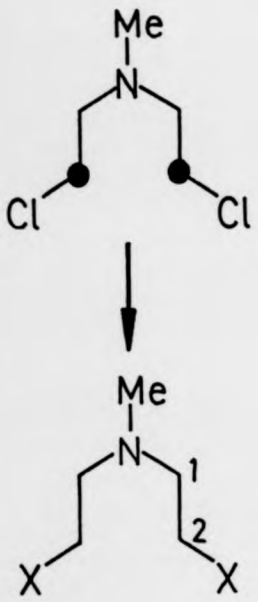


Table 5.1

ISOTOPE DISTRIBUTION IN DI-SUBSTITUTED PRODUCTS DERIVED FROM $^{13}C$-LABELLED NITROGEN MUSTARD




| $X^-$ ($H^+$) | C-1/C-2 |
|---|---|
| $S_2O_3^{2-}$ | 1.09 ± 0.03 |
| $CN^-$ | 1.12 ± 0.05 |
| $MeS^-$ | 1.2 ± 0.1 |
| $NH_3$ | 1.1 ± 0.1 |
| $NH_3$ | 1.15 ± 0.15 |
| | In the presence of 5 equivalents of added $Cl^-$ |

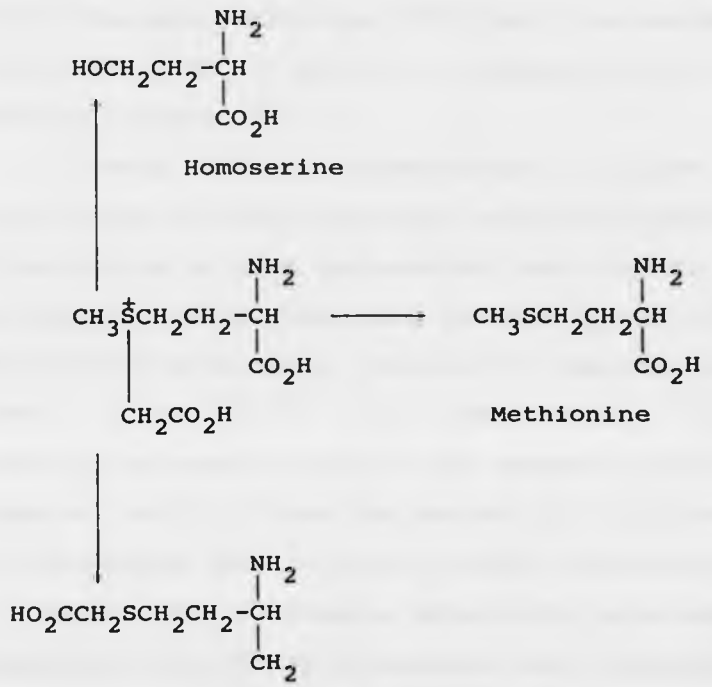
spectral integrals and are therefore likely to be an over-estimate.

## 5.5 REACTION OF NITROGEN MUSTARD WITH [$^{13}$C-METHYL]-*S*-METHIONINE

The studies of Golumbic *et al.* demonstrated that exposure of methionine to sulphur mustard (2) resulted in the formation of the sulphonium salt of methionine rather than alkylation of the amino function. Surprisingly, no such behaviour was observed with nitrogen mustard (1) or ethyl mustard (12), although sulphonium salt formation was reported with the three armed mustard (14). Alkylation of methionine with aziridine (19) showed that, subsequent to sulphonium salt formation, decomposition to homoserine occurred with loss of the sulphur atom[115]. There are obvious implications for de-toxification *in vivo* although the effective replacement of methionine with homoserine may affect the nucleophile's biological function. "Sulphur-stripping" of proteins has been reported and a similar mechanism proposed in the case of busulphan[5]. Inactivation of ribonuclease with iodoacetic acid was found to be pH dependent,[116,117], the fastest reaction at pH 8.5-10 being assigned to alkylation of the ε-amino group of lysine. At an intermediate pH of 5.5-6.0 reaction of a histidine residue was observed, whereas at pH of 2.8 alkylation of methionine sulphur atoms was predominant. Model studies with free methionine indicated a pH dependency and demonstrated three decomposition products of the carboxymethylsulphonium salt (Scheme 5.6). Heating the sulphonium salt for one

Scheme 5.6

Products of the Decomposition of the Methioninecarboxymethylsulphonium Salt

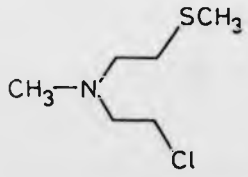


hour at 100 $^{o}$C and pH 6.5 gave almost complete conversion to homoserine, but if the experiment was performed at pH 2.2 significant quantities of homoserine lactone were obtained. Heating the sulphonium salt in strong acid for 24 hours gave methionine (20%) and *S*-carboxymethyl-homocysteine (50%) in addition to homoserine (5%) and homoserine lactone (6%).

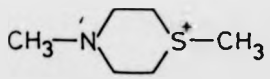
Using $^{1}$H n.m.r. spectroscopy, nitrogen mustard has been shown to form both mono- and *bis*-sulphonium salts with methionine at room temperature (see Chapter 4). These spectra have a great many resonances making their interpretation difficult. Much of the complexity can be avoided by using $^{13}$C{$^{1}$H} n.m.r. spectroscopy, thus eliminating spin-spin coupling and expanding the scale of chemical shifts. When the mustard (1) is allowed to form aziridinium ions in aqueous media, hydroxide ion or (1) may be able to compete effectively with added nucleophile, thus adding resonances due to polymerised or hydrolysed mustard to the spectrum of the desired product. By using stable isotope labelled reagents the fate of specific atoms may be monitored. Therefore, specific labelling of methionine with $^{13}$C allows a considerably simplified spectrum to be observed.

Alkylation of the methionine sulphur atom generates a new chiral centre in addition to that of the amino acid moiety. The $^{1}$H n.m.r. characteristics of diastereomers were considered in Chapter 4; similar behaviour is observed in $^{13}$C n.m.r. spectra but due to the low natural abundance of $^{13}$C such spectra do not exhibit

spin-spin coupling. Therefore, there are three diastereomers of the di-substituted product from the reaction between methionine and nitrogen mustard (Scheme 5.7). Assuming a statistical distribution of products the ratio of (*S*,*S*,*R*,*S*) = (*S*,*R*,*S*,*S*)-: (*S*,*R*,*R*,*S*)-: (*S*,*S*,*S*,*S*)- is predicted as 2:1:1. If the alkylated material breaks down to give homoserine then the labelled methyl groups will be retained in a dithioether of nitrogen mustard (100), which is the product of the reaction between (1) and methanethiolate anion. Also shown in Scheme 5.7 are locations of the label should other reactions occur. If elimination of homoserine is fast then the mono-thioether (101) may be generated. Cyclisation of (101) to an aziridinium ion would eventually give (100) if captured by methionine, but if the nucleophile is hydroxide it is unlikely that the *S*-methyl resonance would be very different from that of (100). However, by analogy with the ammonia situation, an intramolecular alkylation to yield the cyclic sulphonium salt (102) is also possible.

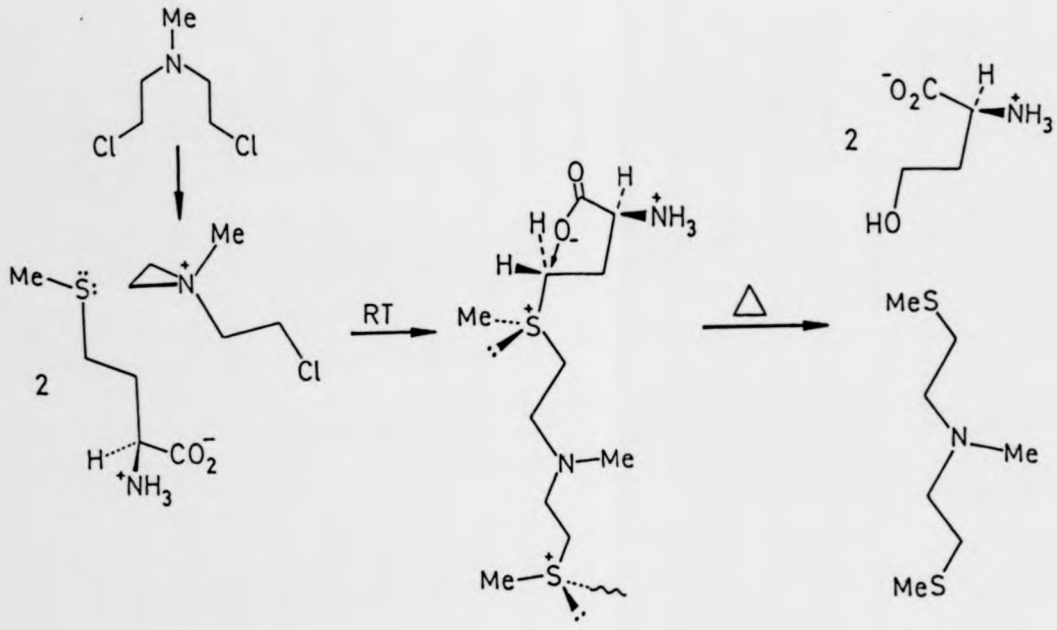



(101)




(102)

Scheme 5.7 Products from the reaction between nitrogen mustard and methionine



The enriched methyl signal from this material would be similar to that of the methionine sulphonium salt and would be quite distinct from that of the dithioether (100). Thus, with reference spectra of methionine and (100) available and no signals observed from species lacking a methionine derived *S*-methyl group, analysis of the reaction is much simplified.

An attempt to follow the reaction at 22.6 MHz was unsuccessful due to the lack of resolution, although sulphonium salt formation was evident. At a higher field strength, 100 MHz, peaks were resolved and the time between observations during the more dynamic early stages of the reaction could be reduced. A high concentration of aziridinium ion (8) was generated from (1) in pH 7.2 citric acid buffer, mixed with an excess of [$^{13}$C-methyl]-(*S*)-methionine and spectral accumulation commenced.

Concentrating on the intense signals derived from labelled methionine, the spectrum (Fig. 5.9) shows two signals after 25 minutes. The more intense of these ($\delta$ 14.90) is assigned to unreacted methionine S-methyl but a broad resonance at $\delta$ 23.52 is characteristic of a sulphonium $\overset{+}{S}$-methyl signal. Comparison of the relative integrals of these species is not meaningful because the tertiary sulphonium $\overset{+}{S}$-methyl will relax at a different rate to the methionine *S*-methyl giving rise to widely different nuclear Overhauser effects. A second spectrum recorded fifty-five minutes from adding the nucleophile shows the methionine peak and considerable structure to the sulphonium signal. The original broad peak exhibits at least three shoulders and a downfield doublet centred

Fig. 5.9a Reaction between nitrogen mustard and (*S*)-[$^{13}CH_3$]-methionine in pH 7.2 buffer monitored by 100 MHz $^{13}C\{^1H\}$ n.m.r. spectroscopy.

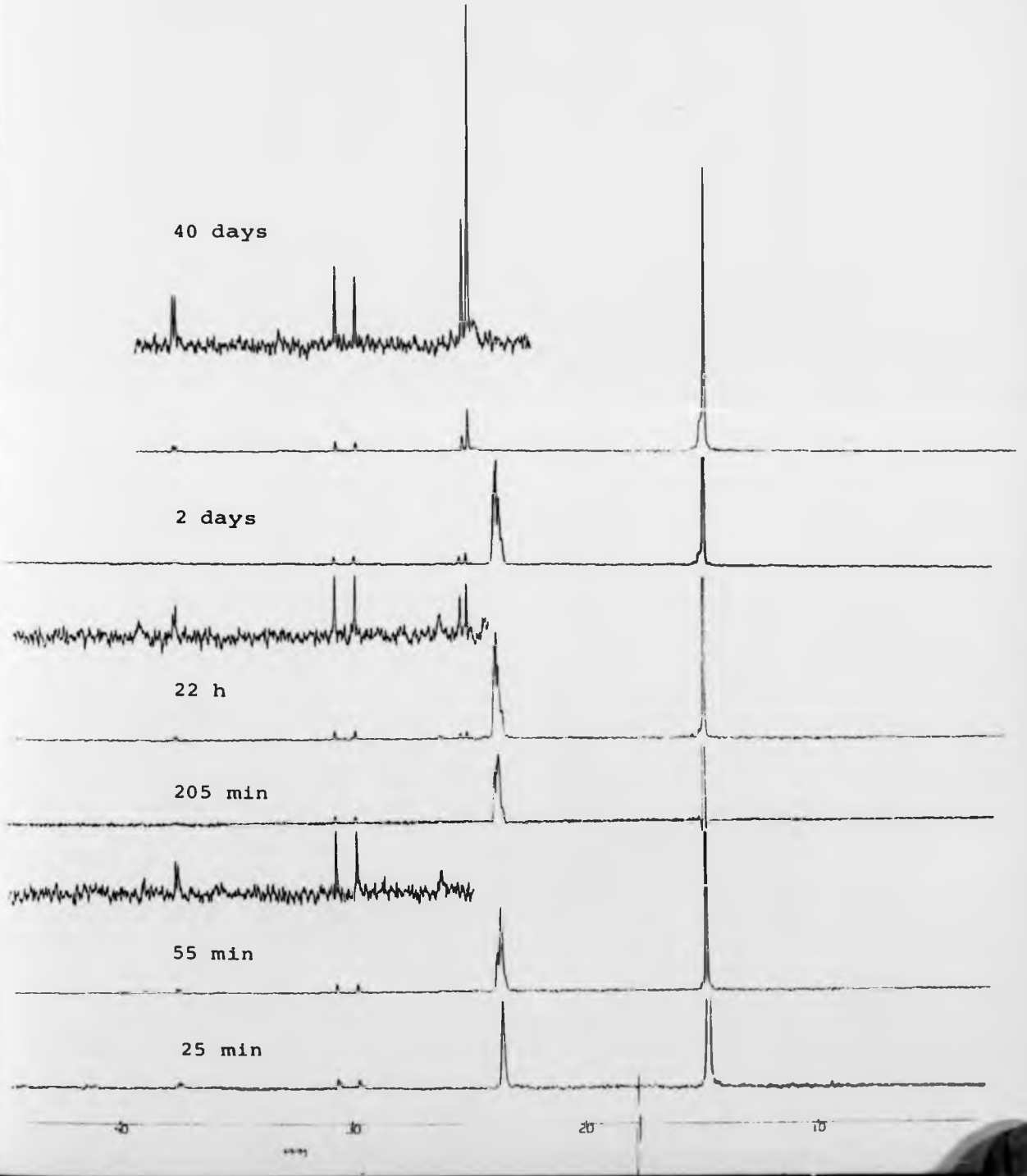
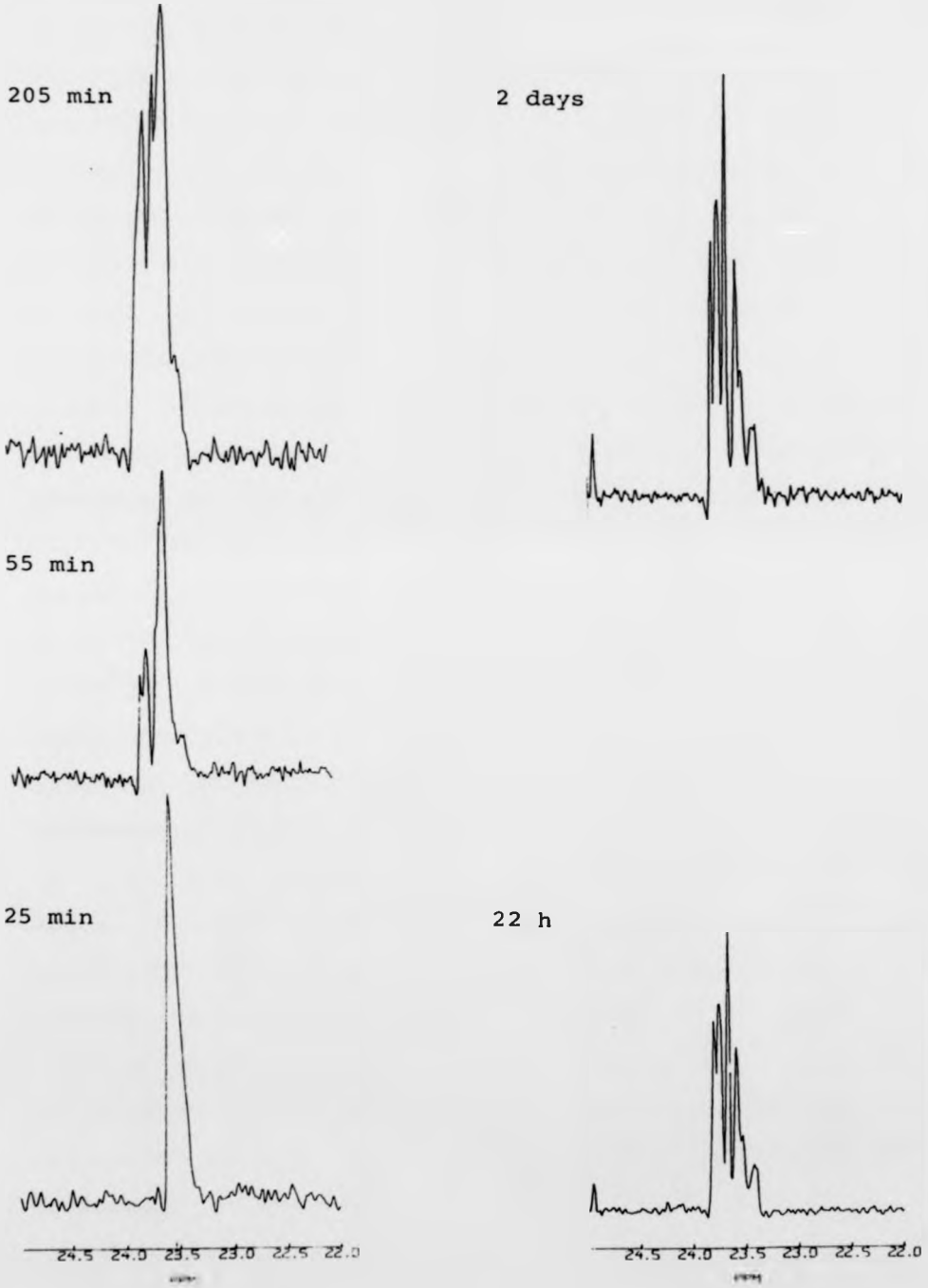


Fig. 5.9b Expansion of $\overset{+}{S}$-Me region of spectra shown in Fig. 5.9a.



at δ 23.8. This doublet is of similar size to the original signal after three hours and shows a prominent upfield shoulder. A spectrum recorded twenty-two hours from mixing shows unreacted methionine, the doublet at δ 23.8, a singlet at δ 23.67, a broad singlet at δ 23.60 with an upfield shoulder and a very broad peak at δ 23.44. The spectrum is essentially unchanged at 48 hours, but 40 days after mixing only 3 signals in the original sulphonium ion region are evident: δ 23.8, 23.73 and 23.58. Two other resonances, probably sulphonium methyls, noticeable after 22 hours, have now grown to significant intensity at δ 25.31 and 24.93. There is also a broad signal slightly downfield from the methionine methyl peak. During this period the sample had been maintained at 20-25 $^{o}$C, but the reaction was now completed by heating at 90 $^{o}$C for five days. The resulting $^{13}$C n.m.r. spectrum showed two signals at δ 25.17 and 24.92 and a slightly broadened methionine methyl signal. All trace of the sulphonium $\overset{+}{S}$-methyl resonances at *ca.* δ 23 has been removed.

These observations are in accord with the predictions above: the first formed sulphonium salts will be mono-alkylated methionines which will give way to the *bis*-alkylated species for which three $\overset{+}{S}$-methyl resonances are predicted (δ 23.8, 23.73 and 23.58 in the 40 day spectrum). It is not possible to be specific in the assignment of other resonances in this region because reference spectra of the transients, (which should include aziridinium derivatives of mono-alkylated methionine), are not available. These three $\overset{+}{S}$-methyl signals are lost on heating because they

can eliminate homoserine and generate a di-thioether in which the *S*-methyl signals overlap those of methionine. The non-heat labile peaks at δ 25.17 and 24.92 are consistent with a diastereomeric sulphonium salt but cannot be assigned.

## 5.6 EXPERIMENTAL

### Reaction of Nitrogen Mustard (1a) with Ammonia Solution

A solution of [2,2'-$^{13}$C]-2-chloro-N-(2-chloroethyl)-N-methylethanamine (30 mg, 10.8 atom % $^{13}$C) in water (4 $cm^3$) was added to a stirring solution of excess ammonia (2 $cm^3$ of 35% $NH_3OH$). After 2 hours the solution was evaporated to dryness and the $^1$H n.m.r. spectrum in 2 M $^2$HCl recorded. This spectrum was consistent with the formation of N-methylpiperazine (96) and 2-amino-N-(2-aminoethyl)-N-methylethanamine(95) and showed no evidence for unreacted starting material. The sample was applied to an acid-washed ion-exchange column (50 g Amberlite CG 120 type I, in a 2 cm column) and eluted with 3 M HCl collecting 10 $cm^3$ fractions of eluant. Each fraction was evaporated to dryness and analysed by $^1$H n.m.r. spectroscopy as a solution in $^2$HCl. The N-methylpiperazine (96) emerged as a sharp band in the seventh and eighth fractions while the amine (95) eluted from the twelfth fraction onwards. Evaporation of the solutions after analysis allowed the yield of each amine to be estimated by weight of the hydrochloride [$C_5H_{14}N_2Cl_2$ 10.0 mg, 5.8 x $10^{-5}$ moles; $C_5H_{18}N_3Cl_3$ 18.0 mg, 8.0 x $10^{-5}$ moles. Total recovery

$1.38 \times 10^{-4}$ moles, 90% of theoretical maximum. Traces of the piperazinium dimer (10) were observed in an early column fraction.] The amines (95) and (96) were individually taken up in $^{2}H_2O$, dioxan added as an internal standard and the label distributions assessed by $^{13}C$ n.m.r. spectroscopy with n.O.e. suppression.

Similarly, a solution of unlabelled nitrogen mustard (30 mg in 4 $cm^3$ of $H_2O$) was added to a stirred solution of ammonia (2 $cm^3$ of 35% $NH_4OH$) and the amines (95) and (96) were separated from the reaction mixture by column ion exchange chromatography as described for the $^{13}C$ labelled material. Each of the amines was then taken up in water (5 $cm^3$) and treated with saturated aqueous $Na_2CO_3$ to pH 9. A solution of N-phenylaminothiocarbonate (1 $cm^3$) in ethanol (8 $cm^3$) was added and the mixture stirred (1 hour) to give an emulsion. The emulsion was extracted with $CH_2Cl_2$ (2 x 80 $cm^3$) and the combined extracts dried over $MgSO_4$ and evaporated to leave the N-phenylaminocarbonyl derivatives of (95) and (96). Comparison with the derivatives of authentic material confirmed the identity of the amines.

(95) *bis*-N-phenylaminothiocarbonate: $\delta_C$ ($C^2H_3O^2H$) 44.28 (N-$CH_3$), 46.95 (C$H_2$-NMe) 57.88 (C$H_2$-N-phenylaminothiocarbonate).

T.l.c. (4:1 ethylacetate:methanol) single spot $R_f$ 0.9

(96) N-phenylaminothiocarbonate: $\delta_C$ ($C^2H_3O^2H$) 46.62 (N-$CH_3$), 49.96 ($CH_2$NMe), 56.3 ($CH_2$-N-phenylaminothiocarbonate)

T.l.c. (4:1 ethylacetate:methanol) single spot $R_f$ 0.1

Reaction of Sodium Thiosulphate with Nitrogen Mustard (1a) at pH 7.2

[2,2'-$^{13}$C]-2-Chloro-N-(2-chloroethyl)-N-methylethanamine (10 mg of 10.7 atom %) was added to an excess of $Na_2S_2O_3$ (21 mg) in 0.5 $cm^3$ of pH 7.2 citrate-phosphate buffer. After standing at room temperature for 24 hours, dioxan internal standard was added and the $^{13}C\{H\}$ n.m.r. spectrum recorded at 100 MHz. The sample showed three resonances at δ 29.33, 41.43 and 56.23. That at 41.43 was of much reduced intensity compared with the outer peaks and was therefore assigned to the unenriched N-methyl of (67). Integration showed the C-2(C-2'):C-1(C-1') ratio to be 1.09 ± .03.

Time-course Reaction between Nitrogen Mustard (1a) and Sodium Thiosulphate

[2,2'-$^{13}$C]-2-Chloro-N-(2-chloroethyl)-N-methylethanamine (13 mg of 10.7 atom %) was added to a solution of $Na_2S_2O_3$ in $^2H_2O$ (2 $cm^3$ of 0.5 M) in an n.m.r. tube. Dioxan was added as an internal standard and $^{13}C\{H\}$ n.m.r. spectra recorded at approximately hourly intervals. One hour from mixing, the spectrum showed 6 peaks in the approximate ratios 2:2:10:1:2:1. These represent unchanged nitrogen mustard δ ($^2H_2O$) 38.2 ($\underline{C}H_2Cl$), 41.5 ($\underline{C}H_3$-N), 57.65 ($\underline{C}H_2$-N) and the *bis*-Bunte salt (67) δ ($^2H_2O$) 29.8 ($\underline{C}H_2$-S) and 55.6 ($\underline{C}H_2$-N). The methyl group of (67) resonates at δ 41.4 and is probably obscured by the methyl signal of (1a). The remaining signal at δ 31.7 is assigned to the half-Bunte salt. Subsequent spectra had increasingly poor signal to noise ratios and showed two

steadily increasing signals for (67) (δ 29.8 and 55.6) and one signal for (1a) (δ 38.2) which decayed and disappeared after 6 hours. After this there were no further spectral changes.

Reaction of Aziridinium Ion (8a) with Sodium Thiosulphate

A solution of [2,2'-$^{13}$C]-2-chloro-N-(2-chloroethyl)-N-methylethanamine (10 mg of 10.0 atom %) in 0.5 cm$^3$ of $^2H_2O$ was treated with $Na_2CO_3$ (27 μl of 1.9 M, 1 mol. equiv.) and allowed to stand for 2 hours. The solution was then added to $Na_2S_2O_3$ (21 mg, 2.5 mol. equiv.), transferred to an n.m.r. tube and allowed to stand overnight. Dioxan internal standard was added and the $^{13}C\{H\}$ spectrum recorded. This showed formation of the Bunte salt (67) δ ($^2H_2O$) 31.67 ($\underline{C}H_2S$), 41.70 ($\underline{C}H_3$-N), 56.30 ($\underline{C}H_2$-N) with a C-1(C-1'):C-2(C-2') integral ratio of 1.07 ± 0.05. Two other intense resonances were assigned to the $CH_2$-Cl and $\overset{+}{N}CH_2$ methylenes of piperazinium dimer (10). *Cis-trans*-isomerism splits each of these signals such that the resonances appear as doublets: δ ($^2H_2O$) 36.00, 36.09 ($CH_2$-Cl), 55.53, 55.61 ($CH_2$-$\overset{+}{N}$).

Reaction of Nitrogen Mustard (1a) with Sodium Cyanide

[2,2'-$^{13}$C]-2-Chloro-N-(2-chloroethyl)-N-methylethanamine (10 mg of 10 atom %) was added to a solution of NaCN in $^2H_2O$ (6.5 mg in 0.5 cm$^3$), rapidly mixed and transferred to an n.m.r. tube. After standing overnight, dioxan internal standard was added and the

$^{13}C\{H\}$ n.m.r. spectrum recorded. Signals consistent with N-2-cyanoethyl-N-methyl-2-cyanoethanamine were observed: δ ($^{2}H_2O$) 15.64 ($\underline{C}H_2CN$), 41.30 ($\underline{C}H_3N$), 51.95 ($\underline{C}H_2N$). The methylene resonances were each *ca.* 10-fold more intense than the methyl resonance with a relative integral C-1(C-1'):C-2(C-2') of 1.12 ± 0.5 :1. Signals at 35.87, 35.97 and 55.47 were characteristic of piperazinium dimer (10). Other minor resonances were observed at δ 15.35, 48.3, 52.59, 53.42, 53.72, 54.18 and 61.24. In a similar spectrum recorded 5 days later the resonances at δ 15.35 and 61.24 had disappeared but no new signals were apparent. This suggests that these peaks should be assigned to the cyanoethyl moiety of a partially reacted mustard, e.g. (69). The other signals may represent partially hydrolysed material.

Reaction of Nitrogen Mustard (1a) with Methanethiol

The procedure was analogous to that used for authentic unlabelled material: [2,2'-$^{13}C$]-2-chloro-N-(2-chloroethyl)-N-methylethanamine (25 mg of 10 atom % $^{13}C$) in de-aerated water was treated with 2 mol. equivs. of $Na_2CO_3$ (28 mg). Immediately the solution at r.t. was saturated with MeSH and left to stand overnight. Basification with $Na_2CO_3$ and extraction with $CH_2Cl_2$ (2 x 25 $cm^3$) gave 2-methylthio-N-[2-(methylthio)ethyl]-N-methylethanemine: b.p. 85 $^{o}C$ at 18 mmHg, yield = 54%. $\delta_C$ ($^{2}H_2O$) 29.95 ($CH_2SMe$), 55.62 ($CH_2$-NMe). A satisfactory combustion analysis and EI mass spectrum were obtained for the unlabelled material.

Calculated for $C_7H_{17}NS_2$: C 46.88%, H 9.55, N 7.81, S 35.76.

Found: C 46.67, H9.54, N 7.70, S 35.03.

The mass spectrum showed the molecular ion at 179 plus:

118 (89%) $CH_3NCH_2CH_2SCH_3$

75 (100) $CH_2CH_2SCH_3$

61 (6.3) $CH_2SCH_3$

47 (9.3) $SCH_3$

42 (14.5) $NCH_2CH_2$

# CHAPTER 6

# FUTURE WORK

The results presented in this thesis have illustrated the use of high-field n.m.r. spectroscopy and specific stable isotope labelling to identify products and intermediates in the reaction of nitrogen mustard with nucleophiles. This work can be extended by kinetic analysis of these reactions, by working with other mustards or by examining the fate of these drugs in a biological environment. A preliminary examination of some of these ideas is presented below.

## 1. REACTION KINETICS BY $^{1}H$ N.M.R. SPECTROSCOPY

Chapter 5 indicated the potential of a $^{13}C$ label for monitoring the consumption of nitrogen mustard by thiosulphate. Because the decay of a single resonance could be followed and because the reaction conditions were chosen to give a long half-life, $^{13}C$ n.m.r. was an ideal tool for this experiment. However, at pH 7.2 this and many other alkylation reactions are too fast for successful monitoring by $^{13}C$ n.m.r. spectroscopy, because of the time required for spectral aquisition. Zon and his co-workers avoided this problem by using $^{31}P$ n.m.r. spectroscopy in kinetic studies of phosphoramide mustard, which enabled selective monitoring without enrichment. With nitrogen mustard we have attempted

some kinetic studies by monitoring the $^1H$ n.m.r. spectrum, concentrating on the N-methyl resonance. Preliminary results indicate that, provided there is no interference from substrate signals, this approach is practical. A good example of this is shown in Fig. 6.1 in which the relative concentrations of nitrogen mustard, mono-thiourea substituted mustard (74) and di-substituted product (73) are plotted against time. Similarly, Fig. 6.2 shows a log plot of mustard concentration against time for reaction with thiourea and thiosulphate. The rate of the two reactions are very similar; in neither set of spectra is an accumulation of azoridinium ion (8) evident, suggesting that the rate limiting factor is formation of (8). However, thiourea is a neutral nucleophile, whereas thiosulphate is charged and this may cause a rate difference. Additionally, although initial pH values were controlled, the pH of the thiourea reaction fell from 7.2 to 6.9 but that of the thiosulphate reaction fell to 6.7.

## 2. REACTIONS OF OTHER MUSTARDS

While it is obviously useful to establish differences in reactivity between various mustards, the identification of aziridinium species from mustards other than (1) would be of considerable interest. Using the procedures already outlined for (1), chlorambucil hydrochloride in $^2H_2O$ (103) HCl was treated with 1 equivalent of $Na_2CO_3$ and the subsequent reaction monitored by $^1H$ n.m.r. spectroscopy (Fig. 6.3).

Fig. 6.1 Plot of concentration against time for nitrogen mustard and mono- and di-substituted products of the reaction with thiourea.

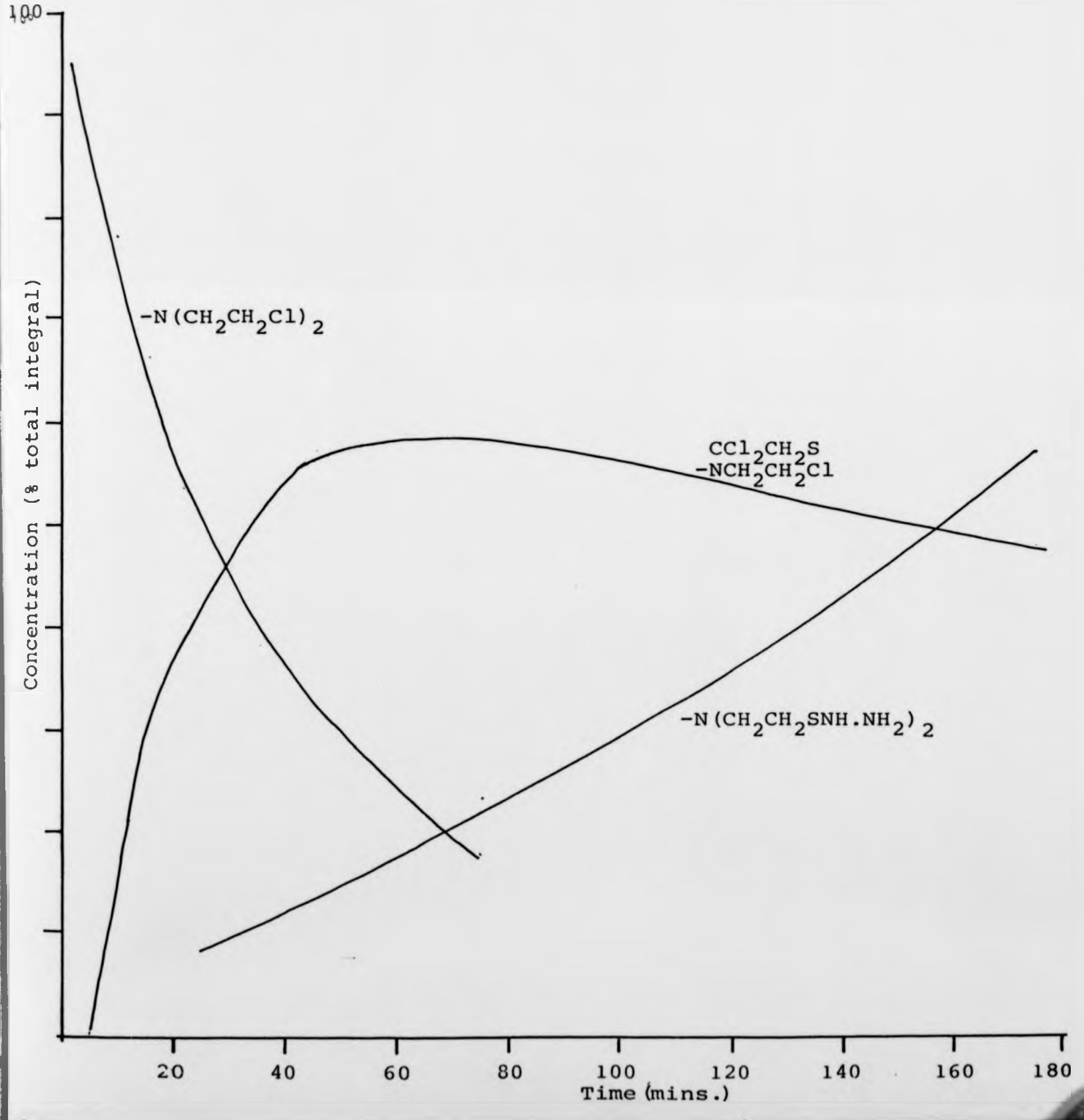


Fig. 6.2 Plot of log[1] against time for the reaction of nitrogen mustard with sodium thiosulphate and thiourea.

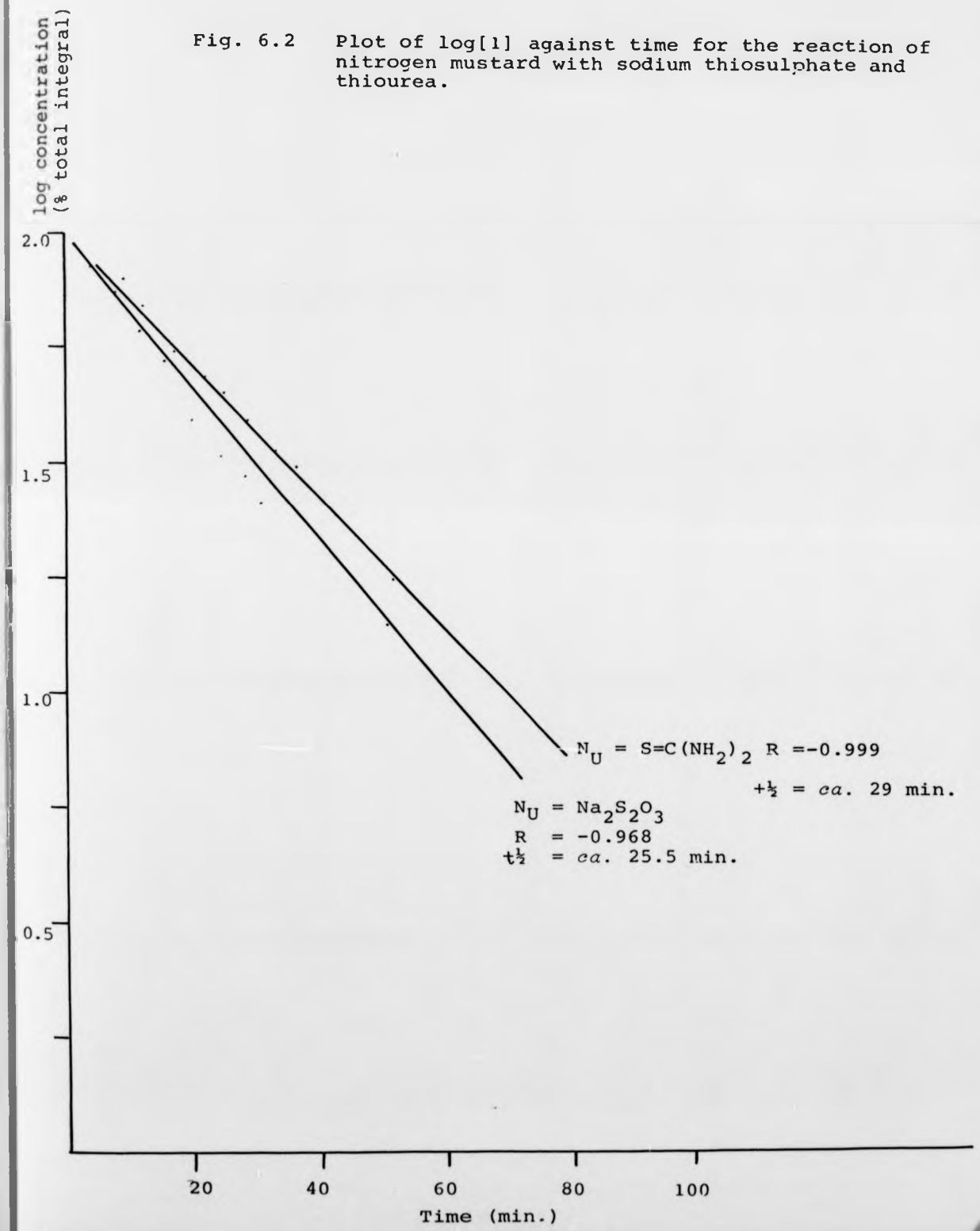
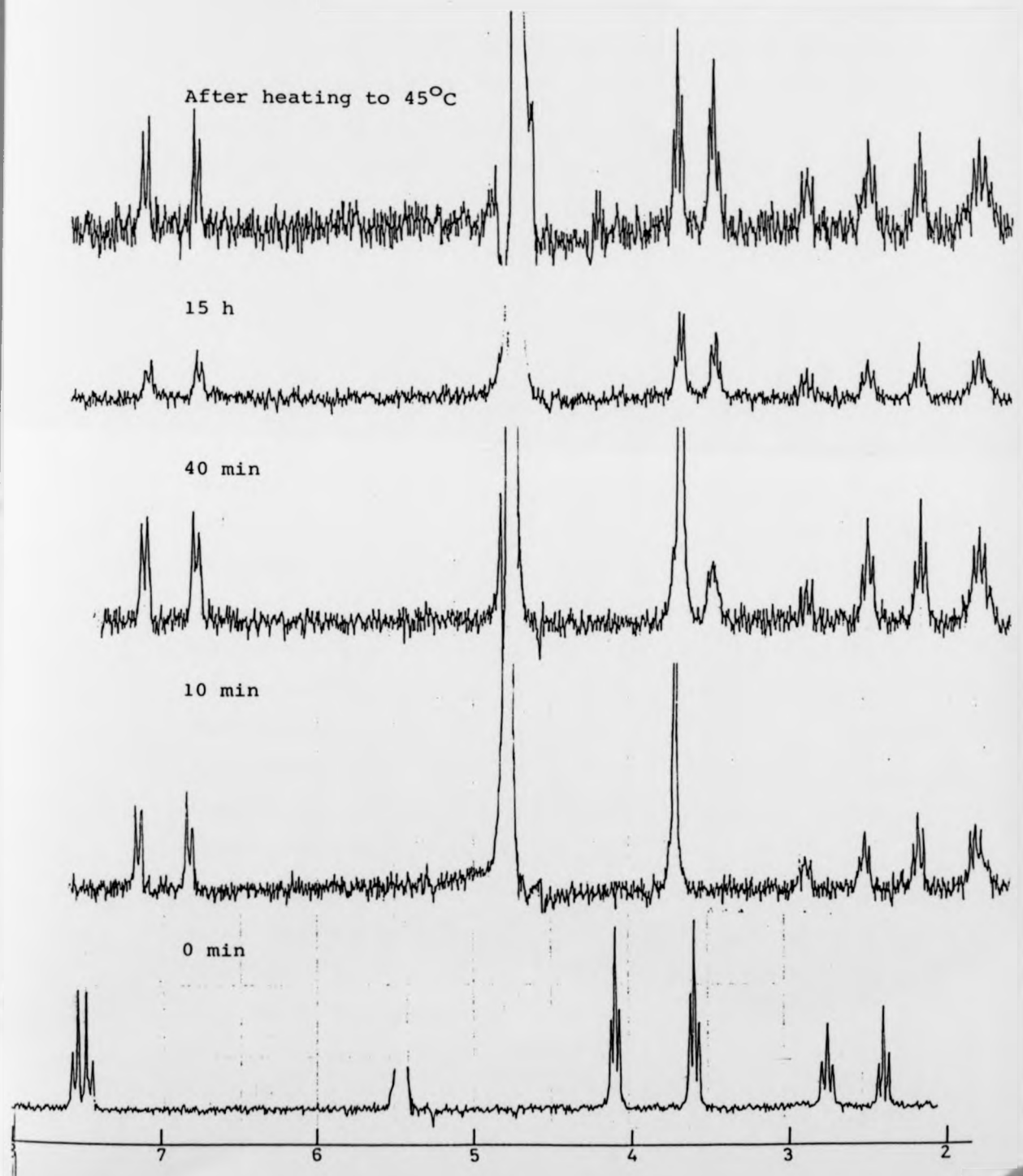


Fig. 6.3 Reaction between chlorambucil hydrochloride and $Na_2CO_3$ monitored by 220 MHz $^1H$ n.m.r. spectroscopy.



A comparison of the spectrum recorded 10 minutes from addition of the base with that of the parent hydrochloride shows that the previously distinct pair of chloroethyl arm triplets have coalesced into a broad singlet and that the aromatic AB system has been affected. Both of these changes are consistent with deprotonation of the amine function; no changes are anticipated or observed for the acid side chain. Analysis of the AB system shows that for (103).HCl in 2 M DCl $H_A$ is at δ 7.514 and $H_B$ resonates at δ 7.597, whereas in the free base $H_A$ is found at δ 6.83 and $H_B$ at δ 7.17. This increased shielding is consistent with the greater electron density on the nitrogen atom of the free base. The spectrum recorded 40 minutes from neutralisation is unchanged compared to that at 10 minutes save for a weak, broad peak at δ 3.49. Four hours from mixing this peak has grown to 20% of the size of the combined chloroethyl resonance at the expense of that signal. The growing resonance is still a broad singlet with no resolvable structure. Although the spectral resolution as judged by the internal standard is still good, there is a significant loss of resolution of the aromatic AB system which is otherwise unchanged. After a further 14 hours, the broad singlet at δ 3.49 is no longer detectable and the signal for the ethyl arms has split into two unequal triplets. The final spectrum was recorded after heating for 2 hours at 45 $^{o}$C and is now well resolved throughout. This spectrum shows the ethyl arms as two equal triplets but shows no other changes from the previous spectrum. Using the spectrum

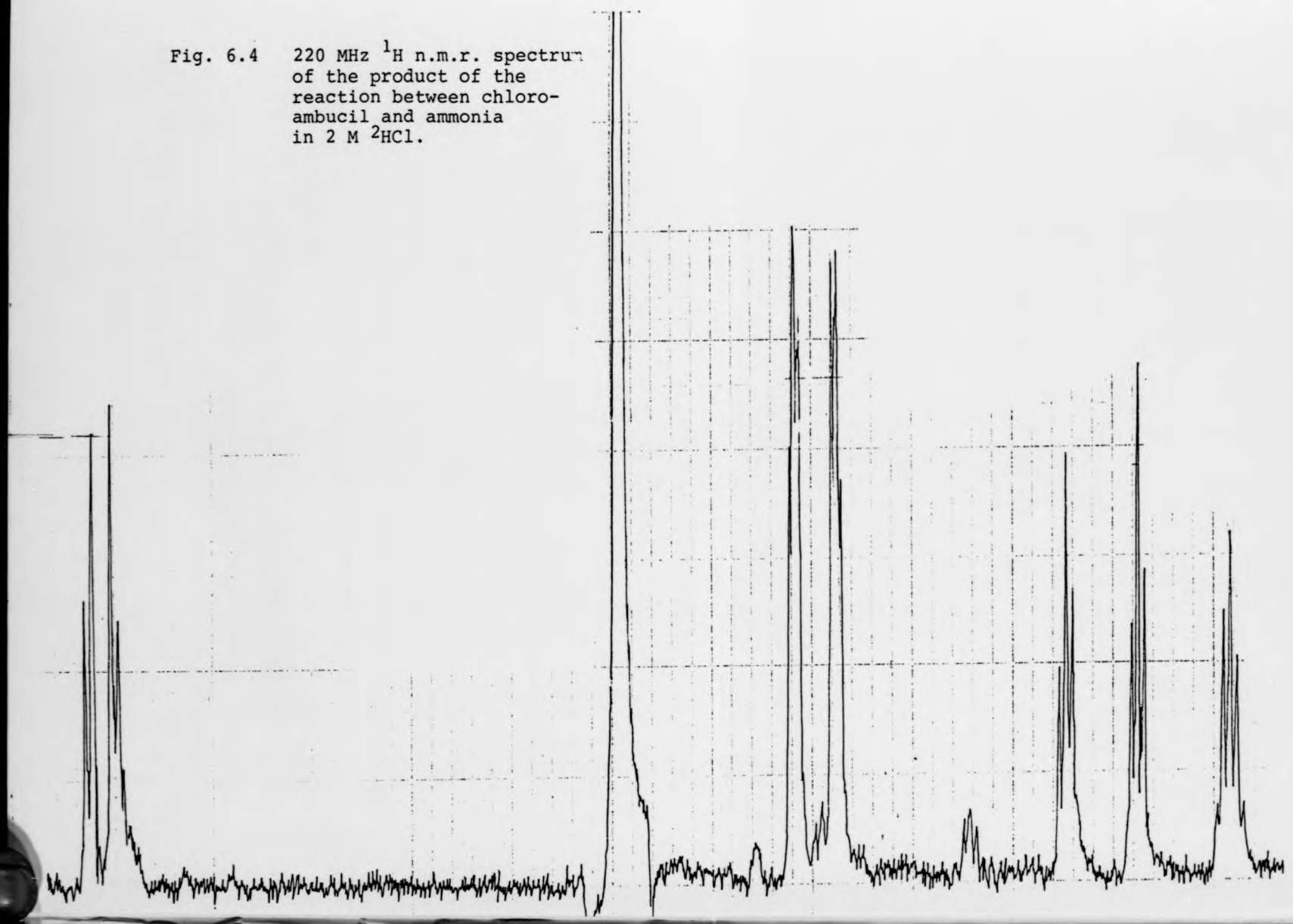
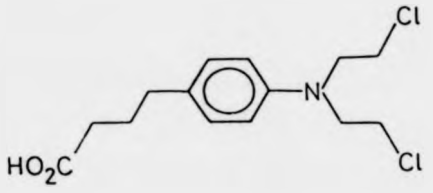


Fig. 6.4 220 MHz $^{1}H$ n.m.r. spectrum of the product of the reaction between chloroambucil and ammonia in 2 M $^{2}HCl$.

recorded 10 minutes from addition of $Na_2CO_3$ as a reference spectrum of the free base at this pH, the reaction product differs primarily in the chemical shift of the ethyl resonances although the aromatic resonances are shifted very slightly upfield.




(103)

This data can be readily interpreted in terms of the reactions of (1) under similar conditions. The spectra must represent conversion of (103) to its aziridinium ions and capture of these by hydroxide to give a diol as the final product. The transient broad singlet at δ 3.49 would then represent the AA'BB' spin system of the intermediate aziridinium ions, but is not resolvable at this field strength (220 MHz). Towards the end of the reaction the sample became cloudy and deposited a white solid, which may be a piperazinium dimer of chlorambucil.

When chlorambucil is allowed to react for 2 days with excess ammonia and the mixture analysed by $^1H$ n.m.r. spectroscopy as a solution in 2 M $^2HCl$, a single product dominates the spectrum (Fig. 6.4).

The aromatic AB system is characterised by

Fig.6.5 Reaction between benzyl mustard (57) and pH 7.2 citrate-phosphate buffer monitored by 220 MHz $^{1}H$ n.m.r. spectroscopy.

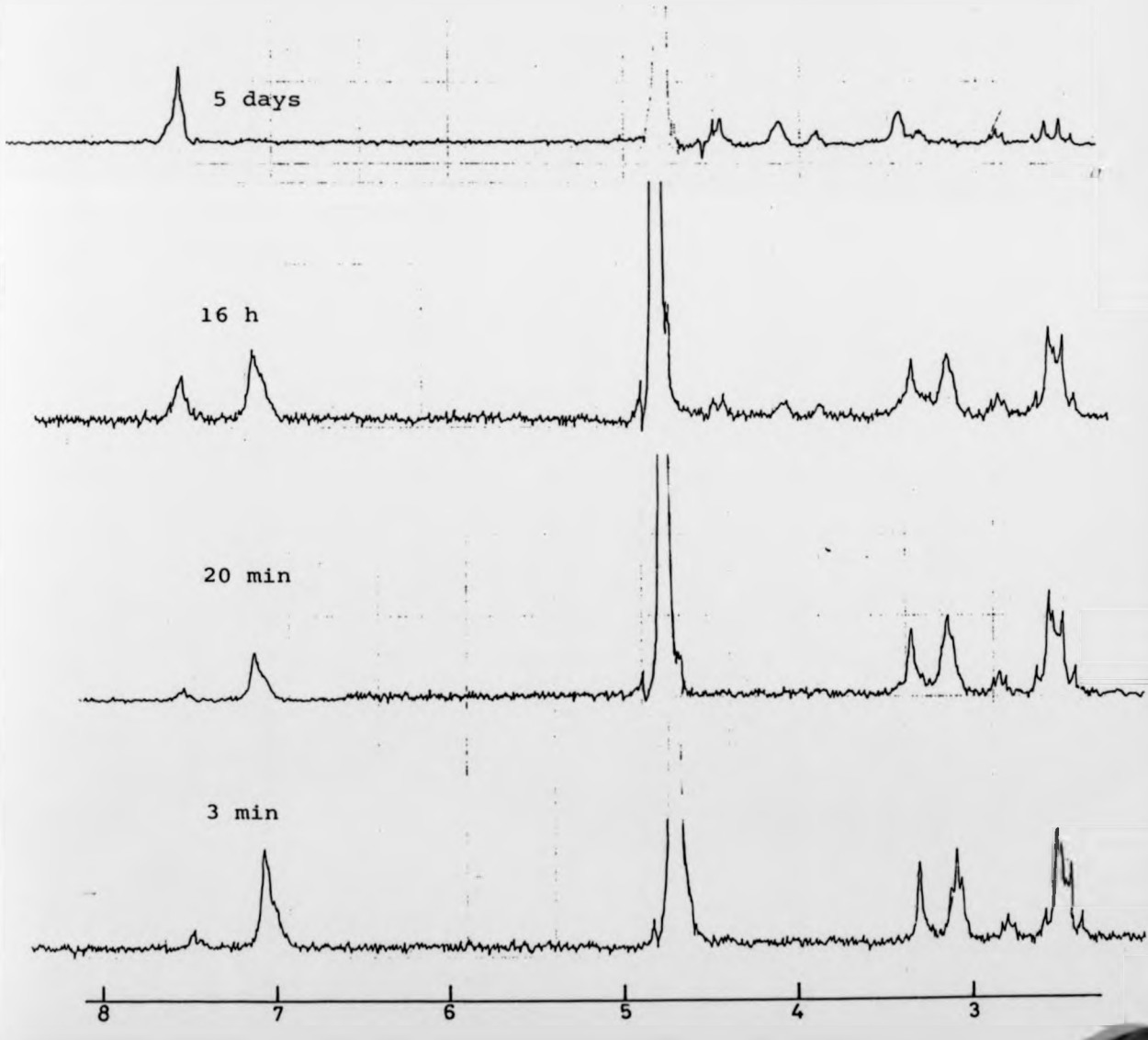
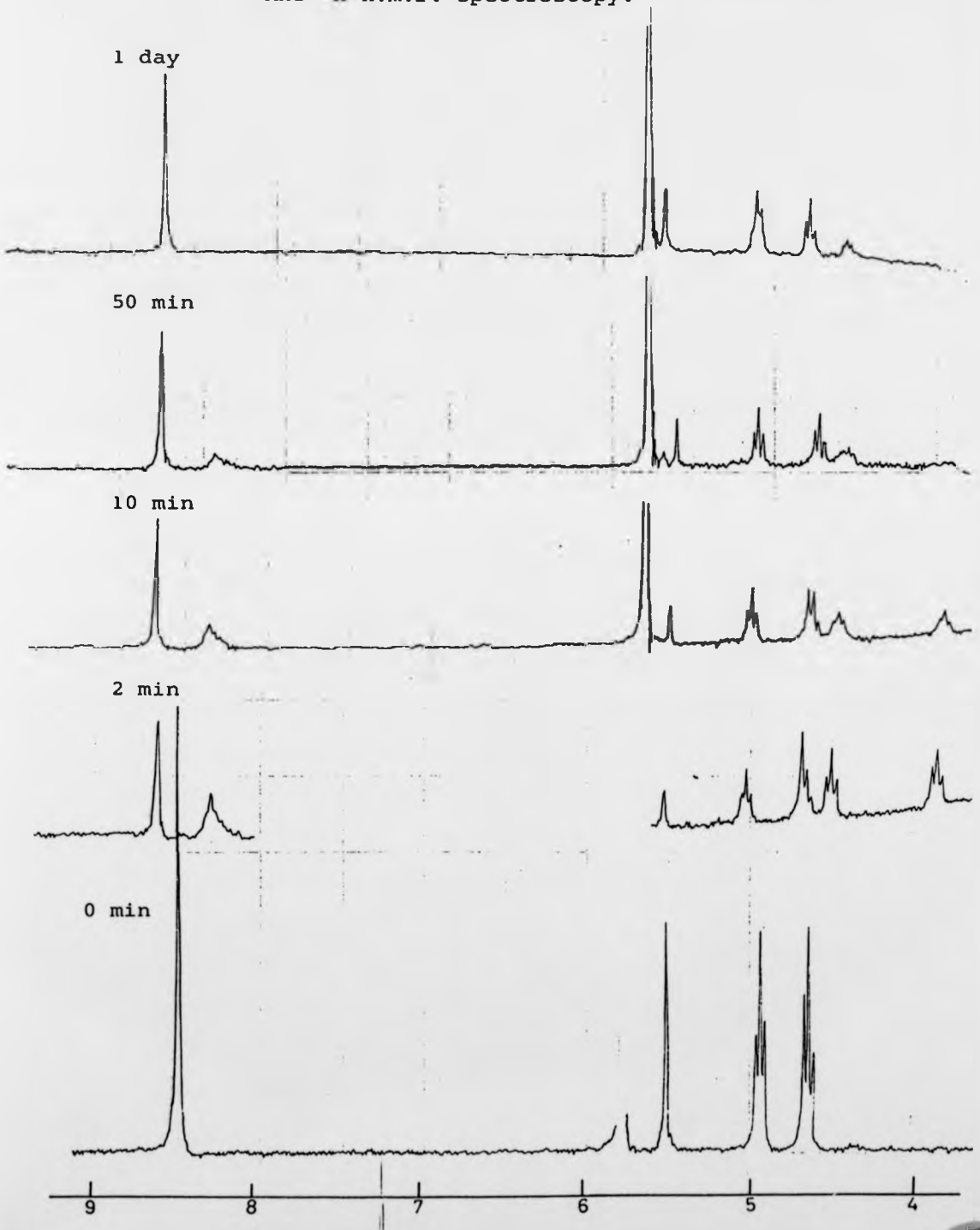
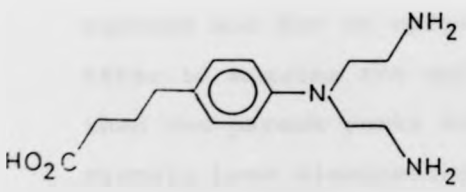


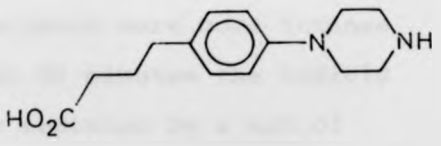
Fig. 6.6 Effect of neutralising benzyl mustard (57) in 1:1 $^{2}H_2O$ acetonitrile monitored by 220 MHz $^{1}H$ n.m.r. spectroscopy.



chemical shifts of δ 7.62 and 7.49, a coupling constant $J_{AB}$ of 9.78 Hz and a chemical shift difference of 0.13 p.p.m., compared to chemical shifts of δ 7.597 and 7.514 and a coupling constant, $J_{AB}$, of 7.33 Hz for unreacted chlorambucil. The ethyl signals are, grossly, a pair of perturbed triplets similar to those observed for the triamine product (95) under the same conditions. This behaviour indicates that the product has a higher pKa than the parent. Minor signals are also observable together with a low intensity broadening of the aromatic signals. From this spectrum it is not possible to distinguish between the two predicted products (104) and (105) but the low basicity of chlorambucil compared to nitrogen mustard should make aziridinium ion formation slower, therefore the piperazine product, which only passes through one aziridinium intermediate, is more plausible than the amino product. Future work to confirm this assignment would be valuable in highlighting a difference in reactivity between chlorambucil and (1). This aim, may be achieved by employing $^{15}NH_3$ as nucleophile, separating the products on an ion exchange resin and examining the $^{15}N$ n.m.r. spectrum.




(104)

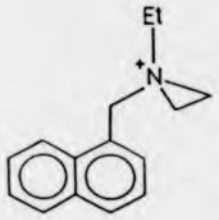



(105)

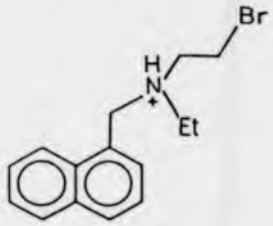
Attempting similar work with the benzyl mustard (57), whose synthesis is described in Chapter 3, is less rewarding due to the lower solubility of this material. Observing the hydrolysis of (57) in pH 7.2 citrate-phosphate buffer clearly showed that some reaction took place, but part of the sample precipitated immediately giving rise to very broad, poorly resolved spectra (Fig. 6.5). Early spectra, up to one hour from the start, showed no difference in the aliphatic region but a new aromatic signal is evident. After 16 hours this new signal is of similar intensity to the original and a poorly resolved doublet at δ 4.5 plus two broad singlets at δ 4.05 and 3.95 are evident, but at low intensity. There is only one aromatic signal visible after 5 days and the signals at δ 4.5, 4.05 and 3.95 are more clear suggesting that the reaction is complete. No transient signals attributable to aziridinium ions were detected. To avoid the solubility problem, the benzyl mustard was neutralised with $Na_2CO_3$ in a 1:1 mixture of $^2H_2O$ and acetonitrile. Within two minutes of neutralising the mustard the spectrum showed a reasonably resolved set of signals for the parent mustard and for an upfield shifted analogue (Fig. 6.6). After 10 minutes the upfield signals were more intense than the parent peaks but after 50 minutes the upfield signals have disappeared to be replaced by a set of resonances very little shifted from those of the parent, but including a small additional benzyl signal. A day from mixing this is the only benzyl signal

observable and there is some loss of resolution of the upfield triplet. At this stage the sample was clear, but it was not homogenous throughout the experiment, although no precipitate was deposited. At no stage could any resonances be assigned to an aziridinium species. The transient species observed may be a partially hydrolysed mustard.

However, in the course of an undergraduate project based on this work, Mary O'Sullivan succeeded in characterising the aziridinium ion (106) derived from the one-armed naphthyl mustard (107) prepared by the method of Chapman *et al.*[118]. The mustard was treated with one equivalent of NaOH in $^{2}H_{2}O/^{2}H_{6}$-acetone (28:72) to generate a long-lived aziridinium ion, which was characterised by $^{1}H$ n.m.r. spectroscopy at 400 MHz. A computer simulation of the AA'BB' portion of the spectrum gave the chemical shifts $H_A$ $\delta$ 3.16, $H_B$ $\delta$ 2.99 and $J_{gem}$ -3.661 Hz, $J_{cis}$ 8.857 Hz and $J_{trans}$ 7.534 Hz, directly comparable to the parameters determined for N-2-chloroethyl-N-methyl aziridinium chloride (Chapter 4). The extended lifetime of (106) is characteristic of monofunctional mustards in accord with the results of Price *et al.*[67]. The crystallisation of aziridinium ion (106) is reported to be possible and would be of interest for an x-ray diffraction study of (106). Such work has to date involved only large ring compounds attached to an aziridine ring[119].




(106)




(107)

Attempts to measure the $^{13}C$ n.m.r. spectrum of aziridinium ion (8) have not led to clear-cut results, even when (1a) labelled at 10.8 atom % $^{13}C$ was employed. A sample of (1a) HCl was taken up in 0.1 M Sorensen's buffer at pH 7.2 and when $^{1}H$ n.m.r. spectroscopy indicates *ca.* 50% conversion to (8) the sample was examined by $^{13}C$ n.m.r. spectroscopy. After 400 seconds accumulation the $^{13}C$ n.m.r. spectrum (Fig. 6.7a) showed two intense, sharp peaks, δ 42.12 and 39.0, and weaker peaks at δ 39.47, 38.20 and 35.94. Another spectrum was recorded after a further 15 minutes of accumulation. This showed (Fig. 6.7b) that the peaks at δ 42.12 and 39.0 were still of approximately equal intensity, the signals at δ 38.2 and 35.94 (doublet) were relatively more intense, but that at 39.47 was weaker and split. A weak new peak was evident at 38.35. A $^{1}H$ n.m.r. spectrum recorded immediately afterwards showed a high concentration of (8), only a trace of (1) and significant amounts of both piperazinium dimer (10) and phosphate ester (64). The sample was returned to the $^{13}C$ probe and a third spectrum recorded (Fig. 6.7c). This showed three equal resonances

Fig. 6.7a $^{13}C\ \{^{1}H\}$ n.m.r. spectrum of neutralised nitrogen mustard containing *ca.* 50% N-2-chloroethyl-N-methylaziridinium chloride.

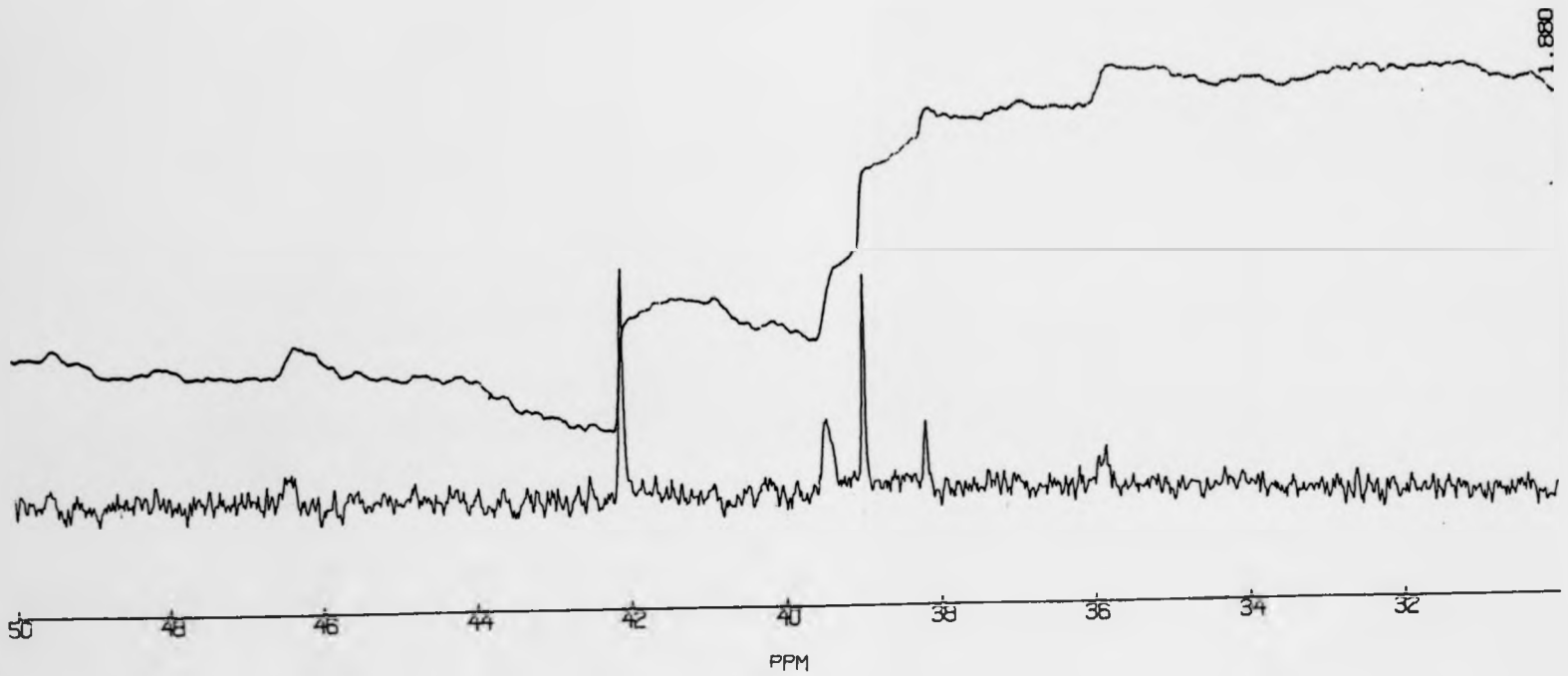


Fig. 6.7b $^{13}C\ \{^{1}H\}$ n.m.r. spectrum of mixture containing N-2-chloroethyl-N-methylaziridinium chloride *ca.* 15 min. from recording 6.7a.

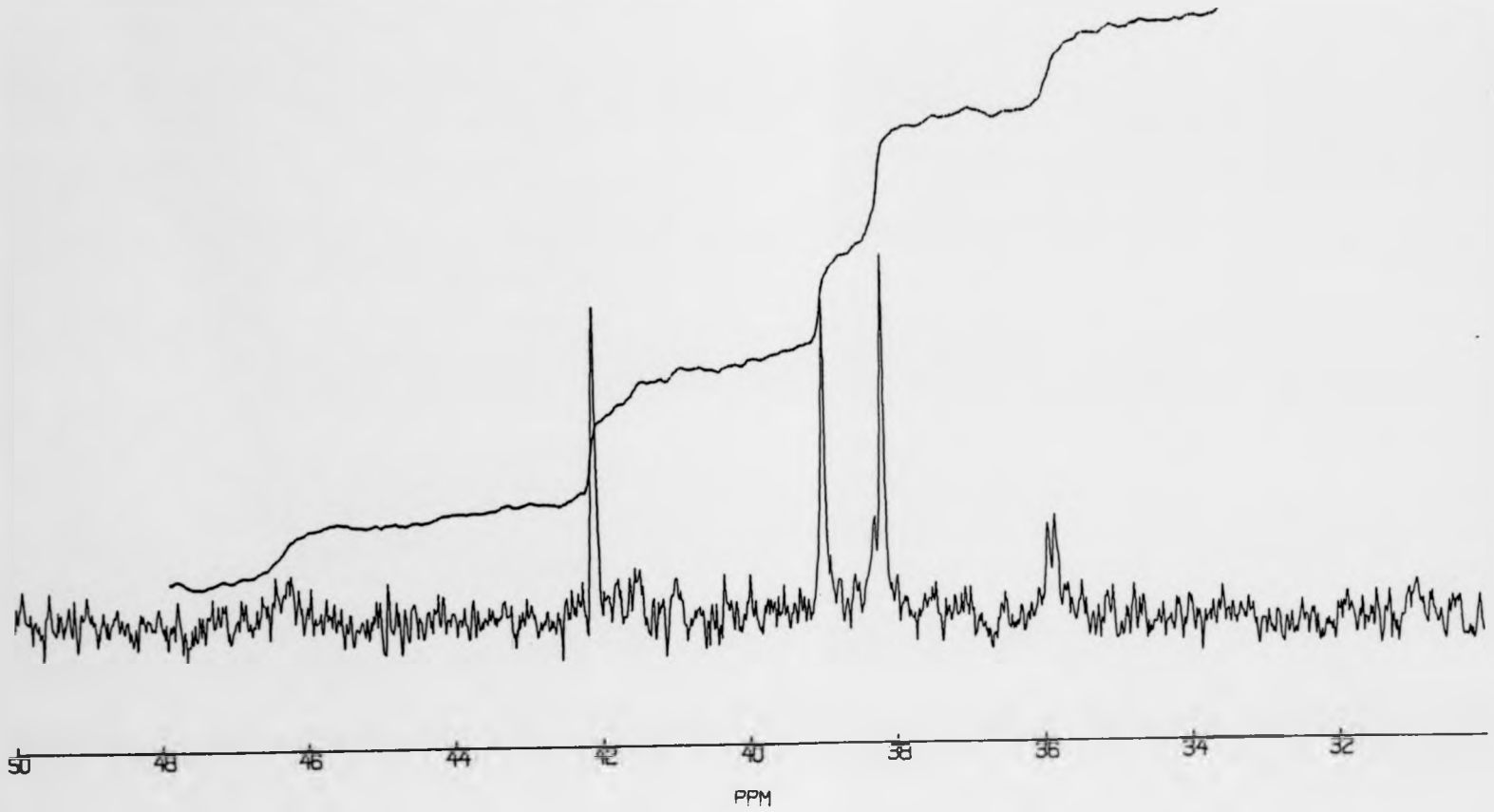
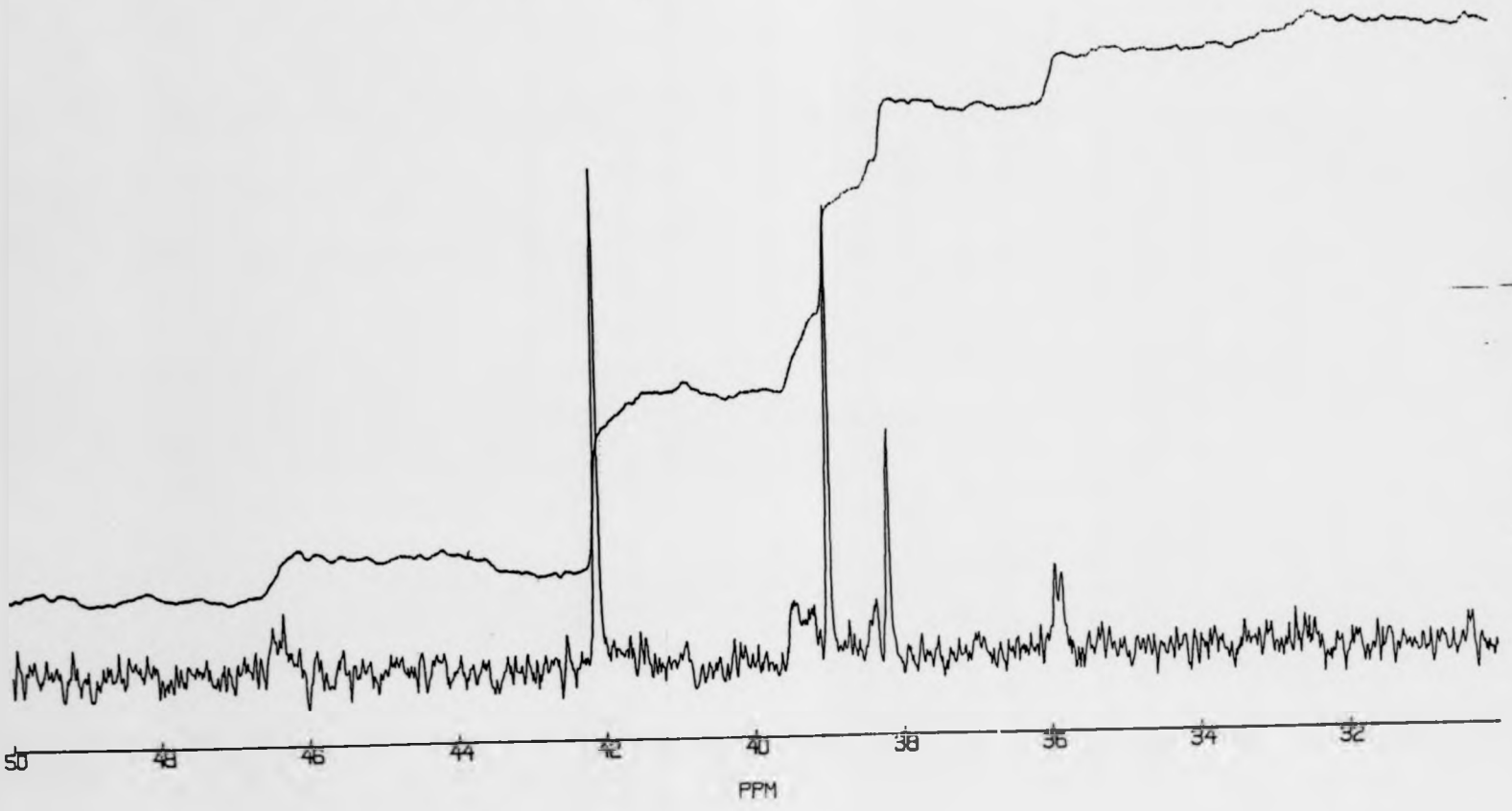


Fig. 6.7c $^{13}C\ \{^{1}H\}$ n.m.r. spectrum of mixture containing N-2-chloroethyl-N-methylaziridinium chloride *ca.* 45 min. from recording 6.7a.



at δ 42.12, 39.0 and 38.20 and a much stronger doublet at δ 35.94. The $^{1}H$ n.m.r. spectrum recorded after this spectrum showed an approximately 1:1:1 mixture of aziridinium ion (8), piperazinium dimer (10) and phosphate ester (64). The perseverence of enriched signals at δ 42.12 and 39.0 throughout the experiment suggest that these are associated with the aziridinium ring methylenes and unchanged chloroethyl arm, respectively. The loss of the signal at δ 39.47 between the second and third $^{13}C$ n.m.r. spectra indicates that this is the methylene adjacent to chlorine in the parent mustard. Similarly the growth of signals at δ 38.2 and 35.94 suggest that they are associated with the phosphate ester product, an interpretation supported by the doublet nature of the latter resonance (J ~ 15 Hz), presumably due to carbon-phosphorous coupling through the ester link.

A logical extension of this work would be a thorough examination of the chemistry of phosphoramide mustard by $^{1}H$ and $^{13}C$ n.m.r. spectroscopy.

## REFERENCES


1. V. Prelog, V. Stepan, *Collection Czechoslov. Chem. Commun.*, (1935) 7, 93
2. A. Gilman, F. S. Philips, *Science*, (1946) 103, 409
3. F. E. Adair, H. J. Bagg, *Ann. Surg.*, (1931) 93, 190
4. C. Golumbic, J. S. Frutton, M. Bergmann, *J. Org. Chem.*, (1946) 11, 518 *et seq.*
5. W. C. J. Ross in 'Biological Alkylating Agents' published by Butterworth, London, 1962
6. T. A. Connors, *Febs. Letts.*, (1975) 57, 223
7. W. B. Pratt and R. W. Ruddon, The Anticancer Drugs, Oxford University Press, New York, 1979
8. T. A. Connors, Ch. 32 of Antineoplastic and Immunosuppresive Agents, Ed. A. C. Sartorelli and D. G. Johns, Springer-Verlag, New York, 1975
9. D. B. Ludlum, *Biochim. Biophys. Acta*, (1965) 95, 674
10. R. J. Goldacre, A. Loveless, W. C. J. Ross, *Nature*, (1949) 163, 667
11. O. M. Friedman, A. M. Seligman, *J. Am. Chem. Soc.*, (1954) 76, 655
12. W. C. J. Ross, *J. Chem. Soc.*, (1949) 183
13. A. Streitivieser, *Chem. Revs.*, (1956) 56, 677
14. W. E. Hanby, G. S. Hartley, E. O. Powell, H. N. Rydon, *J. Chem. Soc.*, (1947) 519

15. P. D. Bartlett, S. D. Ross, C. G. Swain, *J. Am. Chem. Soc.*, (1947) 69, 2971

16. P. D. Bartlett, J. W. David, S. D. Ross, C. G. Swain, *J. Am. Chem. Soc.*, (1947) 69, 2977

17. B. C. Cohen, E. R. van Artsdalen, J. Harris, *J. Am. Chem. Soc.*, (1948) 70, 281

18. B. C. Cohen, E. R. van Artsdalen, J. Harris, *J. Am. Chem. Soc.*, (1952) 74, 1875

19. Rodd's Chemistry of Carbon Compounds, Volume 1, Part B, p.132, Ed. S. Coffey, published by Elsevier Publishing Co., London, 1965

20. S. Gurin, A. M. Delluva, D. I. Crandell, *J. Org. Chem.*, (1947) 12, 606

21. V. B. Schatz, L. B. Clapp, *J. Am. Chem. Soc.*, (1955) 77, 5113

22. G. R. Pettit, J. A. Settepani, R. A. Hill, *Can. J. Chem.*, (1965) 43, 1792

23. S. J. Brois, *J. Org. Chem.*, (19 ) 27, 3532

24. P. L. Levins, Z. B. Papanastassiou, *J. Am. Chem. Soc.*, (1965) 87, 826

25. V. G. Nemetz, G. G. Tzybaeva, *Tr. Leningr. Tekhnol. Inst. im. Lensovetta*, (1960) 60, 56; *Chem. Abs.*, (1962) 56, 7111b

26. J. R. Sowa, C. C. Price, *J. Org. Chem.*, (1969) 34, 474

27. J. A. Stock, Chemistry in Britain, (1970) 6, 11

28. M. H. Benn, P. Kazmaier, C. Watanatada, L. N. Owen, *J. Chem. Soc., Chem. Commun.*, (1970) 1685

29. A. Crist, N. S. Leonard, *Angew Chem. Int. Ed.*, (1962) 8, 962

30. M. Colvin, R. B. Brundrett, M. Khan, I. Jardine, C. Fenselau, *Cancer Res.*, (1976) 36, 1121

31. J. L. Everett, W. C. J. Ross, *J.Chem. Soc.*, (1949) 1972

32. T. J. Bardos, N. Datta-Gupta, P. Hebborn, D. J. Triggle, *J. Med. Chem.*, (1965) 8, 167

33. T. W. Engle, G. Zon, W. Egan, *J. Med. Chem.*, (1979) 22, 897

34. T. W. Engle, G. Zon, W. Egan, *J. Med. Chem.*, (1982) 25, 1347

35. V. T. Vu, C. C. Fenselau, O. M. Colvin, *J. Am. Chem. Soc.*, (1981) 103, 7362

36. P. Brookes, P. D. Lawley, *Biochem. J.*, (1961) 80, 496

37. L. Tenud, S. Farooq, J. Seibl, A. Eschenmoser, *Helv. Chim. Acta*, 1970, 53, 2059

38. J. E. Baldwin, *J. Chem. Soc., Chem. Commun.*, (1976) 734

39. G. Klose, K. Augusten, A. Barth, Z. Samek, *Org. Mag. Res.*, (1982) 19, 15

40. D. B. Ludlum, Ch. 31 of Antineoplastic and Immuno suppressive Agents, Ed. A. C. Sartorelli, published by Springer-Verlag, New York, 1975

41. E. G. Young, R. B. Campbell, *Can. J. Res.*, (1947) 25B, 37

42. B. Reiner, S. Zamenhof, *J. Biol. Chem.*, (1957) 228, 475

43. D. B. Ludlum, *Biophys. et Biochem. Acta*, (1965) 95, 674

44. P. D. Lawley, P. Brookes, *Biochem. J.*, (1963) 89, 127

45. P. D. Lawley, P. Brookes, *Nature*, (1961) 192, 1081

46. J. W. Jones, R. K. Robins, *J. Am. Chem. Soc.*, (19 ) 85, 193

47. J. W. Jones, R. K. Robins, *J. Am. Chem. Soc.*, (19 ) 85, 242

48. P. D. Lawley, *Proc. Chem. Soc.*, (1957) 290

49. I. J. Mizrahi, P. Emmelot, *Biophys. et Biochem. Acta*, (1964) 91, 362

50. J. A. Butler, L. Gilbert, P. W. James, *J. Chem. Soc.*, (1952) 3268

51. J. A. Butler, L. Gilbert, P. W. James, *Biochem. J.*, (1964) 92, 19

52. K. W. Kohn, C. L. Spears, *Biophys. et Biochem. Acta*, (1967) 145, 734

53. P. D. Lawley, W. Warren, *Chem. Biol. Interact.*, (1976) 12, 211

54. D. B. Ludlum, *J. Biol. Chem.*, (1970) 245, 477

55. C. J. Chetsango, M. Lozon, *Biochemistry*, (1981) 20, 5201

56. T. P. Brent, *Biochemistry*, (1979) 18, 911

57. T. Lindahl, S. Lungquist, W. Siegert, B. Nyberg, B. Sperens, *J. Biol. Chem.*, (19 ) 252, 3286

58. P. D. Lawley, S. A. Shah, *Biochem. J.*, (1972) 128, 117

59. J. A. Haines, C. B. Reese, A. R. Todd, *J. Chem. Soc.*, (1962) 5281

60. G. Hems, *Nature*, (1958) 187, 1721

61. C. J. Chetsanga, B. Bearie, C. Markaroff, *Chem. Biol. Interact.*, (1982) 41, 217

62. P. D. Lawley, J. H. Lethbridge, P. A. Edwards, K. V. Shooter, *J. Molec. Biol.*, (1969) 39, 181

63. A. Loveless, J. C. Stock, *Proc. Roy. Soc. B*, (1959) 150, 423

64. A. Ronen, *Biochim. Biophys. Res. Commun.*, (1968) 33, 190

65. W. G. Verly, L. Brakier, *Biochim. Biophys. Acta*, (1969) 174, 674

66. L. Brakier, W. G. Verly, *Biochim. Biophys. Acta*, (1970) 213, 296

67. C. C. Price, G. M. Gaucher, P. Koneru, R. Shibakawa, J. R. Sowa, M. Yamaguchi, *Biochim. Biophys. Acta*, (1968) 166, 327

68. J. T. Lett, G. M. Parkins, P. Alexander, *Arch. Biochem. Biophys. Acta*, (1962) 97, 180

69. B. Strauss, T. Hill, *Biochim. Biophys. Acta*, (1970) 213, 14

70. E. Dennis, F. Westheimer, *J. Am. Chem. Soc.*, (1966) 88, 3432

71. R. J. Rutman, E. H. L. Chun, J. Jones, *Biochim. Biophys. Acta*, (1969) 174, 663

72. C. C. Price, M. L. Yip, *J. Biol. Chem.*, (1974) 249, 6849

73. R. Fussganger, V. Dold, H. Holzer, *Z. Hebforsch.*, (1967) 69, 275

74. G. P. Wheeler, *Cancer Res.*, (1962) 22, 651

75. E. Liss, G. Palme, *Z. Krebforsch.*, (1964) 66, 196

76. E. F. Roth, R. L. Nagel, R. M. Bookchin, A. L. Grayzel, *Biochim. Biophys. Res. Commun.*, (1972), 48, 612

77. L. W. Fung, Chein Ho, E. F. Roth, R. L. Nagel, *J. Biol. Chem.*, (1975) 250, 4786

78. W. J. Goux, A. Allerhand, *J. Biol. Chem.*, (1979) 254, 2210

79. H. Yamada, T. Imoto, S. Noshita, *Biochemistry*, (1982) 21, 2187

80. See e.g. R. J. Rutman, W. J. Steele, C. C. Price, *Cancer Res.*, (1961) 21, 1134 and R. A. G. Ewig, K. W. Kohn, *Cancer Res.*, (1977) 37, 2114

81. C. B. Thomas, K. W. Kohn, W. M. Bonner, *Biochemistry*, (1978) 17, 3954

82. G. R. Pettit, E. I. Saldana, *J. Med. Chem.*, (1974) 17, 896

83. J. W. Lown, C. C. Hanstock, *J. Am. Chem. Soc.*, (1982) 104, 3213

84. G. H. Denny, M. A. De Marco, R. D. Babson, *J. Med. Chem.*, (1976) 19, 426

85. A. B. Foster, M. Jarman, R. W. Kinas, J. van Maanen, G. N. Taylor, *J. Med. Chem.*, (1981) 24, 1398

86. A. Leo, A. Panthananickal, C. Hansch, *J. Med. Chem.*, (1978) 21, 16

87. A. Leo, A. Panthananickal, C. Hansch, J. Theiss, M. Shimkin, A. Andrews, *J. Med. Chem.*, (1981) 24, 859

74. G. P. Wheeler, *Cancer Res.*, (1962) 22, 651

75. E. Liss, G. Palme, *Z. Krebforsch.*, (1964) 66, 196

76. E. F. Roth, R. L. Nagel, R. M. Bookchin, A. L. Grayzel, *Biochim. Biophys. Res. Commun.*, (1972), 48, 612

77. L. W. Fung, Chein Ho, E. F. Roth, R. L. Nagel, *J. Biol. Chem.*, (1975) 250, 4786

78. W. J. Goux, A. Allerhand, *J. Biol. Chem.*, (1979) 254, 2210

79. H. Yamada, T. Imoto, S. Noshita, *Biochemistry*, (1982) 21, 2187

80. See e.g. R. J. Rutman, W. J. Steele, C. C. Price, *Cancer Res.*, (1961) 21, 1134 and R. A. G. Ewig, K. W. Kohn, *Cancer Res.*, (1977) 37, 2114

81. C. B. Thomas, K. W. Kohn, W. M. Bonner, *Biochemistry*, (1978) 17, 3954

82. G. R. Pettit, E. I. Saldana, *J. Med. Chem.*, (1974) 17, 896

83. J. W. Lown, C. C. Hanstock, *J. Am. Chem. Soc.*, (1982) 104, 3213

84. G. H. Denny, M. A. De Marco, R. D. Babson, *J. Med. Chem.*, (1976) 19, 426

85. A. B. Foster, M. Jarman, R. W. Kinas, J. van Maanen, G. N. Taylor, *J. Med. Chem.*, (1981) 24, 1398

86. A. Leo, A. Panthananickal, C. Hansch, *J. Med. Chem.*, (1978) 21, 16

87. A. Leo, A. Panthananickal, C. Hansch, J. Theiss, M. Shimkin, A. Andrews, *J. Med. Chem.*, (1981) 24, 859

88. D. M. Jerina, J. W. Daley, *Science*, (1974) 185, 573

89. D. E. Cane, T.-C. Liang, H. Hasler, *J. Am. Chem. Soc.*, (1982) 104, 7274

90. N. Boden, R. J. Bushby, L. D. Clark, *J. Chem. Soc., Perkin Trans. I*, (1983) 543

91. A. H. Blatt (ed.) Organic Syntheses, Collective Vol. 2, 397, John Wiley, London, 1959

92. D. C. Billington, B. T. Golding, *J. Chem. Soc., Perkin Trans. II*, (1982) 341

93. H. S. Mosher, J. Cornell, O. L. Stafford, T. Roe, *J. Am. Chem. Soc.*, (1953) 75, 4949

94. K. Harada, T. Yoshida, *Bull. Chem. Soc. Jpn.*, (1970) 43, 921

95. E. J. Corey, R. J. McCaully, H. S. Sachdev, *J. Am. Chem. Soc.*, (1970) 92, 2476

96. U. E. Diner, M. Worsley, J. R. Lown, J. Forsythe, *Tetrahedron Letters*, (1972) 3145

97. T. P. Dang, H. B. Kagan, *J. Chem. Soc., Chem. Commun.*, (1971) 487

98. R. Graham, W. M. STanley, *Analyt. Biochem.*, (9172) 47, 310

99. B. D. Andersan, *J. Labelled Compds.*, (1978) 14, 580

100. D. Dolphin, K. Endo, *Analyt. Biochem.*, (1970) 36, 338

101. H. Gunther in NMR Spectroscopy, John Wiley, Chichester, 1980

102. H. Gunther, *Angew Chem. Int. Ed. Engl.*, (1972) 11, 861

103. Ching-jer Chang, Chi-Gen Lee, *Biochemistry*, (1981) 20, 2657

104. R. W. Alder, R. Baker, J. M. Brown, Mechanism in Organic Chemistry, John Wiley, Chichester, 1978

105. R. Marshall, S. M. Birnbawm, J. P. Greenstein, *J. Am. Chem. Soc.*, (1956) 78, 4636

106. Ching-jer Chang, Gomes, Byrn, *J. Am. Chem. Soc.*, (1981) 103, 2892

107. R. J. Kaldeney, A. Karlin, *J. Biol. Chem.*, (1983) 258, 6232

108. I. Ugi, D. Lenoir, R. El Gormati, *Angew Chem. Internat. Ed.*, (1975) 14, 59

109. C. A. Manyanoff, F. Ogura, K. Mislow, *Tetrahedron Lett.*, (1975) 4095

110. H. Yamaguchi, K. Kawada, T. Okanoto, E. Egert, H. J. Linder, M. Braun, R. Damman, M. Liesner, H. Neumann, D. Seebach, *Chem. Ber.*, (1976) 109, 1589

111. R. W. Gray, C. B. Chapeto, T. Veryani, A. Dreiding, M. Liesner, D. Seebach, *Helv. Chimica Acta*, (1976) 59, 1547

112. J. F. King, M. J. McGarrity, *J. Chem. Soc., Chem. Commun.*, (1979) 1140

113. A. C. Fry in Ch. 6 of Isotope Effects in Chemical Reactions, Eds. C. J. Collins, N. S. Bowman, A. C. S. Monograph No. 167, published by Van Nostrand, London, 1970

114. A. J. Kesge, N. N. Lichtin, K. N. Rao, *J. Am. Chem. Soc.*, (1963) 85, 1210

115. W. A. Schroeder, J. R. Shelton, B. Robberson, *Biochim. Biophys. Acta*, (1967) 147, 590

116. H. G. Gundlach, W. H. Stein, S. Moore, *J. Biol. Chem.*, (1959) 234, 1754

117. H. G. Gundlach, W. H. Stein, S. Moore, *J. Biol. Chem.*, (1959) 234, 1761

118. N. B. Chapman, J. W. James, *J. Chem. Soc.*, (1953) 1865

119. L. M. Trefonas, R. Towns, R. Majeste, *J. Heterocyclic Chem.*, (1967) 4, 511