Chemistry of Nitrogen Mustard [2-Chloro-*N*-(2-chloroethyl)-*N*-methylethanamine] studied by Nuclear Magnetic Resonance Spectroscopy

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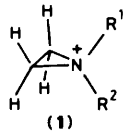
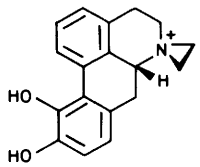
Reactions of the nitrogen mustard drug 2-chloro-*N*-(2-chloroethyl)-*N*-methylethanamine with nucleophiles in aqueous solution have been studied by ^{1}H and ^{13}C n.m.r. spectroscopy. Conditions have been devised for converting the mustard into the *N*-2-chloroethyl-*N*-methylaziridinium ion which has been characterised by ^{1}H n.m.r. spectroscopy. To assist the studies of reactions of the mustard by ^{13}C n.m.r. spectroscopy, it has been prepared labelled at both C-2 atoms by ^{13}C. It is shown that reactions of the mustard with strong nucleophiles (*e.g.* thiosulphate) proceed to a product of disubstitution, without the aziridinium ion being detected spectroscopically, although its intermediacy is inferred by examining the distribution of ^{13}C in product from ^{13}C-labelled mustard. Less reactive nucleophiles (*e.g.* thiourea) yield a product of disubstitution *via* spectroscopically detected intermediates (aziridinium ion and monosubstituted intermediate). Relatively weak nucleophiles (*e.g.* guanosine) did not give detectable products of substitution; *cis*- and *trans*-*NN'*-2-chloroethyl-*NN'*-methylpiperazinium dichloride were formed *via* the aziridinium ion. The reaction of the mustard with excess of ammonia gives a 3:2 ratio of 2-amino-*N*-(2-aminoethyl)-*N*-methylethanamine and *N*-methylpiperazine. The distribution of ^{13}C label in these products derived from ^{13}C-labelled drug shows that the triamine is formed *via* aziridinium intermediates, whilst the piperazine arises *via* intramolecular cyclisation of the intermediate 2-amino-*N*-(2-chloroethyl)-*N*-methylethanamine.

Several aziridinium ions of the ethyleneimmonium type (**1**), usually generated *in situ* from 2-halogenoethylamines, are used as reagents for the alkylation of biological nucleophiles (nucleic acids, proteins).[1] Thus, the formation of aziridinium ion (**1a**) from 2-chloro-*N*-(2-chloroethyl)-*N*-methylethanamine (**2a**) is believed to be the first step in the cross-linking of double-stranded nucleic acids and consequent anti-tumour action of this drug of the nitrogen mustard class.[1d] Nitrogen mustards can also alkylate proteins, cause protein–nucleic acid cross-linking, and inhibit key cellular enzymes. The aziridinium ion (**1b**) from *N*-(2-chloroethyl)norapomorphine (**2b**) is a dopamine



(**1**)

a; R^1 = Me, R^2 = $^1CH_2{}^2CH_2Cl$
c; R^1 = Et, R^2 = CH_2CH_2OH
d; R^1 = $H_2NPO_2^-$, R^2 = CH_2CH_2Cl
e; R^1 = Et, R^2 = 
f; R^1 = Me, R^2 = $CH_2CH_2NH_2$



(**1b**)

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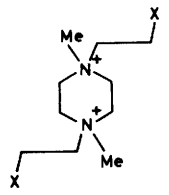
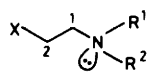


a; *cis*, X = Cl
b; *trans*, X = Cl
c; X = $NHCHMeCO_2^-$
(stereochemistry not defined)

(**3**)

(**2a**,**c**,**d**) X = Cl
R^1, R^2 as in structures (**1a**,**c**,**d**)
(**2b**) X = Cl, 6-membered rings as in (**1b**)
(**2e**) X = Br, R^1, R^2 as in (**1e**)
(**2f**) X = Cl, R^1, R^2 as in (**1a**)
^{13}C (10 atom %) at each C-2

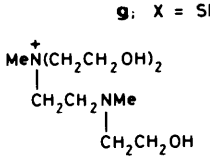


(**2g**) X = Cl, R^1 = Me, R^2 = $^{13}CH_2CH_2Cl$. ^{13}C at C-2
(**2h**) X = Cl, R^1 = Me, R^2 = $CH_2{}^{13}CH_2Cl$, ^{13}C at C-2

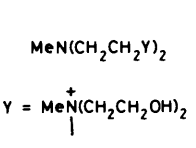
$MeN(^1CH_2{}^2CH_2X)_2$

(**4**)

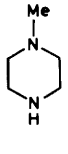
a; X = $S-\overset{+}{C}(NH_2)_2$
b; X = OH
c; X = NH_2
d; X = Cl in one arm
X = $S_2O_3^-$ in the other
e; X = $S_2O_3^-$
f; X = CN
g; X = SMe



(**5**)



(**6**)



(**7**)

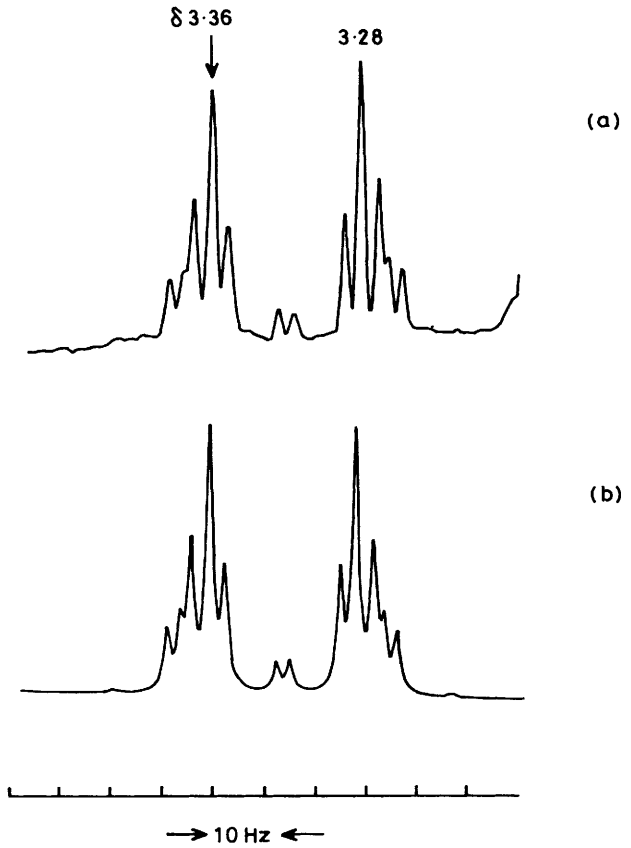


Figure 1. (a) Experimental spectrum (δ 3.2—3.4) for aziridinium ion (**1a**). (b) Computer-simulated spectrum using the parameters given in the text.

antagonist because of its ability to alkylate irreversibly components of a membrane receptor.[2] The ion (**1c**), prepared from (**2c**), is structurally related to choline. It causes a prolonged cholinergic hypofunction in mice, probably by virtue of its alkylation of nucleophilic sites on the choline carrier protein.[3] Numerous studies of 2-halogenoethylamines have shown that these substances usually react with nucleophiles *via* an intermediate aziridinium ion.[1a,4] The techniques for designating aziridinium ions have included kinetic investigations,[5] trapping experiments,[6] electrophoretic measurements,[7] and isolations of crystalline aziridinium salts from reaction mixtures.[6a,8]

Surprisingly few attempts have been made to characterise aziridinium ions in solution spectroscopically. Thus, in a ^1H n.m.r. study, the resonances from the aziridinium protons of (**1a**) were stated to be a doublet,[9] whilst those of (**1c**) were described as a singlet.[3] In another study only the resonance from the methyl group of (**1a**) was referred to.[10] We thought that the methylene protons of (**1a**) should exhibit an AA'BB' pattern,[11] characterisation of which would be of value for monitoring directly reactions of (**2a**) and other 2-halogenoethylamines in solution. Olah and Szilagyi[12] determined ^1H n.m.r. spectra for a series of protonated *N*-substituted aziridines (substituent = various alkyl, phenyl) and for the ethylmethylaziridinium ion. The AA' BB' X character of the former species and the AA' BB' of the latter were recognised, but δ and *J* parameters were not evaluated. The ability to follow reactions of drugs like (**2a**) directly by n.m.r. spectroscopy would complement analyses by other methods (*e.g.* h.p.l.c.;[2] see also ref. 4). Reactions of phosphoramide mustard (**2d**) have been examined by ^{31}P n.m.r. spectroscopy, and a particular singlet resonance that appeared and disappeared was assigned to an intermediate aziridinium ion (**1d**).[13] However, this method of identification is not generally applicable.

We have found that reactions of drug (**2a**) with nucleophiles in dilute aqueous solution at pH *ca.* 7 can be directly and conveniently studied by high-field ^1H n.m.r. spectroscopy of unlabelled drug and by $^{13}C\{^1H\}$ n.m.r. spectroscopy of ^{13}C-enriched drug. From the spectra we can deduce the structures of products and the mechanisms of their formation. Furthermore, the relative rates of reactions of drug (**1a**) with a range of model compounds for biological nucleophiles can be determined.

Results and Discussion

^1H N.m.r. Spectroscopic Analysis of Aziridinium Ion (**1a**) *and Drug* (**2a**).—The 400 MHz ^1H n.m.r. spectrum of drug (**2a**) in 2M-^2HCl–^2H_2O shows resonances at δ 3.05 (3 H, s, Me), 3.66 (2 H, ddd, 2 × NCH), 3.78 (2 H, ddd, 2 × NCH), and 4.03 (4 H, m, 2 × CH_2Cl). Irradiation at the CH_2Cl resonance caused the pattern from protons of the NCH_2 groups to collapse to an AB system exhibiting J_{AB} 13.7 Hz. When (**2a**)·HCl was dissolved in pH 7.2 citrate–phosphate–^2H_2O buffer the NCH_2 protons were observed as a sharp triplet at δ 3.29 along with resonances for CH_2Cl protons at 3.84 (t) and Me at 2.70 (s). These observations arise because the protons in each CH_2 are diastereotopic. At low pH values separate resonances are observed for the members of each pair, whereas at pH values near to or above the pK_a of (**2a**) (6.45),[5b] the diastereotopic protons are exchanged by nitrogen inversion (*cf. NN*-dibenzylmethylamine[14]). This behaviour was also observed with other 2-substituted *N*-(2-substituted ethyl)-*N*-methylethanamines (*e.g.* with hydroxy substituent).

When a 0.1M solution of (**2a**)·HCl in ^2H_2O was treated with 1 mol equiv. Na_2CO_3, or when (**2a**)·HCl was dissolved in pH 7.2 citrate–phosphate buffer, monitoring by ^1H n.m.r. spectroscopy showed the development within 2 h at *ca.* 20 °C of new species. Resonances observed at δ 3.46, 3.48 (2 × NMe), and 4.15 (methylene protons) are assigned (comparison with authentic material)[15] to the piperazinium salts (**3a** and **b**). The other signals at δ 3.16 (s, Me), 3.2—3.4 (m, 2 × aziridinium CH_2) (see Figure 1), 3.64 (t, CH_2N) and 4.08 (t, CH_2Cl) are assigned to the aziridinium ion (**1a**). These resonances are not observed if a sufficiently powerful nucleophile (*e.g.* thiosulphate) is added to citrate–phosphate buffer *before* (**2a**)·HCl. They appear and disappear in the presence of less strong nucleophiles such as thiourea, with the formation of adducts [*e.g.* (**4a**)] as well as piperazinium salts (**3a** and **b**). With very weak nucleophiles (*e.g.* guanosine) the main reaction of (**2a**) leads slowly to the (**3a**) + (**3b**) mixture *via* (**1a**). Reactions of (**1a**) with nucleophiles are discussed fully below.

To assist the analysis of the ^1H n.m.r. spectrum of aziridinium ion (**1a**) we prepared the ion (**1e**) essentially as described in ref. 5*c*. Thus, neutralisation with NaO^2H of 2-bromo-*N*-ethyl-*N*-(1-naphthylmethyl)ethanamine hydrobromide [(**2e**)·HBr] in ^2H_2O–[$^2H_6$]acetone (28:72 v/v) caused the disappearance of resonances from (**2e**) and the appearance of resonances at δ 5.03 (naphthyl CH_2), 3.35 (MeCH_2), 2.9—3.2 (AA' BB' pattern for aziridinium methylenes), and 1.41 (Me), as well as naphthyl resonances. An n.O.e. experiment (irradiation at δ 5.03) showed that the high-field component of the AA' BB' pattern belongs to protons *syn* to the naphthyl methylene. Analysis of the pattern by the PANIC program[16] on the Aspect 2000 data system of the Bruker WH400 instrument, gave $\nu_A - \nu_B$ 66.1 Hz and J_{12} 8.86, J_{34} 8.96, $J_{14} = J_{23} = 7.53$, and $J_{13} = J_{24} = -3.66$ Hz, *i.e.* $J_{cis} > J_{trans}$, as expected for vicinal coupling in a three-membered ring. With the aid of this data, the AA' BB' system for (**1a**) was solved to give the parameters $\nu_A - \nu_B$ 32.2 Hz and J_{12} 8.66, J_{34} 8.61, $J_{14} = J_{23} = 7.81$, and $J_{13} = J_{24} = -3.66$ Hz. This set is very similar to that of (**1e**), with $J_{cis} > J_{trans}$. To avoid

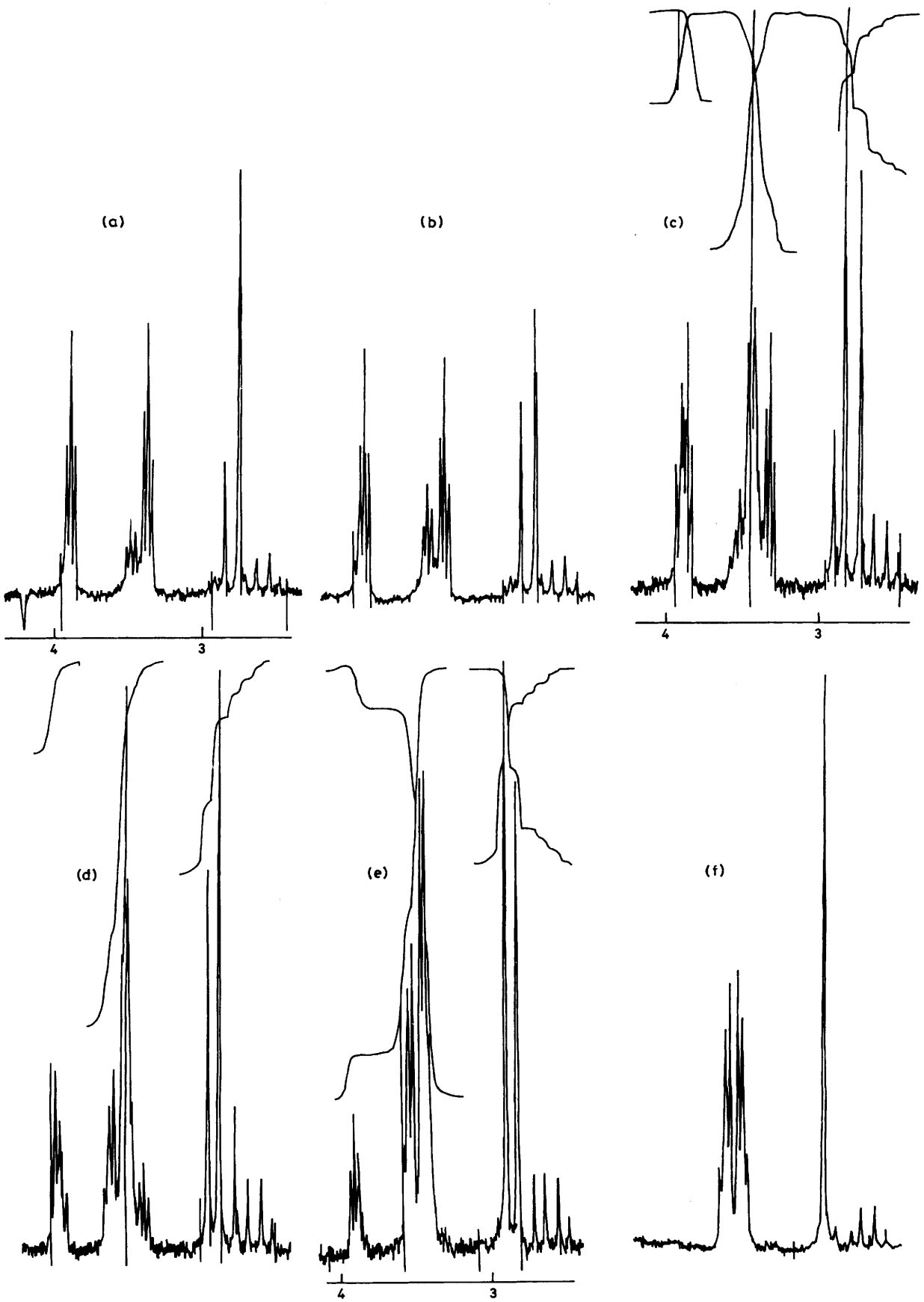


Figure 2. Reaction between nitrogen mustard (**2a**) and $Na_2S_2O_3$ in pH 7.2 buffer monitored by 220 MHz ^1H n.m.r. spectroscopy. (*a*) 10 min, (*b*) 15 min, (*c*) 31 min, (*d*) 64 min, (*e*) 101 min, (*f*) 370 min

convergence to a false solution, it was important to include low-intensity peaks to the outside of the main multiplet, which were clearly visible in (**1e**), but almost hidden by noise in (**1a**). The appearance of AA′ BB′ spectra changes drastically as the shift difference $\nu_A - \nu_B$ varies, for a given set of coupling parameters.[17] It should be possible to simulate the spectrum of the methylene protons of any aziridinium ion of the ethyleneimmonium type using J parameters similar to those derived above and an appropriate value for $\nu_A - \nu_B$. An n.O.e. experiment showed that the protons of the aziridinium ring that are *syn* to the methyl group contribute to the upfield portion of the AA′ BB′ system of (**1a**).

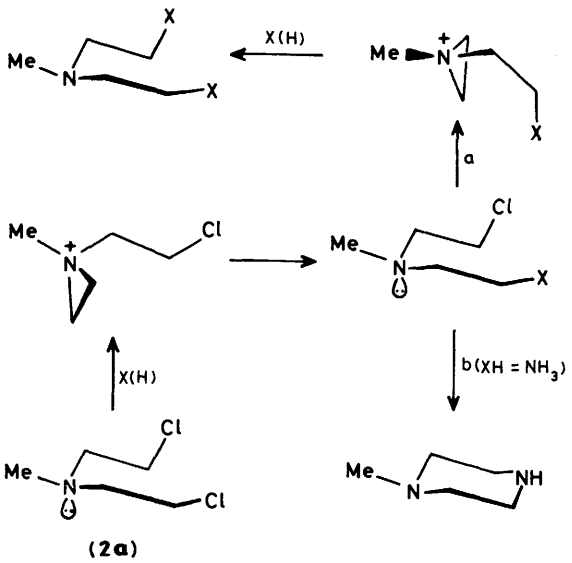
Reactions of Drug (**2a**) *with Nucleophiles monitored by* ^{1}H *N.m.r. Spectroscopy.*—The ^{1}H n.m.r. *N*-methyl resonance of nitrogen mustard (**2a**) is observed at δ *ca.* 2.6 in the free base and at δ *ca.* 3.05 in the protonated species. At a pH where the nitrogen is partially protonated, a weighted average signal will be observed between these extremes. To minimise pH-dependent shifts and to maintain the reactivity of basic functions during reactions, a buffer of low nucleophilicity was needed. Sorensen's citrate–phosphate buffer was chosen, because it is the only common buffer for the required pH range, was of low reactivity towards (**1a**) and (**2a**), and contains only a small quantity of an organic component. To avoid heating the solutions in n.m.r. experiments at high-field strengths a buffer of low ionic strength, but consequently poor buffering capacity, was used. However, the maximum observed pH change was from 7.2 to 6.6.

The preferred experimental procedure was to dissolve the nucleophile (2.5 mol equiv.) in buffer and record the ^{1}H n.m.r. spectrum; the solution was then mixed with (**2a**)·HCl and a series of spectra were recorded. In earlier experiments, (**2a**)·HCl was neutralised with sodium carbonate and the formation of aziridinium ion (**1a**) was allowed to proceed. The solution of (**1a**) was then treated with a nucleophile in pH 7.2 buffer. However, this permitted some trapping of the aziridinium ion (**1a**) by hydroxide and nitrogen mustard base, before the nucleophile was present, and the spectra were consequently more complex. The advantage of this mode of reaction was that consumption of aziridinium ion could be watched. Furthermore, the demonstration of identical products from both (**1a**) and (**2a**) is consistent with the proposal that (**1a**) lies on the reaction pathway from (**2a**).

Solutions (0.1M) of aziridinium ion (**1a**), generated as described in ^2H_2O, decompose over several hours at 20 °C to the piperazinium dimers (**3a** and **b**). These substances arise by reversion of (**1a**) to (**2a**), which reacts with (**1a**) to give, after cyclisation, dimer (**3a** or **b**). Addition of ^{2}HCl to a solution of (**1a**) immediately gave (**2a**), trapped by protonation (*cf.* ref. 18). Solutions of citrate, phosphate, acetate, alanine, amine (ammonia, propylamine), *N*-acetylhistidine, and guanosine monophosphate at pH *ca.* 7 are not efficient trapping agents for (**1a**) at 20 °C. However, reactions which compete effectively with the dimerisation to (**3a** and **b**) are observed in the presence of 0.25M-thiol (methanethiol, *N*-acetylcysteine methyl ester, 2-mercaptoethanol), thiourea, methionine, azide, cyanide, and thiosulphate at 20 °C. The most effective trapping agent was thiosulphate, whilst methionine was the least effective of the compounds examined. The better trapping agents (*e.g.* thiosulphate and thiolates) prevent the accumulation of (**1a**), and hence the formation of (**3a** and **b**), when they are added to citrate–phosphate buffer *before* (**2a**)·HCl. Products were identified by the observation of appropriate resonances in the ^{1}H n.m.r. spectrum and by isolation for the case of methanethiol.

Addition of excess of hydroxide (either NaOH or >2M-Na_2CO_3) to aziridinium ion (**1a**) gave the diol (**4b**). Lower concentrations of Na_2CO_3 (*ca.* 1M) gave a mixture of (**4b**), (**3a**), (**3b**), and two products, tentatively assigned structures (**5**) and (**6**). Addition of excess of concentrated ammonia to (**2a**) gave a 3:2 mixture of 2-amino-*N*-(2-aminoethyl)-*N*-methylethanamine (**4c**) and *N*-methylpiperazine (**7**). The reason that these products were not observed at pH *ca.* 7 is that ammonia (pK_a 9.2) is almost completely protonated at this pH, and so the predominant nucleophile is nitrogen mustard base, which captures (**1a**) and leads to the (**3a,b**) mixture. Similar reasoning applies to the failure of propylamine (pK_a 10.7) and alanine (pK_a 9.87) to scavenge (**1a**) at pH 7. Golumbic *et al.*[6a] found that (**1a**) with alanine at pH 7.5—8.0 gives the piperazinium dimer (**3c**).

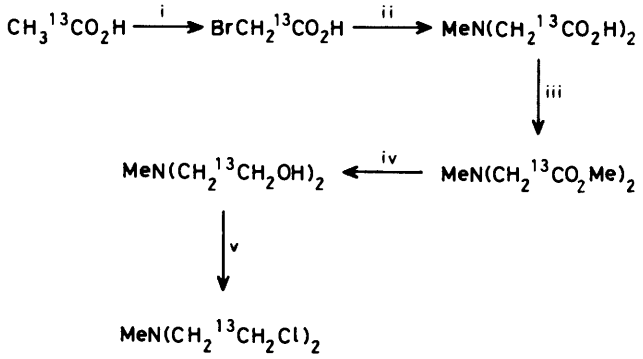
Reactions of (**2a**) with cyanide, thiolates, thiourea, and thiosulphate showed the stepwise replacement of chloride (*cf.* Figure 2). Thus, at short reaction times, (**2a**) with thiosulphate showed resonances at δ 2.47, 3.08, 3.27, and 3.76 which were assigned to the Bunte salt (**4d**) of monosubstitution. These resonances gradually disappeared as the Bunte salt (**4e**) of disubstitution was formed. Thiourea was proposed as an effective scavenger of aziridinium ions derived from drug (**2a**) and phenylalanine mustard.[19] However, Engle *et al.*[13] did not observe any reaction between an aziridinium ion produced from phosphoramide mustard (**2d**) and thiourea. We observed a stepwise reaction between thiourea and (**2a**) according to Scheme 1, in agreement with the proposal of ref. 19.



Scheme 1. Reaction between nitrogen mustard (**2a**) and nucleophiles. Pathway a is followed by nucleophile X = thiourea or ammonia. Pathway b was only observed with ammonia

Mixing either *N*-acetylhistidine or guanosine monophosphate with (**2a**)·HCl in pH 7.2 citrate–phosphate–^2H_2O buffer showed the formation of (**1a**), followed by (**3** and **b**), in a manner similar to that observed when either of these potential nucleophiles was absent. It has been claimed[20] that drug (**2a**) reacts (pH 6.2) with a histidine residue in hemoglobin S, and reports of the cross-linking of nucleic acids *via* N-7 of guanine residues are legion.[1] The reactivities of functional groups of model components for proteins and nucleic acids may not exactly mimic the reactivities of these groups in the macromolecules. Furthermore, inter-strand cross-linking of only a small fraction of guanine residues in a nucleic acid may be lethal. We would not have detected <5% alkylation of guanosine monophosphate or *N*-acetylhistidine by (**1a**).

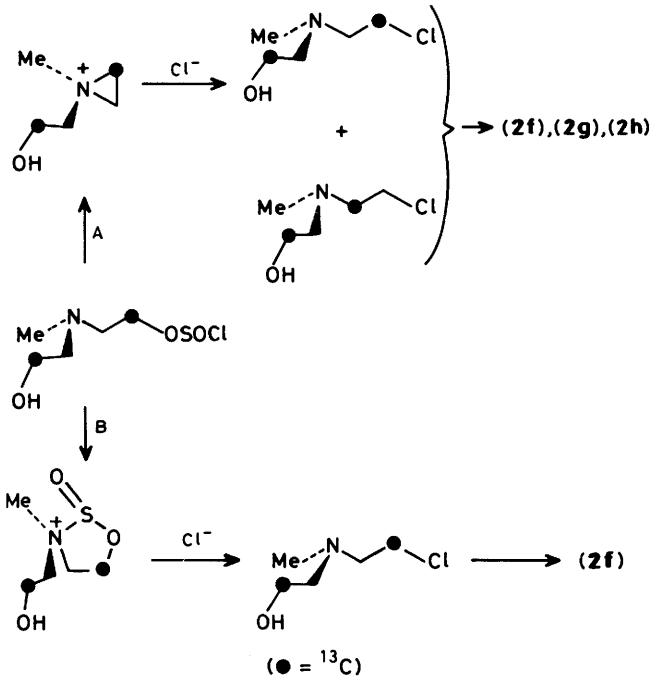
Synthesis of [2,2-$^{13}C_2$]-2-*Chloro*-N-(2-*chloroethyl*)-N-*methylethanamine*.—2-Chloro-*N*-(2-chloroethyl)-*N*-methylethanamine (**2a**) labelled with ^{14}C was employed to determine the distribution of the drug in normal and leukemic mice.[21] Specifically deuteriated mustards, *e.g.* [2,2-$^2H_4$]-*NN*-bis-(2-chloroethyl)phosphorodiamidic acid [*cf.* (**2d**)], have been used to investigate the mechanisms of their reactions with nucleophiles. In one study,[22] mass spectrometry was used to assay the deuterium content of products, whilst in the other ^1H n.m.r. spectroscopy was utilised.[23] Proton-decoupled ^{13}C n.m.r. spectroscopy of ^{13}C-labelled substrates enables their reactions to be monitored directly, by observing the changes in intensity of a few well resolved resonances. To study reactions of drug (**2a**) with nucleophiles directly by ^{13}C n.m.r. spectroscopy, and to obtain mechanistic information about these reactions, the specifically labelled derivative [2,2-$^{13}C_2$]-2-chloro-*N*-(2-chloroethyl)-*N*-methylethanamine (**2f**) (10 atom % ^{13}C at each C-2) was synthesised (see Scheme 2) by adapting established procedures for the corresponding unlabelled compounds.



Scheme 2. *Reagents:* i, $P-Br_2$; ii, $MeNH_2$, NaOH; iii, MeOH–HCl; iv, $LiAlH_4$; v, $SOCl_2$

It was anticipated that chlorination of labelled diol (**4b**) might proceed *via* aziridinium ions and yield a mixture of labelled mustards, (**2f**—**h**). It has been shown that chlorination of [1-^{13}C]-2-(methylthio)ethanol gives a 1:1 mixture of [1-^{13}C]- and [2-^{13}C]-1-chloro-2-(methylthio)ethane, *via* an intermediate thiiranium ion, irrespective of the chlorinating agent employed.[24] In practice, reaction of labelled diol (**4b**) with thionyl chloride in either benzene or chloroform gave specifically [2,2-$^{13}C_2$]-2-chloro-*N*-(2-chloroethyl)-*N*-methylethanamine (**2f**). Alkyl chlorosulphites can be isolated from mixtures of thionyl chloride and alcohols.[25] A chlorosulphite produced from labelled diol (**4b**) could be converted either into an aziridinium ion (Scheme 3, path A) or into a five-membered-ring intermediate (Scheme 3, path B). Product formation *via* path B ensures that all the ^{13}C label will be at C-2. Alternatively, protonation of the nitrogen by hydrogen chloride, produced in the formation of chlorosulphite, will prevent aziridinium ion formation.

Reactions of Drug (**2a**) *with Nucleophiles monitored by* ^{13}C *N.m.r. Spectroscopy*.—The $^{13}C\{^1H\}$ n.m.r. spectrum of (**2a**)·HCl in 2M-^2HCl–^2H_2O shows resonances at δ 38.02 (C-2), 41.27 (Me), and 57.65 (C-1) p.p.m. Under similar conditions, (**2f**) showed three resonances at the same chemical shifts as for (**2a**), but that for C-2 was *ca.* 10 times more intense than the resonance for C-1. Reactions of (**2f**) in pH 7.2 citrate–phosphate buffer with thiosulphate, cyanide, and methanethiol were investigated by ^{13}C n.m.r. spectroscopy. The final products from these reactions [(**4e**—**g**) respectively] showed C-1:C-2 ratios of 1.09 ± 0.03 (**4e**), 1.12 ± 0.05 (**4f**) (both measured *in situ*), and 1.2 ± 0.1 (**4g**) (determined on isolated material).



Scheme 3. Alternative pathways for the formation of [2,2-$^{13}C_2$]-2-chloro-*N*-(2-chloroethyl)-*N*-methylethanamine by reaction of [2,2-$^{13}C_2$]-2-hydroxy-*N*-(2-hydroxyethyl)-*N*-methylethanamine with thionyl chloride

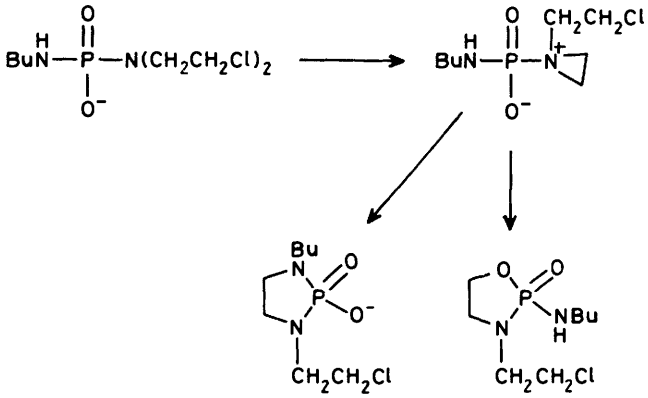
Addition of (**1a**) or (**2a**) to an excess of ammonia gave 2-amino-*N*-(2-aminoethyl)-*N*-methylethanamine (**4c**) (60%) and *N*-methylpiperazine (**7**) (40%). These products were identified after separation by ion-exchange chromatography, by comparing their ^1H and ^{13}C n.m.r. spectra with spectra for authentic samples. Treatment of (**2f**) with excess of ammonia gave these products containing ^{13}C in the following ratios: 1.15 ± 0.05 [C-1(C-1′):C-2(C-2′)] for (**4c**) and 2.04 ± 0.12:1 [C-2(C-6):C-3(C-5)] for (**7**).

The observation that ^{13}C is approximately equally distributed between C-1(C-1′) and C-2(C-2′) of triamine (**4c**) does not prove that this compound is formed *via* aziridinium ions (**1a** and **f**) (both labelled with ^{13}C: see Scheme 1). It is conceivable that (**2f**) equilibrates with the isomeric labelled nitrogen mustards (**2g** and **h**) *via* ^{13}C-labelled (**1a**). The mustards then react with ammonia by direct displacement. Two items of evidence exclude this possibility. The ratio of ^{13}C at C-1(C-1′) and C-2(C-2′) of (**4c**) was not altered in an experiment to which NaCl was added (total $[Cl^-]$ at the start of the experiment increased from 0.1M to 0.6M). This shows that the capture of labelled (**1a**) by chloride to give (**2f**—**h**) does not effectively compete with its capture by ammonia. Of decisive relevance is the fact that the two ethylene fragments of *N*-methylpiperazine are differentially labelled with ^{13}C. If (**2f**) had equilibrated with (**2g** and **h**) 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.

The formation of (**4c**) and (**7**) and the distribution of ^{13}C described is explained by Scheme 1. The deviation of the ^{13}C ratio from unity can be explained by postulating a primary kinetic isotope effect in the S_N2-type cleavage of each intermediate aziridinium ion. The magnitude of carbon isotope effects (k_{12}/k_{13}) in S_N2 displacements falls in the range 1.04—1.08.[26] For S_N1 reactions k_{12}/k_{13} is closer to unity. The formation of (**4c**) and (**7**) occurs *via* aziridinium ion (**1a**) (*cf.* Scheme 1). The resulting 2-amino-*N*-(2-chloroethyl)-*N*-methylethanamine can cyclise either to aziridinium ion (**1f**) or to

piperazine (7). If this is the sole route to (7) and provided that aziridinium formation is irreversible, than the ratio of ^{13}C at C-2(C-6):C-3(C-5) should be 2.03:1, in excellent agreement with the ratio found.

An alternative route to (7) is *via* aziridinium ion (**1f**). However, this would require a front-side S_N2 displacement at carbon (or 5-*endo-tet* displacement using the terminology of ref. 27) and would lead to a ^{13}C ratio [C-2(C-6):C-3(C-5)] of *ca.* 1.1, at variance with that observed. Previous attempts to observe front-side S_N2 displacements have failed.[28] Engle *et al.*[13] proposed the endocyclic displacements (4-*endo-tet*) of Scheme 4, but in view of our findings these reactions are



Scheme 4. Endocyclic displacement proposed in ref. 13

almost certainly 5-*exo-tet* displacements at the CH_2Cl group, followed by cleavage of the aziridinium ring by chloride.

Studies with ^2H-labelled mustards have indicated the intermediacy of aziridinium ions in reactions with nucleophiles.[22,23] However, these species were not directly observed and the effect of the reversibility of aziridinium formation was not considered. A very important finding of ref. 23 was that aryl nitrogen mustards can react either *via* an intermediate aziridinium ion or by direct displacement, depending on the strength of the nucleophile.

Conclusions.—The methodology described in this paper complements the use of mass spectrometric techniques for determining sites of alkylation by *e.g.* phosphoramide mustard.[22,29] [^{13}C]Methyl toluene-*p*-sulphonate and $^{13}C\{^1H\}$ n.m.r. spectroscopy have been used to determine sites of methylation in nucleic acids and their constituents.[30] We believe that ^{13}C-enriched mustards have a future in the direct determination by n.m.r. spectroscopy of the sites of alkylation of biological macromolecules. Present methodology for determining such sites is inadequate, being either tedious (radiolabelling techniques) or imprecise. For example, a recent study of the alkylation of the nicotonic acetylcholine receptor of *Torpeda californa* by a quinacrine mustard was unable to specify the nucleophilic amino acid residue that was alkylated, although some evidence to exclude a thiol grouping was provided.[31]

Experimental

Unlabelled nitrogen mustard hydrochloride was obtained commercially and was recrystallised twice from acetone before use. 2-Bromo-*N*-ethyl-*N*-(1-naphthylmethyl)ethanamine was prepared from 1-(chloromethyl)naphthalene as described.[5c] Barium chloride dihydrate was purified by precipitation from aqueous solution with ethanol, followed by drying *in vacuo*. ^1H n.m.r. spectra were recorded either at 220 MHz (Perkin-Elmer R34 instrument) or at 400 MHz (Bruker WH400 n.m.r. spectrometer). Typical spectral conditions with the Bruker instrument for 24 scans: 45° pulse, no delay between acquisitions, 4.85 s acquisition time, data collection using 32 K data points giving a digital resolution of 0.2 Hz per point, processing with a Gaussian multiplication window to narrow the lines by 0.8 Hz. Samples were examined as 0.1M solutions in ^2H_2O using dioxane (δ 3.55) as external reference. The temperature of all measurements was ambient (*ca.* 295 K). $^{13}C\{^1H\}$ N.m.r. spectra were measured either at 22.63 MHz (Bruker WH90 instrument) or at 100.26 MHz (Bruker WH400 instrument), with dioxane (67.4) as internal reference. Sorensen's citrate–phosphate buffer (0.1M) was prepared by dissolving citric acid monohydrate (3.8 mg) and disodium hydrogen phosphate dihydrate (290 mg) in ^2H_2O (10 cm^3). The 'p^{2}H' measured with a Pye–Unicam 405 combined electrode was 7.2.

[1-^{13}C]*Bromoacetic Acid* (55 *atom* % ^{13}C).—To a mixture of [1-^{13}C]acetic acid (3.6 g, 0.06 mol, 91.1 atom % of ^{13}C; BOC Prochem), unlabelled acetic acid (2.4 g, 0.04 mol), acetic anhydride (1 drop), and purified red phosphorus (35 mg), was added bromine (18.7 g, 0.23 mol), dropwise over 4.5 h, whilst boiling under reflux with the exclusion of atmospheric moisture. Heating was continued for a further 1.5 h. The mixture was cooled, treated with water (20 cm^3), and allowed to stand overnight at room temperature. The resulting solution was fractionally distilled under reduced pressure to give [1-^{13}C]bromoacetic acid (55 atom % ^{13}C) as a solid (10.6 g, 76%), b.p. 110—111 °C at 30 mmHg, m.p. 30 °C. A similar procedure was described in ref. 32.

[1-^{13}C,$^{13}CO_2H$]-N-*Carboxymethyl*-N-*methylglycine*.—To [1-^{13}C]bromoacetic acid (10.6 g, 0.075 mol, 55 atom % of ^{13}C) in water (20 cm^3), cooled in an ice–salt bath, was added dropwise aqueous 6.65M-sodium hydroxide (11.4 cm^3, 0.075 mol) at such a rate that the temperature did not exceed 12 °C. A mixture of 6.65M-NaOH (11.45 cm^3) and methylamine (4.2 cm^3 of 28% w/v aqueous solution containing *ca.* 0.038 mol $MeNH_2$) was added dropwise with cooling to maintain a maximum temperature of 15 °C. The mixture was allowed to stand at room temperature for 3 days. A boiling solution of purified barium chloride dihydrate (9.7 g, 0.04 mol) in water (30 cm^3) was added with vigorous shaking. The resulting suspension was boiled under reflux for 30 min and the barium salt of the title compound was removed by filtration and dried (6.86 g, 0.024 mol, 63%). A boiling aqueous suspension of the salt was treated with 2.73M-sulphuric acid (9.25 cm^3, 0.025 mol H_2SO_4) over 45 min. The resulting suspension was cooled and filtered through Celite, which was washed with boiling water (2 × 15 cm^3). The filtrate was evaporated to *ca.* 15 cm^3 and crystallisation was allowed to proceed overnight at 4 °C after addition of an excess of methanol to give [1-^{13}C,$^{13}CO_2H$]-*N*-carboxymethyl-*N*-methylglycine (2.8 g, 50% from methylamine), $\delta_H(^2H_2O)$ 3.02 (s, CH_3) and 4.0 (s, 2 × NCH_2). The above procedure is based on that described for preparing *N*-carboxymethyl-*N*-methylglycine from methylamine and chloroacetic acid.[33]

[1-^{13}C,$^{13}CO_2H$]-N-*Carboxymethyl*-N-*methylglycine Dimethyl Ester*.—The solution obtained by passing an excess of dry hydrogen chloride through a suspension of [1-^{13}C,-$^{13}CO_2H$]-*N*-carboxymethyl-*N*-methylglycine (2.8 g, 0.019 mol) in dry methanol was boiled under reflux for 2 h with exclusion of atmospheric moisture. Evaporation gave a thick oil which was taken up in dry methanol, evaporated, and pumped in high vacuum for 30 min. Dissolution of the residue in methanol and evaporation was repeated twice more, with final

drying *in vacuo* for 2 h. The free base was liberated from its hydrochloride by addition of 3.57M-sodium methoxide solution in methanol (5.25 cm^3, containing 0.019 mol NaOMe), followed by dropwise addition of the sodium methoxide solution until a single drop caused a sharp rise in pH. After filtration of the suspension through Celite and evaporation of the filtrate, the remaining sodium chloride was removed by extracting the residue with diethyl ether and filtering. The filtrate was concentrated and pumped in high vacuum to leave a clear viscous oil of the title compound (2.54 g, 77%), $\delta_H(CDCl_3)$ 2.5 (s, NMe), 3.45 (s, 2 × CH_2), and 3.7 (s, 2 × OMe).

[2,2-$^{13}C_2$]-2-*Hydroxy*-N-(2-*hydroxyethyl*)-N-*methylethanamine*.—To a stirred solution of lithium aluminium hydride (0.695 g, 0.018 mol) in dry diethyl ether (30 cm^3) under a static pressure of dry nitrogen was added dropwise over 1 h a solution of [1-^{13}C,$^{13}CO_2H$]-*N*-carboxymethyl-*N*-methylglycine dimethyl ester (2.54 g, 0.0145 mol) in diethyl ether (30 cm^3), maintaining the temperature below −5 °C. The resulting suspension was boiled under reflux for 2 h and then the excess of lithium aluminium hydride was destroyed by successive addition of water (0.7 cm^3), 10% NaOH (1 cm^3), and water (2.1 cm^3). The mixture was filtered and evaporation gave a sticky solid, which was transferred to a Soxhlet thimble. Extraction overnight with diethyl ether gave a solution that was dried (Na_2SO_4) and concentrated to an oil. This was distilled to give [2,2-$^{13}C_2$]-2-hydroxy-*N*-(2-hydroxyethyl)-*N*-methylethanamine as a liquid (1.5 g, 86%), b.p. 138—142 °C at 20 mmHg, $\delta_H(^2H_2O)$ 2.38 (s, CH_3), 2.71 (t, 2 × CH_2N), and 3.71 (t and dt, 2 × CH_2OH) (lit.,[15] b.p. 123—125 °C at 4 mmHg).

[2,2-$^{13}C_2$]-2-*Chloro*-N-(2-*chloroethyl*)-N-*methylethanamine* (**2f**).—To thionyl chloride (2.11 g, 0.018 mol) in benzene (3 cm^3), boiled under reflux in a closed apparatus connected to a static nitrogen supply, was added over 1 h [2,2-$^{13}C_2$]-2-hydroxy-*N*-(2-hydroxyethyl)-*N*-methylethanamine (0.184 g, 1.54 × 10^{-3} mol, 55 atom % ^{13}C) and unlabelled *N*-2-hydroxyethyl-2-hydroxy-*N*-methylethanamine (0.819 g, 6.88 × 10^{-3} mol) as a suspension in benzene (2 cm^3). The resulting mixture was boiled under reflux for 2 h and cooled overnight to yield a mass of off-white crystals. These were washed with light petroleum (b.p. 40—60 °C) and recrystallised from acetone to give [2,2-$^{13}C_2$]-2-chloro-*N*-(2-chloroethyl)-*N*-methylethanamine hydrochloride as crystals (1.185 g, 73%); $\delta_H(CDCl_3)$ 2.38 (s, CH_3N), 2.82 (t, 2 × CH_2N), 3.57 [dt, J (^{13}C-^1H) 151 Hz, 0.1 × 4 H, $^{13}CH_2Cl$, and t, 0.9 × 4 H, $^{12}CH_2Cl$]; δ_C (2M-^{2}HCl in ^2H_2O) 38.0 (2 × C-2), 41.3 (Me), and 57.7 p.p.m. (2 × C-1) [resonance for C-2 *ca.* 10 times as intense as that for C-1]; 10 atom % ^{13}C at each C-2 (calculated from starting percentage, using the given dilutions).

Generation of an Aqueous Solution of the N-2-*Chloroethyl*-N-*methylaziridinium Ion* (**1a**).—^2H_2O (0.5 cm^3) was added to a vial containing nitrogen mustard hydrochloride (10 mg, 0.052 mmol). Treatment of the resulting solution with 1.7M-sodium carbonate in ^2H_2O (31 μl; 1 mol equiv. Na_2CO_3) gave a briefly opaque solution as the mustard base was liberated. Monitoring by ^1H n.m.r. spectroscopy showed the title species to be present 2 min after mixing, $\delta_H(^2H_2O)$ 3.16 (s, MeN), 3.2—3.4 (m, 2 × ring CH_2), 3.64 (t, CH_2N), and 4.08 (t, CH_2Cl) (for detailed analysis see text), growing to a maximum concentration of 80—90% of starting mustard after *ca.* 2 h. *NN'*-2-Chloroethyl-*NN'*-methylpiperazinium dichloride (**3a** and **b**) was detectable 20 min after neutralisation and increased while the mustard base was still present. After reaching its maximum concentration, aziridinium ion (**1a**) was slowly converted into the piperazinium dimers (**3a** and **b**) (conversion virtually complete after 48 h). At this time a ^1H n.m.r. spectrum showed traces of diol (**4b**) and the linear dimer (**5**) derived therefrom, $\delta_H(^2H_2O)$ for (**4b**) 2.40 (s, MeN), 2.76 (t, 2 × CH_2N), and 3.74 (t, 2 × CH_2OH).

Warming a solution of aziridinium ion (**1a**) to 30 °C for 1 h converted it into piperazinium dimers (**3a** and **b**), δ_H 3.46 (s, 2 × Me of *cis*-isomer), 3.48 (s, 2 × Me of *trans*-isomer), 4.15 (br s, 8 × CH_2 groups). The *cis*-isomer was prepared by synthesis,[15] $\delta_H(^2H_2O)$ 3.46 (s, 2 × MeN) and 4.16 (br s, 8 × CH_2).

Reaction between the N-2-*Chloroethyl*-N-*methylaziridinium Ion* (**1a**) *and Nucleophiles in the Absence of Buffer*.—In all cases, nitrogen mustard hydrochloride (10 mg) in ^2H_2O (0.5 cm^3) was treated with 1.7M-Na_2CO_3 in ^2H_2O (31 μl, 1 mol equiv.) and the resulting solution was allowed to stand at room temperature (18—20 °C) for 2 h, when ^1H n.m.r. analysis showed at least 80% conversion into aziridinium ion (**1a**). The nucleophile (2.5 mol equiv.) in ^2H_2O (0.5 cm^3) was added and ^1H n.m.r. spectra were recorded at *ca.* 1 min, 5 min, 1 h, 2 h, and 24 h from mixing.

(a) Sodium thiosulphate gave predominantly the mono-Bunte salt (**4d**) after 5 min, $\delta_H(^2H_2O)$ 2.47 (s, MeN), 3.08 (m, 2 × CH_2N), 3.27 (t, $CH_2S_2O_3$), and 3.76 (t, CH_2Cl). The 24 h spectrum showed only piperazine dimers (**3a** and **b**) and the bis-Bunte salt (**4e**), δ_H 2.47 (s, CH_3N) and 3.18 (m, 2 × CH_2CH_2-S_2O_3).

(b) Sodium hydroxide gave after 24 h a 2:1 mixture of diol (**4b**), δ_H 2.27 (s, MeN), 2.60 (t, 2 × CH_2N), and 3.70 (t, 2 × CH_2OH), and compound (**5**), δ_H 2.33 (s, MeN), 2.98 (m, 2 × CH_2N), 3.21 (s, MeN), 3.60 (m, 3 × CH_2N), and 4.02 (m, 3 × CH_2OH).

(c) Sodium azide gave a 24 h spectrum consistent with $MeN(CH_2CH_2N_3)_2$, δ_H 2.56 (s, MeN), 2.98 (t, 2 × CH_2N), and 3.65 (t, 2 × CH_2N_3).

(d) Sodium cyanide gave a 24 h spectrum consistent with $MeN(CH_2CH_2CN)_2$, δ_H 2.32 (s, MeN) and 2.75 (m, 2 × CH_2CH_2).

(e) 2-Mercaptoethanol gave after 24 h a spectrum consistent with $MeN(CH_2CH_2SCH_2CH_2OH)_2$ as the main product, δ 2.78 (t) and 2.93 (m) [4 × SCH_2], 2.97 (s, MeN), 3.47 (m, 2 × NCH_2), and 3.76 (t, 2 × CH_2OH).

(f) Guanosine monophosphate (added as disodium salt) gave only piperazinium dimers (**3a** and **b**).

Reactions between the N-2-*Chloroethyl*-N-*methylaziridinium Ion* (**1a**) *and Added Nucleophile in Sorensen's Buffer*.—In each experiment, nitrogen mustard hydrochloride (10 mg) was dissolved in ^2H_2O (0.5 cm^3) was treated with 1.7M-sodium carbonate in ^2H_2O (31 μl, 1 mol equiv.) and the resulting solution was left for 2 h at room temperature, when ^1H n.m.r. analysis showed a high concentration of aziridinium ion (**1a**). 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 ^1H n.m.r. spectroscopy. All data refer to the ^1H n.m.r. spectrum recorded 24 h from mixing unless otherwise indicated:

(a) Sodium thiosulphate gave the bis-Bunte salt (**4e**).

(b) Ammonia gave a poor spectrum in which only the piperazinium dimers (**3a** and **b**) could be identified.

(c) Sodium azide gave $MeN(CH_2CH_2N_3)_2$.

(d) 2-Mercaptoethanol gave a spectrum indicative of $MeN(CH_2CH_2SCH_2CH_2OH)_2$. Earlier spectra, *e.g.* 3 h from mixing, showed the intermediacy of $MeN(CH_2CH_2Cl)(CH_2CH_2SCH_2$-$CH_2OH)$.

Reactions between Nitrogen Mustard Hydrochloride and Added Nucleophile in Sorensen's Buffer.—An excess (2.5 mol. equiv.) of the nucleophile was dissolved in pH 7.2 citrate–phosphate buffer (0.5 cm^3) and the ^1H n.m.r. spectrum was

recorded. The contents of the tube were added to a vial containing nitrogen mustard hydrochloride (10 mg). Addition of the buffer gave an opaque solution, which was transferred back to the n.m.r. tube, and vigorously shaken until the opacity had disappeared. Spectra were recorded up to 24 h or a week later.

(a) Sodium thiosulphate consumed the mustard, δ_H 2.76—2.71 (MeN), between spectra recorded at 64 and 107 min. The mono-Bunte salt (**4d**), δ_H 2.82—2.84 (NMe), was present from 2 min; the bis-Bunte salt (**4e**), δ_H 2.89 (NMe), appeared after 15 min. The concentrations of the Bunte salts were about equal after 1.5 h. The spectrum after 6 h displayed resonances for buffer and Bunte salt (**4e**) only. The pH of this system dropped from 7.2 to 6.9 during 6 h.

(b) *Thiourea.* The methyl groups of a monosubstituted intermediate, δ 2.42, and aziridinium ion (**1a**) were discernible 8 min after adding the alkylating agent to this nucleophile. Concentrations of mustard and monoalkylated product were equal 28 min after the addition. $MeN(CH_2CH_2Cl)[CH_2CH_2SC^+(NH_2)_2]$ gave $\delta_H(^2H_2O)$ 2.42 (s, MeN), 2.98 (m, 2 × NCH_2), 3.29 (m, CH_2S), and 3.74 (m, CH_2Cl). Nearly all the starting material had been consumed by 175 min when mono- and di-substituted products were present in equal amounts. The final spectrum (24 h) showed <2% conversion into piperazinium dimers (**3a** and **b**), the dominant feature being resonances assigned to structure (**4a**), $\delta_H(^2H_2O)$ 2.37 (s, MeN), 2.9 (t, 2 × CH_2N), and 3.31 (t, 2 × SCH_2).

(c) 2-Mercaptoethanol gave a stepwise substitution which allowed only brief observation of the aziridinium ion (**1a**) and did not give rise to any piperazine dimers (**3a** and **b**). The final product was $MeN(CH_2CH_2SCH_2CH_2OH)_2$ (for spectra data, see above). The methyl signal of this was evident 6 min after mixing. Transitory resonances were consistent with a mono-substituted intermediate, $MeN(CH_2CH_2Cl)(CH_2CH_2SCH_2CH_2OH)$, $\delta_H(^2H_2O)$ 2.78 (t, SCH_2), 2.91 (s, MeN), 2.98 (t, SCH_2), 3.36 (t, NCH_2), 3.43 (t, NCH_2) 3.76 (t, CH_2OH), and 3.93 (t, $ClCH_2$). Formation of the final product was complete after 4 h.

(d) *N*-Acetylcysteine methyl ester showed simultaneous production of 1:1 and 2:1 adducts without any accumulation of aziridinium intermediates. The fully reacted material was characterised as $MeN[CH_2CH_2SCH_2CH(NHAc)CO_2Me]_2$, $\delta_H(^2H_2O)$ 2.07 (s, 2 × MeCO), 2.91 (s, MeN), 2.98 (m, 2 × SCH_2), 3.13 (d, 2 × CH_2CH), 3.39 (t, 2 × NCH_2), 3.78 (s, 2 × CO_2Me), and 4.66 (m, 2 × CH).

Reaction of Nitrogen Mustard (**2f**) *with Ammonia.*—The following reaction was initially conducted with unlabelled mustard (**2a**) and products (**4a**) and (**7**) were identified by comparison with authentic samples. A solution of [2,2-$^{13}C_2$]-2-chloro-*N*-(2-chloroethyl)-*N*-methylethanamine (**2f**) (30 mg, 0.156 mmol) in water (4 cm^3) was added to a stirred solution of an excess of ammonia (2 cm^3 of 35% aqueous ammonia; *ca.* 40 mmol NH_3). After 2 h the solution was evaporated to dryness and the ^1H n.m.r. spectrum in 2M-^2HCl was recorded. This spectrum showed the formation of *N*-methylpiperazine (**7**) and 2-amino-*N*-(2-aminoethyl)-*N*-methylethanamine (**4c**). There was no unchanged starting material. The sample was applied to an acid-washed ion-exchange column (50 g Amberlite CG 120 type I, in a 2 cm diam. column) and eluted with 3M-HCl, collecting 10 cm^3 fractions. Each fraction was evaporated to dryness and analysed by ^1H n.m.r. spectroscopy as a solution in ^2HCl. The *N*-methylpiperazine (**7**) emerged as a sharp band in the seventh and eighth fractions while the amine (**4c**) eluted from the twelfth fraction onwards. Evaporation of the solutions gave the hydrochlorides [$C_5H_{14}N_2Cl_2$ (10.0 mg, 5.8×10^{-5} mol); $C_5H_{18}N_3Cl_3$ (18.0 mg, 8.0×10^{-5} mol); total recovery 1.38×10^{-4} mol, 90% of theoretical maximum]. Traces of the piperazinium dimers (**3a** and **b**) were observed in an early column fraction. The amines (**7**) and (**4c**) were individually taken up in ^2H_2O, dioxane was added, and the distributions of ^{13}C assessed by n.m.r. spectroscopy with gated pulse for n.O.e. suppression.

Reaction of Sodium Thiosulphate with Nitrogen Mustard (**2f**) *at pH* 7.2.—[2,2-$^{13}C_2$]-2-Chloro-*N*-(2-chloroethyl)-*N*-methylethanamine (10 mg) was added to $Na_2S_2O_3$ (21 mg, 2.5 mol equiv.) in 0.5 cm^3 of pH 7.2 citrate–phosphate buffer. After standing at room temperature for 24 h, dioxane was added and the $^{13}C\{^1H\}$ n.m.r. spectrum was recorded, δ_C 29.33, 41.43, and 56.23 p.p.m. The resonance at 41.43 was of much reduced intensity compared with the other peaks and was therefore assigned to the non-enriched *N*-methyl group. Integration showed the C-2(C-2′):C-1(C-1′) ratio to be 1.09 ± 0.03.

[2,2-$^{13}C_2$]-2-Chloro-*N*-(2-chloroethyl)-*N*-methylethanamine (13 mg) was added to a solution of 0.5M-$Na_2S_2O_3$ in ^2H_2O (2 cm^3). Dioxane was added and $^{13}C\{^1H\}$ n.m.r. spectra were recorded at approximately hourly intervals. One hour from mixing, the spectrum showed six peaks in the approximate ratios 2:2:10:1:2:1. These are derived from mustard (**2f**), δ_C 38.2 (CH_2Cl), 41.5 (MeN) and 57.65 p.p.m. (CH_2N); the bis-Bunte salt (**4e**), δ_C 29.8 (CH_2S) and 55.6 (CH_2N). [*N.B.* the methyl group of (**4e**) resonates at δ 41.4 and is probably obscured by the methyl signal of (**2f**).] A signal at δ 31.7 was assigned to the mono-Bunte salt (**4d**). Subsequent spectra had increasingly poor signal to noise ratios and showed two steadily increasing signals for (**4e**) (δ 29.8 and 55.6 p.p.m.) and one signal for (**2f**) (δ 38.2 p.p.m.) which decayed and disappeared after 6 h.

Reaction of Aziridinium Ion with Sodium Thiosulphate.—A solution of [2,2-$^{13}C_2$]-2-chloro-*N*-(2-chloroethyl-*N*-methylethanamine (10 mg) in ^2H_2O (0.5 cm^3) was treated with aqueous 1.9M-Na_2CO_3 (27 μl, 1 mol equiv.). After 2 h the resulting solution was added to $Na_2S_2O_3$ (21 mg, 2.5 mol equiv.), transferred to a n.m.r. tube, and allowed to stand overnight. Dioxane was added and the $^{13}C\{^1H\}$ spectrum was recorded. This showed formation of the Bunte salt (**4e**), δ_C 31.67 (CH_2S), 41.70 (MeN), and 56.30 p.p.m. (CH_2N) with a C-1(C-1′):C-2(C-2′) ratio of 1.09 ± 0.05. Other resonances were from the CH_2Cl and NCH_2 groups of piperazinium dimers (**3a** and **b**).

Reaction of Nitrogen Mustard (**2f**) *with Sodium Cyanide.*—[2,2-$^{13}C_2$]-2-Chloro-*N*-(2-chloroethyl)-*N*-methylethanamine (10 mg) was rapidly mixed with a solution of NaCN (6.5 mg) in ^2H_2O (0.5 cm^3). After standing the resulting solution overnight, dioxane was added and the $^{13}C\{^1H\}$ n.m.r. spectrum was recorded. Signals consistent with *N*-2-cyanoethyl-*N*-methyl-2-cyanoethanamine were observed, δ_C 15.64 (CH_2CN), 41.30 (MeN), and 51.95 p.p.m. (CH_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 ± 0.05 : 1.

Reaction of Nitrogen Mustard (**2f**) *with Methanethiol.*—[2,2-$^{13}C_2$]-2-Chloro-*N*-(2-chloroethyl)-*N*-methylethanamine (25 mg) in deaerated water was treated with Na_2CO_3 (28 mg, 2 mol. equiv.). Immediately, the solution at room temperature was saturated with MeSH and left to stand overnight. Addition of Na_2CO_3 and extraction with CH_2Cl_2 (2 × 25 cm^3) gave 2-methylthio-*N*-[2-(methylthio)ethyl]-*N*-methylethanamine (54%), b.p. 85 °C at 18 mmHg, $\delta_C(^2H_2O)$ 29.95 (CH_2SMe) and 55.62 p.p.m. (CH_2NMe) (Found: C, 46.65; H, 9.55; N, 7.7; S, 35.05. Calc. for $C_7H_{17}NS_2$: C, 46.9; H, 9.55; N, 7.8; S, 34.75%). (*N.B.* The combustion analysis was carried out on unlabelled material similarly prepared from mustard (**2a**)].

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