Mechanism and reactivity of chlorambucil and chlorambucil–spermidine conjugate

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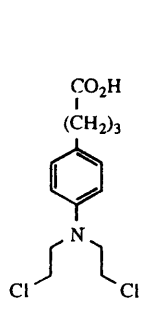
The mechanism and kinetics of hydrolysis of chlorambucil and chlorambucil–spermidine conjugate in aqueous buffered solutions have been compared. In the absence of added chloride ion the reactions are shown to be first-order in the nitrogen mustard and independent of the nucleophile concentration. In the presence of high concentrations of sodium chloride the reaction is reversible and is subject to a significant common-ion effect. The rates of hydrolysis of both compounds are independent of pH in the range 8 to 3.5, and both rates begin to drop rapidly below pH 3.5 which corresponds to the pK_as of the aryl amine groups. The relative rates of alkylation of a range of nucleophiles by chlorambucil have been deduced from the isokinetic points, and have shown that the phosphate dianion, imidazole base and particularly thiolates are all capable of competing with water for the aziridinium ion at comparatively low concentrations. The rates of reaction of chlorambucil and the chlorambucil–spermidine conjugate have been shown to be sensitive to the medium, and, in particular, there is a large micellar inhibition of the hydrolysis of chlorambucil (60-fold reduction in rate) in the presence of hexadecyltrimethylammonium chloride that is not seen for the conjugate. These data are all accounted for in terms of a rate limiting formation of the aziridinium ion intermediate in each case. No evidence for any other mechanistic pathways was found.

The nitrogen mustard chlorambucil (**1**) is widely used in the treatment of chronic lymphocytic leukemia, lymphomas and ovarian carcinoma. We have exploited this anticancer agent to explore strategies for drug delivery and targeting, and we recently reported the synthesis and *in vitro* and *in vivo* evaluation of the effects of conjugation of the aromatic nitrogen mustard to the polyammonium carrier spermidine.[1,2] It was



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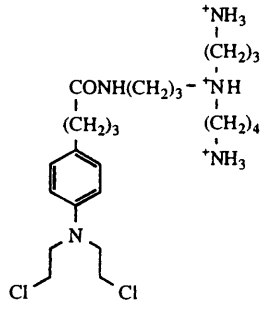

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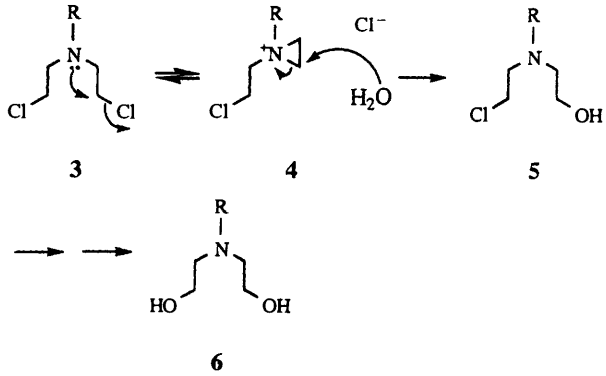
anticipated that the conjugate (**2**) could exploit two distinct functions of the polyamine. Firstly, the presence of an active polyamine uptake system(s) in many cell types,[3] but particularly in malignant cell lines, might facilitate selective accumulation in tumour tissue. Secondly, the high affinity of polyamines for DNA might concentrate the cytotoxic agent near to the intracellular target. Summarising the results of these initial studies: (1) the chlorambucil–spermidine conjugate showed a remarkable increase in reactivity with naked DNA by a factor of 10^4 as compared to chlorambucil; (2) *in vitro* cytotoxicity of the conjugate in polyamine depleted ADJ/PC6 cells was increased by *ca.* 200-fold compared to chlorambucil; (3) the *in vivo* activity of the conjugate increased by only four-fold. The present study comparing the intrinsic reactivity of chlorambucil and the chlorambucil–spermidine conjugate was initiated to address both the origin of the 10^4 increase in reactivity of the conjugate with DNA and to address the likely bioavailability and half-life of the conjugate in the *in vitro* and *in vivo* studies previously reported.

A considerable body of work on the mechanism of hydrolysis of both aliphatic and aromatic nitrogen mustards including chlorambucil itself has led to the usual acceptance of a mechanism involving the intramolecular nucleophilic participation of the nitrogen, with the reaction proceeding *via* the aziridinium ion intermediate (**4**) (Scheme 1), despite earlier suggestions of both S_N1 and/or S_N2 pathways.[4–11]



3 **4** **5** **6**

Scheme 1

Perhaps the most persuasive evidence in favour of such a mechanism is the demonstration that alkylation reactions using appropriate isotopically-labelled nitrogen mustards are usually accompanied by complete scrambling of the two methylene groups in the alkylated products which would be expected if the reaction proceeds *via* the aziridinium ion.[4,5] An exception to this is the alkylation of a thiol nucleophile by an aryl nitrogen mustard in tertiary butanol reported by Benn *et al.* which occurred without scrambling of the methylene groups, ruling out reaction *via* a free aziridinium ion intermediate in this case.[4] A very recent study on a series of substituted aryl nitrogen mustards has also suggested that a general-base-catalysed direct hydrolysis of the chloroethyl moieties may compete with the reaction *via* the aziridinium ion.[12] In view of this surprising

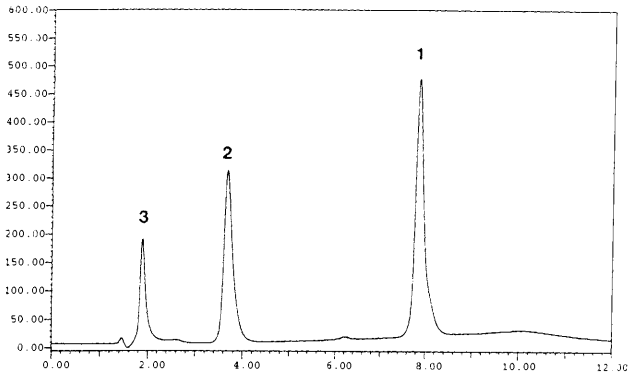


Fig. 1 Typical HPLC trace for chlorambucil hydrolysis in borate buffer at pH 8. Peaks 1, 2 and 3 correspond to chlorambucil (**1**), the half-mustard (**5**) and the diol (**6**), respectively.

observation we have looked for evidence for this in our systems.

Results

pH–rate profile

The hydrolysis of both chlorambucil (**1**) and the chlorambucil–spermidine conjugate (**2**) could be conveniently monitored by HPLC, which also allowed the monitoring of the consecutive displacements of both chlorines of these nitrogen mustards. A typical HPLC elution profile for the hydrolysis of chlorambucil itself is shown in Fig. 1. In some of the buffer systems used the buffer competes with water as the nucleophile leading to mixtures of products which significantly complicates the quantitation of the data. However, even in these cases the rate of disappearance of the nitrogen mustard can be readily determined by HPLC for both chlorambucil and the chlorambucil–spermidine conjugate. The hydrolysis of chlorambucil is clearly first-order throughout the pH range studied. Fig. 2 shows the pH–rate profile for the hydrolysis of chlorambucil and it is clear that the observed rate is independent of pH throughout the range 8 to 3.5. The observed rate rapidly drops off below pH 3.5. A very similar pH-rate profile is also observed for the chlorambucil–spermidine conjugate, Fig. 3 and Table 1. The k_{obs} values for chlorambucil and the chlorambucil–spermidine conjugate are comparable, demonstrating that the rate of reaction of the nitrogen mustard is unaffected by the conjugation to the polyamine.

Since the mechanism of hydrolysis of aromatic nitrogen mustards potentially involves the ionisable arylamino group, in order to explain these pH–rate profiles it is necessary to know the pK_as of this group. There are surprisingly diverse literature values for the pK_a of chlorambucil and related structures ranging from 5.8 and 8 for chlorambucil,[13] 5.85 to 6.5 for a series of *para*-substituted aromatic nitrogen mustards[12] to 2.2 for *N*,*N*-dichloroethylaniline.[14] In view of this we have independently determined the pK_a by UV spectroscopy. The value of the pK_a of chlorambucil (**1**) at room temperature in buffered aqueous solution was found to be 2.3 and that for the spermidine–chlorambucil conjugate (2) under the same conditions was 2.0. An independent determination of the pK_a of the spermidine–chlorambucil conjugate (**2**) by ^{1}H NMR spectroscopy was possible for this nitrogen mustard because the material was sufficiently soluble across the entire pH range to allow NMR spectra to be recorded throughout the range 0–10. The apparent pK_a determined from pH-dependent chemical shift changes was 2.3, it should however be noted that no correction has been made for the measurement of pH of D_2O buffer solutions using a standard combination electrode and there may well be an isotope effect on the arylamine

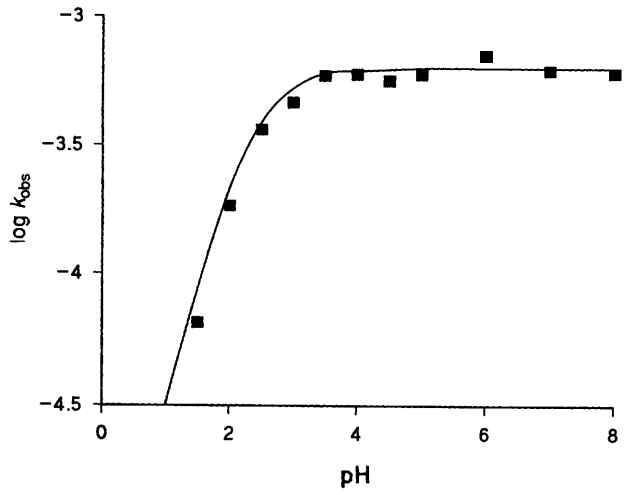


Fig. 2 pH–rate profile for the hydrolysis of chlorambucil (**1**) based on the data summarised in Table 1. The experimental data points are plotted together with the theoretical curve derived from the mechanism involving only reaction of the neutral amine occurring *via* rate-limiting formation of the aziridinium ion.

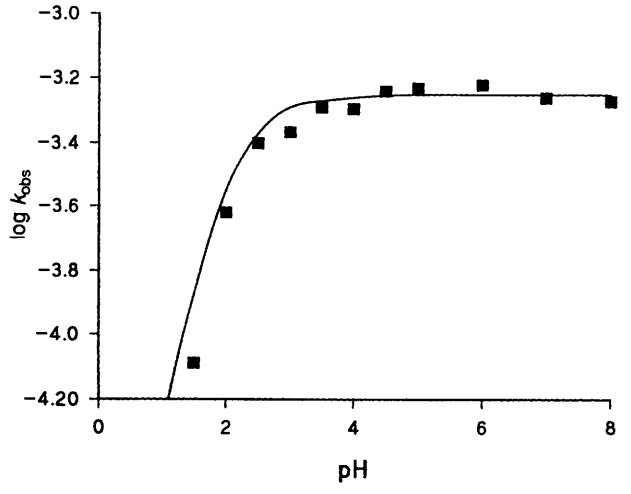


Fig. 3 pH–rate profile for the hydrolysis of chlorambucil–spermidine conjugate (**2**) based on the data summarised in Table 1. The experimental data points are plotted together with the theoretical curve derived from the mechanism involving only reaction of the neutral amine occurring *via* rate-limiting formation of the aziridinium ion.

protonation. However, the values determined by UV and by NMR are comparable and both are consistent with expectation. All of the relevant pK_a values that we have determined together with relevant reference compounds from the literature are summarised in Table 2.

It is apparent that the drop in the observed rate of hydrolysis coincides with the pK_as of the nitrogen mustards supporting the expectation that the reactive form of the aromatic nitrogen mustards is the unprotonated amine and that protonation of the nitrogen mustard leads to a dramatic decrease in the rate of hydrolysis. Assuming the minimal kinetic scheme for the hydrolysis of the mustard to involve reaction only through the neutral amine (**3**) one can derive the corresponding kinetic scheme below.

$$\text{Rate} = k_{obs}[\text{RCl}] \quad (1)$$

If the rate of reaction of the protonated mustard (**7**) ($RHCl^+$) is negligible then the observed rate will depend on pH since the amount of RCl will depend on the K_a.

Table 1 First order rate constants for the hydrolysis of chlorambucil (**1**) and the chlorambucil–spermidine conjugate (**2**) at 37 °C in various aqueous buffers at different pH

Chlorambucil				Spermidine–chlorambucil conjugate		
pH	$k_{obs}/10^{-4}$ s^{-1}	log k_{obs}	[RCl]/[RCl]$_{tot}$	pH	$k_{obs}/10^{-4}$ s^{-1}	log k_{obs}
8[a]	6.08	−3.22		8[a]	5.34	−3.27
7[a]	6.12	−3.21		7[a]	5.52	−3.26
6[a]	7.13	−3.15		6[a]	6.06	−3.22
5[a]	6.62	−3.18		5[a]	5.59	−3.25
5[b]	5.66	−3.25	0.998	5[b]	6.15	−3.21
4.5[b]	5.62	−3.25	0.994	4.5[b]	5.77	−3.24
4[a]	5.89	−3.23		4[a]	5.48	−3.26
4[b]	6.04	−3.21	0.983	4[b]	4.73	−3.33
3.5[b]	5.90	−3.23	0.947	3.5[b]	5.08	−3.29
3[a]	4.31	−3.37		3[a]	4.08	−3.39
3[b]	5.02	−3.30	0.849	3[b]	4.55	−3.34
2.5[b]	3.59	−3.44	0.640	2.5[b]	4.01	−3.40
2[b]	1.84	−3.74	0.360	2[b]	2.41	−3.62
1.5[b]	0.65	−4.19	0.151	1.5[b]	0.81	−4.09

[a] Citrate–phosphate buffer. [b] Perchlorate–perchlorate buffer.

Table 2 pK_a values for the various nitrogen mustards of relevance to this study

Structure	pK_a
Chlorambucil (**1**)	2.3[a]
Spermidine–chlorambucil conjugate (**2**)	2.0,[a] 2.3[b]
Chlorambucil–dialcohol (**6**)	4.8[a]
Spermidine–chlorambucil conjugate dialcohol	3.4[a]
Aniline	4.80,[a] 4.75,[b] 4.87,[c] 4.70,[d] 4.61[e]
N,*N*-bis(2-chloroethyl)aniline	2.2[f]
N-Ethyl-*N*-(2-chloroethyl)aniline	3.5[f]
N,*N*-Diethlyaniline	5.84,[f] 6.57[c]
N,*N*-bis(2-hydroxyethyl)aniline	4.8[e]
Aromatic nitrogen mustard (**11**)	6.05[a]

[a] This study, determined by UV spectroscopy. [b] This study, determined by NMR spectroscopy. [c] Ref. 13. [d] Ref. 17. [e] Ref. 18. [f] Ref. 14. [g] Ref. 12.

Hence

$$k_{app} = k_{obs}\frac{K_a}{K_a + [H^+]} \quad (2)$$

where k_{obs} is the observed first-order rate constant for the neutral nitrogen mustard (*i.e.* at pH $\gg$ 2.3) and k_{app} is the actual rate constant at each pH. Thus the apparent rate constant will depend on the pH and the ionisation constant (K_a) for the mustard. Fig. 2 presents the experimental data together with the theoretical pH–rate profile based on this kinetic scheme which shows very good agreement.

To demonstrate that the protonated species (**7**) does not react at a significant rate with respect to the rate of reaction of the neutral amine (**3**) one can expand eqn. (1) to take explicit account of any reaction occurring through the protonated form of the mustard.

$$\text{Rate} = k_{obs}([RCl] + [RHCl^+]) \quad (3)$$

$$\text{Rate} = k_u[RCl] + k_p[RHCl^+] = k_{obs}[RCl]_{total} \quad (4)$$

where k_u is the rate constant for the unprotonated form and k_p is the rate constant for the protonated form.

Hence

$$k_{obs} = \frac{k_u[RCl]}{[RCl]_{total}} + \frac{k_p[RHCl^+]}{[RCl]_{total}} \quad (5)$$

From eqn. (5) it can be seen that a plot of k_{obs} *vs.* [RCl]/[RCl]$_{total}$ should give a straight line with a slope of k_u and a y intercept of k_p[RHCl$^+$]/[RCl]$_{total}$. Using the pK_a for chlorambucil of 2.3 determined by UV spectroscopy the fraction of neutral mustard was determined and the plot is shown in Fig. 4. If the rate of reaction of the protonated mustard is insignificant k_p approaches zero and therefore the intercept term k_p[RHCl$^+$]/[RCl]$_{total}$ must also approach zero. As can be seen in Fig. 4 the plot of k_{obs} *vs.* [RCl]/[RCl]$_{total}$ is indeed a straight line which passes through the origin, that is to say the rate of reaction of the protonated mustard is insignificant.

We have looked at the rate of the reaction of chlorambucil in different buffers and observe that there are small differences in rate arising from the change in buffer at a given pH, Table 3. The interpretation of these differences becomes difficult since some of these common buffer systems contain chloride ion and as discussed below the common ion rate suppression leads to significant rate changes. However, for buffer systems that do not contain chloride ion the observed rates of reaction of chlorambucil and the chlorambucil–spermidine conjugate are the same within experimental error, and independent of pH in the range 4 to 9. Also for buffers containing chloride where the chloride ion concentration does not change with changing pH the rates are pH independent.

Effects of salts and buffers

The solvolysis of chlorambucil and the conjugate in phosphate buffer each showed a mixture of final products (Scheme 2) that included the diol (**6**) (identified by comparison with the sole

Table 3 First order rate constant for the hydrolysis of chlorambucil (**1**) at 37 °C in different buffer systems

Buffer	pH	$[Cl^-]$/mol dm^{-3}	$k_{obs}/10^4$ s^{-1}	log k_{obs}
Phosphate	7	—	7.07	−3.15
	8	—	7.06	−3.15
	8.5	—	7.27	−3.14
Citrate–phosphate	5	—	6.62	−3.18
	6	—	7.13	−3.15
	7	—	6.12	−3.24
	8	—	6.08	−3.22
Borate	8	0.05	3.94	−3.40
	9	0.05	4.31	−3.37
Tris	6	0.07	3.74	−3.43
	7	0.05	4.13	−3.37
	8	0.03	5.64	−3.25
	9	0.005	6.76	−3.17

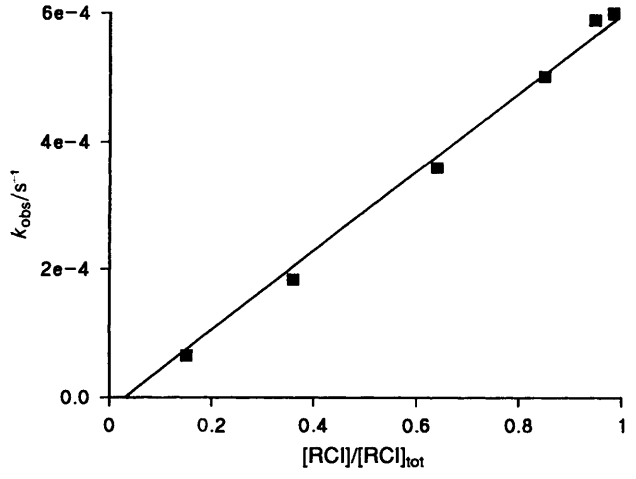


Fig. 4 Plot of the observed rate constant for hydrolysis of chlorambucil (**1**) *vs.* the fraction of the unprotonated form present

product obtained from hydrolyses conducted in aqueous NaOH), and two other products that increased with increasing phosphate buffer concentration that are presumed to correspond to the hydroxy monophosphate (**9**) (Y = $-OPO_3^{2-}$) and the bisphosphate (**10**) (Y = $-OPO_3^{2-}$). During the courses of these reactions for each the expected two intermediates (**5**) and (**8**) in which only one branch of the mustard had reacted either with water or phosphate were seen to build up then react further to give the three final products. The rate of disappearance of chlorambucil and the conjugate at pH 7 in phosphate buffer was shown to be independent of the concentration of phosphate nucleophile over a range of almost three orders of magnitude, Table 4. The reaction is therefore zero order with respect to the nucleophile, consistent with a rate determining formation of the aziridinium ion followed by a fast reaction with the nucleophile. Approximately equal amounts of the phosphate esters and the hydroxy products are obtained at a buffer concentration of approximately 0.03 mol dm^{-3}. Since these are derived from a direct competition for the common aziridinium ion intermediate by phosphate or by water then it follows that at the buffer concentration where equal amounts of products derived from the two competing nucleophiles are produced then $k_2[Y] = k_3[H_2O]$, where Y = phosphate and $[H_2O]$ = 55 mol dm^{-3}. From this it can be seen that the ratio of k_2/k_3 = 55/0.03 = 1833, *i.e.* the rate of reaction of the aziridinium ion with phosphate dianion is over three orders of magnitude greater than with water (Scheme 2). In the above experiments in which the phosphate concentration was varied, the ionic strength was also allowed to vary. Since the rate did not change significantly it is clear that the rate of reaction of these nitrogen mustards is not significantly affected by ionic strength in buffered aqueous solutions.

Table 4 Effects of increasing phosphate ion concentration on the first order rate constants for the hydrolysis of chlorambucil (**1**) and chlorambucil–spermidine conjugate (**2**) at 37 °C in phosphate buffer at pH 7

[Phosphate]/mol dm^{-3}	Chlorambucil $k_{obs}/10^{-4}$ s^{-1}	Conjugate $k_{obs}/10^{-4}$ s^{-1}
0.5	7.45	5.93
0.2	7.07	—
0.1	6.00	5.48
0.05	7.19	5.46
0.025	7.79	5.47
0.01	7.39	5.39
0.005	7.31	5.25
0.001	7.22	4.98

If the mechanism of hydrolysis of the nitrogen mustard does indeed involve the rate limiting formation of the aziridinium ion then addition of chloride ion would be expected to suppress the rate by virtue of the added chloride ion competing for the aziridinium ion and making the back reaction to give the neutral mustard significant. We have shown that the hydrolysis of chlorambucil is subject to significant common ion effects (Table 5). In phosphate buffer pH 7.0 the second step of the reaction is a competition between reaction of the aziridinium ion with water and with the phosphate dianion, and in this system the observed rate is decreased by a factor of *ca.* 3.5 in the presence of 0.5 mol dm^{-3} NaCl. Given that we have already shown that there is a negligible general ionic strength effect (see above) this rate suppression is most easily accounted for in terms of the common ion effect and provides supporting evidence for the reaction proceeding *via* the aziridinium ion. Shown below is the derivation of the kinetics describing this mechanism that has allowed us to demonstrate unambiguously that the rate suppression is indeed due to the common ion effect. We have also been able to extract the relative values of some of the individual rate constants. If the first step is reversible and rate limiting then

$$\text{Rate} = \frac{-\text{d}[\text{RCl}]}{\text{d}t} = k_1[\text{RCl}] - k_{-1}[\text{R}^+][\text{Cl}^-] \quad (6)$$

Applying the steady state approximation to the aziridinium ion intermediate (R^+)

$$\frac{-\text{d}[\text{R}^+]}{\text{d}t} = k_1[\text{RCl}] - k_{-1}[\text{R}^+][\text{Cl}^-] - k_2[\text{R}^+][\text{Y}] - k_3[\text{R}^+][\text{H}_2\text{O}] = 0 \quad (7)$$

$$k_1[\text{RCl}] = [\text{R}^+](k_{-1}[\text{Cl}^-] + k_2[\text{Y}] + k_3[\text{H}_2\text{O}]) \quad (8)$$

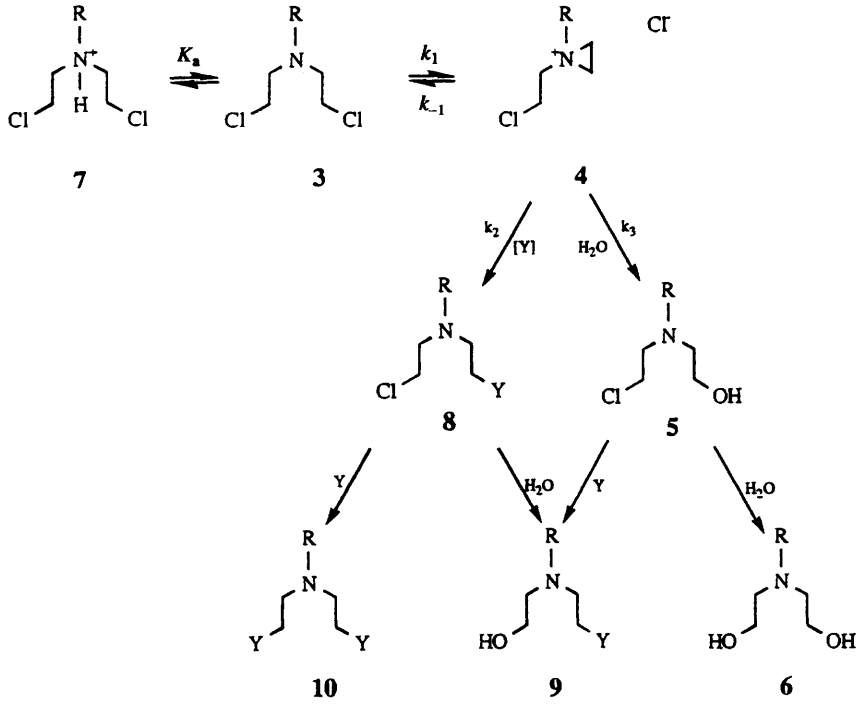
$$[\text{R}^+] = \frac{k_1[\text{RCl}]}{(k_{-1}[\text{Cl}^-] + k_2[\text{Y}] + k_3')} \quad (9)$$

where $k_3' = k_3[H_2O]$.

Substituting eqn. (9) into (6)

$$\frac{-\text{d}[\text{RCl}]}{\text{d}t} = k_1[\text{RCl}] - \frac{k_1k_{-1}[\text{RCl}][\text{Cl}^-]}{k_{-1}[\text{Cl}^-] + k_2[\text{Y}] + k_3'} \quad (10)$$

$$\Longrightarrow \text{Rate} = \frac{k_1[\text{RCl}](k_2[\text{Y}] + k_3')}{k_{-1}[\text{Cl}^-] + k_2[\text{Y}] + k_3'} \quad (11)$$



Scheme 2

Table 5 Effects of increasing chloride ion concentration on the first order rate constant for the reaction of chlorambucil (**1**) in phosphate buffer (0.025 mol dm^{-3}, pH 7)

$[Cl^-]$ mol dm^{-3}	$k_{obs}/10^{-4}$ s^{-1}	$(1/k_{obs})$/s
0	7.79	1284
0.15	4.25	2353
0.25	3.45	2894
0.3	3.09	3236
0.35	2.91	3436
0.4	3.02	3311
0.5	2.34	4274
0.6	2.02	4950

since rate = k_{obs} [RCl] then

$$k_{obs} = \frac{k_1(k_2[Y] + k_3')}{k_{-1}[Cl^-] + k_2[Y] + k_3'} \quad (12)$$

$$\frac{1}{k_{obs}} = \frac{k_{-1}[Cl^-]}{k_1(k_2[Y] + k_3')} + \frac{1}{k_1} \quad (13)$$

From eqn. (13) it can be seen that varying the concentration of Cl^- and plotting $1/k_{obs}$ against $[Cl^-]$ gives an intercept of $1/k_1$ and a slope of $k_{-1}/k_1(k_2[Y] + k_3')$. As $[Cl^-]$ tends to zero then $1/k_{obs} = 1/k_1$. The plot is shown in Fig. 5 from which is derived the value of 7.3×10^{-4} s^{-1} for k_1 from the intercept. Using the value of k_2/k_3 of 1833 derived above we can extract the remaining relative rates from the slope of the plot in Fig. 5; $k_{-1}/k_2 = 0.23$ and $k_{-1}/k_3 = 422$. As can be seen from these relative rates the extent of the competition of external ion return depends on the nature of the nucleophile.

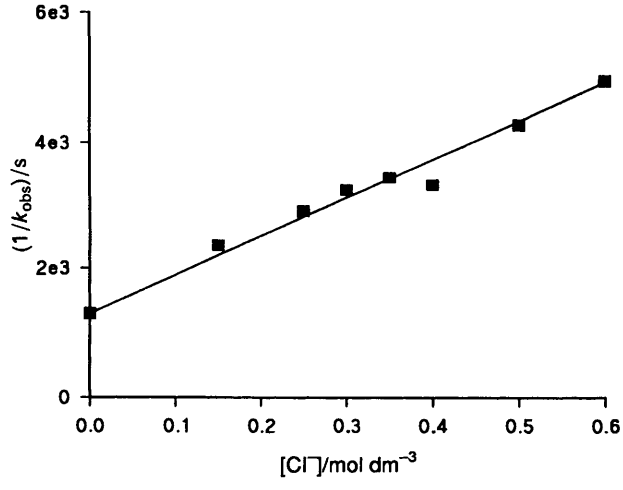


Fig. 5 The effect of increasing chloride ion concentration on the rate of hydrolysis of chlorambucil (**1**) in phosphate buffer (0.025 mol dm^{-3}, pH 7)

Nature of the nucleophile

Although we have found no evidence of any pH–rate dependence in the range of 8 to 3.5 and indeed no significant rate dependence on buffer concentration in a variety of different buffer systems, we have looked specifically at the effect of imidazole on the rate of hydrolysis in part because of the recent study that appeared to detect a small rate acceleration due to the imidazole that was assigned to a general-base-catalysed pathway.[12] In the range 0–0.1 mol dm^{-3} imidazole in 0.2 mol dm^{-3} phosphate buffer (pH 7) the rate of loss of chlorambucil was the same within experimental error and therefore independent of the imidazole concentration, Table 6. At the higher concentrations of imidazole, alkylation products of both imidazole and phosphate were observed. At 0.01 mol dm^{-3} imidazole and 0.03 mol dm^{-3} phosphate (pH 8) the products of the competing reactions of the aziridinium ion with the alternative nucleophiles (water, phosphate and imidazole base) were present in approximately equal amounts which allows the relative rates of the competing second steps to be simply deduced. Clearly imidazole is a three-fold better nucleophile than phosphate under these conditions.

Despite not being able to find evidence of any general-base-catalysed pathway the results of this study with imidazole are interesting in view of the specificity for nitrogen mustards

Table 6 Effects of imidazole on the first order rate constant for the reaction of chlorambucil in phosphate buffer (0.03 mol dm^{-3}, pH 8)

[Imidazole]/mol dm^{-3}	$k_{obs}/10^{-4}$ s^{-1}
0	7.07
0.01	7.24
0.025	6.54
0.05	6.68
0.075	7.16
0.1	6.87

to alkylate DNA at N-7 of the guanine bases. Alkylation of imidazole may therefore be seen as a model alkylation reaction of a nitrogen heterocycle. Interestingly, attempts to demonstrate direct reaction of chlorambucil or the conjugate with 2-deoxyguanosine were unsuccessful. At 0.01 mol dm^{-3} 2-deoxyguanosine in sodium perchlorate buffer pH 5, and phosphate buffer pH 7, only the hydrolysis and in the case of the latter buffer alkylation products could be detected. This puts a rough upper limit on the relative rate of reaction with N-7 of 2-deoxyguanosine for which the rate of reaction of the aziridinium ion with 2-deoxyguanosine must be less than a tenth of that observed with imidazole.

In view of the fact that the original studies of Benn *et al.*[4] showed that a thiol nucleophile reacted with an aromatic nitrogen mustard without exchange of the methylene groups of the chloroethyl group we looked at the kinetics of reaction with a thiol nucleophile. Because of the relevance to biologically important thiols, such as cysteine residues in proteins, we chose 2-sulfanylethanol (2-mercaptoethanol) as a model. The rate of reaction was again independent of the concentration of the thiol nucleophile, suggesting that this reaction proceeds by a mechanism involving the rate-limiting formation of the aziridinium ion. The products from alkylation of the sulfur were detectable at comparatively low levels of thiol (0.2–2 mmol dm^{-3}) and the relative rate of the competing second steps was again deduced by determining the concentration of thiol required to produce equal amounts of product from alkylation of phosphate, water and 2-sulfanylethanol. At pH 7 this concentration was 2×10^{-3} mol dm^{-3} whereas at pH 8 the concentration required falls to 2×10^{-4} mol dm^{-3}. This is entirely expected if the nucleophilic species is the thiolate since the pK_a for 2-sulfanylethanol is 9.7.

Medium effects

Because the rate limiting step involves formation of the aziridinium ion it is reasonable to expect that the overall rate of reaction might be strongly dependent on the medium since the energetics of this are likely to be dominated by the ability of the medium to solvate the ionic products. We were interested in the magnitude of such effects since this may also be pertinent to the half-lives of these drugs in biological systems. Furthermore, the recent study on the hydrolysis of a series of related aromatic nitrogen mustards by O'Connor *et al.*[12] appeared to observe significantly lower rates than we have observed in this study despite the fact that their nitrogen mustards appear to be significantly more basic than chlorambucil and the chlorambucil–spermidine conjugate.

For comparison with the results of O'Connor *et al.*[12] we initially looked at rate differences in a non-chloride-containing buffer (0.05 mol dm^{-3} $HClO_4/NaClO_4$; pH 5.0) on addition of 50% v/v acetone. The k_{obs} in the aqueous buffer at 37 °C was 5.66×10^{-4} s^{-1} which corresponds to a $t_{\frac{1}{2}}$ of *ca.* 20 min, Table 1, as compared to a k_{obs} in 50% acetone under otherwise identical conditions of 3.6×10^{-5} s^{-1} which corresponds to a $t_{\frac{1}{2}}$ of 320 min. Thus the rate is reduced by a factor of 16 and the observed rate of reaction in 50% aqueous acetone at 37 °C is comparable to that reported by O'Connor for their related series of aromatic nitrogen mustards.

The rates of hydrolysis of chlorambucil (**1**) and the chlorambucil–spermidine conjugate (**2**) in biological systems may be differentially affected by the potentially different interactions with cellular components. In particular it is clear that chlorambucil (**1**) enters cells by passive diffusion whereas the conjugate (**2**) is presumed to require the active polyamine uptake system since it is assumed that the polycation cannot passively diffuse across the lipid bilayer. These considerations together with the observation of the pronounced effects of added cosolvent on the rate led us to look at the rate of hydrolysis in the presence of cationic micelles. The rate of reaction of chlorambucil (**1**) and chlorambucil–spermidine conjugate (**2**) were both determined in buffered aqueous solution (0.2 mol dm^{-3} phosphate, pH 7) containing hexadecyltrimethylammonium chloride (CTAC; 0.015 mol dm^{-3}). The observed rate constants for disappearance of nitrogen mustards were clearly first order in each case. For chlorambucil the observed rate in the presence of CTAC micelles was 1×10^{-5} s^{-1} and for conjugate (**2**) the rate was 1.34×10^{-4} s^{-1}. This corresponds to a $t_{\frac{1}{2}}$ of 1155 and 90 min respectively, as compared to a $t_{\frac{1}{2}}$ for both chlorambucil and the conjugate of approximately 20 minutes in the absence of micelles.

Effect of temperature

For chlorambucil we have determined the first order rate constants over a 20 degree temperature range to determine the Arrhenius parameters. The plot of ln k_{obs} against $1/T$ gave a reasonable straight line with intercept of ln A and a gradient of $-E_a/R$, from which we have determined the activation energy to be 97.5 kJ mol^{-1} and the value of ln A is 30. The derived activation energy is similar to values reported by O'Connor *et al.*[12] for their series of aromatic nitrogen mustards which ranged from 86 to 118 kJ mol^{-1}.

Discussion

We initiated this study into the instrinsic reactivity of chlorambucil and the chlorambucil–spermidine conjugate as a result of the observation of a 10^4 increased reactivity with DNA *in vitro* for the conjugate. In principle this dramatic increase in reactivity could either arise from an increase in the intrinsic reactivity of the chlorambucil–spermidine conjugate compared to chlorambucil or it could reflect the favourable electrostatic interaction between the polyammonium moiety of the conjugate leading to localisation of the alkylating agent close to the DNA. In this latter case the conjugate functions in much the same way as an affinity-based enzyme inhibitor except that it is not clear that polyamines have a unique binding site on the DNA. The observed rates of reaction of chlorambucil and the chlorambucil–spermidine conjugate presented above are clearly very similar thus ruling out the possibility that the increased cross-linking reactivity of the conjugate arises from an increase in intrinsic reactivity and supporting our belief that this increased cross-linking reactivity arises solely from the affinity of polyammonium cations for DNA.

The pH–rate profiles for the hydrolysis of chlorambucil and the conjugate are virtually identical. Both show rates of reaction that are independent of pH in the range of 4–8 and for both the rate drops dramatically in the pH range 1–3. The steep decrease in rate corresponds in both cases to the pK_a of the aryl amino group which is in accord with a mechanism or reaction that explicitly involves the neutral amino group in both systems. The simplest explanation of the pH–rate profile would involve significant reaction only through the neutral mustard, with the nitrogen involved in nucleophilic participation and the reaction proceeding *via* the rate-limiting formation of the aziridinium

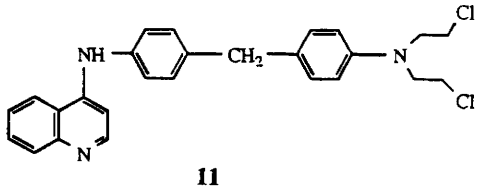
ion intermediate. The observed pH–rate profiles for both the reaction of chlorambucil and the conjugate show excellent fits to theoretical curves derived for a mechanism that assumes that the formation of the aziridinium ion intermediate is rate determining and that the rate of reaction of the protonated aromatic mustard is insignificant. Two important further observations support this mechanistic scheme. Firstly, the rate of disappearance of the starting nitrogen mustard is clearly first order with respect to the nitrogen mustard and, in the absence of chloride ion, is independent of the concentration of the nucleophile (zero order) over a range of three orders of magnitude in concentration of the nucleophile. Secondly, the rate of the reaction is suppressed by addition of chloride ion as a result a competing back reaction to the starting nitrogen mustard, reducing the steady state amount of the aziridinium ion.

Although the rate limiting step is the first step of the reaction leading to the formation of the aziridinium ion it has been possible to deduce the relative rates of the competing second steps involving the ring opening of the aziridinium ion intermediate. In terms of the site of alkylation by aromatic nitrogen mustards *in vivo* the fact that a phosphate dianion reacts *ca.* 2×10^3 times faster than water, imidazole reacts *ca.* 6×10^3 times faster and a simple thiol greater than 2×10^5 times faster is clearly very important. The latter if corrected for the amount of thiolate present at pH 8 would increase to 1×10^7. The apparent chemoselectivity in favour of thiols has obvious importance in terms of alkylation sites in cells.

With respect to our *in vitro* cytotoxicity studies and the *in vivo* antitumour activities these studies show that in terms of anticipated losses of drug due to chemical hydrolysis in free solution, chlorambucil and the conjugate behave similarly and both show a $t_{\frac{1}{2}}$ of *ca.* 20 min at 37 °C at all physiologically relevant pHs. Of course there may be major differences in the metabolism of the two species and indeed preliminary observations suggest that the normal β-oxidation of the side chain seen for chlorambucil is blocked in the conjugate and that the latter survives apparently unmetabolised for longer than the parent drug. Clearly there is a pronounced medium effect on the rate of reaction of chlorambucil and the chlorambucil–spermidine conjugate. Addition of 50% acetone reduces the rate of reaction of chlorambucil by a factor of 16 ($t_{\frac{1}{2}}$ = 320 min) as a result of disfavouring the formation of the aziridinium ion.

Pursuing this further we reasoned that there was likely to be a significant difference between chlorambucil and the chlorambucil–spermidine conjugate in terms of potential interactions with a variety of biological macromolecules and this might have an effect on the rate of reaction. In particular, in view of the relatively hydrophobic nature of the neutral form of chlorambucil this molecule may, for example, distribute into lipid bilayers. Indeed, the fact that chlorambucil can readily enter cells by passive diffusion suggests that this distribution must be reasonably favourable. In contrast, the conjugate (**2**) bears three positive charges and is therefore unlikely to be able to cross the lipid bilayer. In a preliminary model for this we have shown as part of this study that the reaction of chlorambucil shows marked micellar inhibition in CTAC (0.015 mol dm^{-3}, cmc for CTAC 1×10^{-3} mol dm^{-3}), with the rate being reduced by a factor of 60. Although the surfactant counter ion is chloride the rate reduction cannot be due to the common ion effect since 0.5 mol dm^{-3} NaCl only reduces the rate by 3.5 in the same buffer system and the chloride ion concentration in the micellar system is only 0.015 mol dm^{-3}. The rate of reaction of the conjugate (**2**) shows a rate difference in the presence of CTAC of a only a factor of three which could in part be due to a chloride ion effect and/or a general medium effect. The very large rate reduction for chlorambucil in the presence of CTAC micelles is most easily explained in terms of the partitioning of the mustard into the micelle, and the unfavourable formation of the aziridinium ion within this hydrophobic environment. Clearly both the medium effect and the micellar effects have important implications for both the *in vitro* and *in vivo* studies since the cytosol is not strictly a dilute aqueous buffer and there are opportunities for either of these drugs to become associated with proteins and lipids which, based on this preliminary observation in a micellar system, is capable of having a differential effect on the relative half-lives in cellular systems. This observation provides a possible explanation for the earlier report that the hydrolysis of chlorambucil is significantly slower in the presence of serum.[14]

Putting our results in context with other studies in this field, the conclusion that these aromatic nitrogen mustards react *via* an aziridinium ion intermediate and that its formation is rate limiting is entirely consistent with related systems and the results reported by others. The most recent study by O'Connor *et al.*[12] draws a similar conclusion although they report a more complex pH–rate profile and appear to suggest competition from a general-base-catalysed hydrolysis of the protonated nitrogen mustard by imidazole. The validity of their conclusion rests heavily upon the pK_as of the nitrogen mustards studied by O'Connor *et al.*[12] since the kinetic analysis fractionates the observed rates into those parts occurring *via* the protonated and unprotonated forms of the mustard. The mustards used in the O'Connor's study are significantly different from chlorambucil and the chlorambucil–spermidine conjugate making direct comparisons difficult. However in the case of the mustard (**11**)



11

shown below, the *para*-substituent is not conjugated to the aromatic nitrogen mustard and therefore must exert its effect through a purely inductive effect. The pK_as of simple aromatic nitrogen mustards are expected to be below 3 because of effects of the two electronegative chlorine substituents, and our measured pK_as of 2.3 and 2.0 for chlorambucil and the chlorambucil–spermidine conjugate respectively are as expected very similar to the parent *N*,*N*-dichloroethylaniline.[13] It seems improbable that the *para*-benzylic substituent in (**11**) would have a sufficiently profound inductive effect to shift this pK_a to 6.05. Since this represents a pK_a that was measured by UV spectroscopy it is possible that this pK_a is associated with one of the other ionisable groups in the molecule. If the pK_a of (**11**) is significantly lower than assumed then the amount of the protonated mustard present in the pH range studied would be insignificant and the proposed general-base-catalysed direct attack of water on the alkyl chloride would seem unreasonable. The rate differences reported for the hydrolysis of (**11**) in the presence of imidazole and at different pHs are very small but there does appear to be a small concentration dependence in the case of imidazole which requires some explanation. If the buffer used by O'Connor *et al.* contained chloride ion then the observed rate will depend on the relative concentration of chloride ion and nucleophile (in this case imidazole), see eqn. (13), and the rate becomes dependent on the nucleophile concentration This may be sufficient to account for the observed dependence of k_{obs} on imidazole concentration seen by O'Connor.

In our study we have looked at the reactions of chlorambucil

and the chlorambucil–spermidine conjugate over a wide range of buffer concentrations and pHs and we can find no evidence for any pathway other than that involving the formation of the aziridinium ion. Given that it is apparent that the formation of the aziridinium ion is very sensitive to the nature of the medium, and that the presence of added chloride ion which often forms a part of many common buffer systems suppresses the rate significantly, the interpretation of very small differences in rate should perhaps be approached with caution.

Experimental

Materials and methods

Unless otherwise stated chemicals were from the Sigma Chemical Co. Ltd. and were used as supplied. Concentrated hydrochloric acid, acetone, HPLC grade acetonitrile and HPLC grade methanol were obtained from Fisons plc. Deuterium oxide was from Goss Scientific Instruments Ltd. Water was purified by a Maxima Ultra Pure unit to 18.2 MΩ (Elga Ltd., High Wycombe).

Spermidine–chlorambucil conjugate (**2**) was synthesised and characterised as previously reported.[1]

Chlorambucil diol and Spermidine–chlorambucil diol. Chlorambucil (20 mg) or the conjugate in 1.5 cm^3 of 0.3 mol dm^{-3} NaOH was left for four days to convert to the corresponding diol, and characterised by mass spectrometry. The solution was neutralised with HCl and the diol concentration determined from the chlorambucil UV calibration curve.

Measurement of pK_a by UV

The UV spectra of chlorambucil (**1**) and the conjugate (**2**) at high pH were recorded on a Beckman DU 7500 Spectrophotometer and found to show maxima at 258 and 304 nm, with extinction coefficients of ε = 15 200 (258) and ε = 1600 (304). These values are reasonable since they agree favourably with the literature values for *N*,*N*-diethylaniline ε = 15 800 (258) and ε = 2500 (304).[16] On protonation of the aryl nitogen the intensities of these absorption bands are greatly reduced to ε = 274 (258), and these pH-dependent changes in the UV spectra have been used to determine the pK_a values. Since chlorambucil has poor solubility in water, a solution of 1 mg cm^{-3} in acetonitrile (HPLC grade) was diluted 1 : 30 with the appropriate buffer to give a final concentration of 0.12 mmol dm^{-3}. Buffers made from a mixture of HCl and KCl were used to adjust the pH over the range 0.2–4.8, and citric acid–Na_2HPO_4 buffer was used over the range 2.6–7.6. In all cases the A_{258} was followed as a function of pH, and the pK_a value was calculated according to the method of Albert and Serjeant.[13] As a control, the pK_a of aniline was determined and found to agree with the literature values. The pK_a values of spermidine–chlorambucil conjugate (**2**), chlorambucil diol (**6**), and spermidine–chlorambucil diol were also determined by the same method (Table 2).

Measurement of pK_a by NMR spectroscopy

The NMR spectra were recorded on a Bruker ARX 250 MHz spectrometer. The spectra for both chlorambucil (**1**) and the conjugate (**2**) show two major changes on protonation of the aromatic nitrogen. Firstly, the two aromatic doublets (due to the *ortho* and *meta* protons) move downfield and closer together (AX→AB). Secondly, the multiplet due to the $ClCH_2CH_2N$ protons moves downfield and separates into two triplets ($A_2B_2 \rightarrow A_2X_2$). Only the conjugate could be studied throughout the pH range by NMR spectroscopy since chlorambucil is only sufficiently soluble in aqueous solution at the two extremes of pH where it bears either a negative or a positive charge, in the region where the molecule is neutral chlorambucil is insufficiently soluble in aqueous solution to record the ^1H NMR spectrum. For the conjugate using the above two regions of the ^1H NMR spectrum, the pH-dependent differences in chemical shifts were determined from which the pK_a could be determined. For each buffered sample, 20 mg conjugate (**2**) was added to 0.5 cm^{-3} of 0.5 mol dm^{-3} citric acid (in D_2O) and the pH was adjusted with NaOD. Samples were also made in 1 mol dm^{-3} DCl and 1 mol dm^{-3} NaOD. Again, the pK_a of aniline was also determined and found to agree with the literature.

Kinetic analysis

Chlorambucil samples were made with a stock of 1 mg cm^{-3} in MeCN diluted to 0.1 mmol dm^{-3} with buffer. Spermidine–chlorambucil (**2**) samples were made with a stock of 10 mmol dm^{-3} in water, diluted to 0.34 mmol dm^{-3} with buffer. These were incubated at 37 °C in a shaking water bath unless otherwise stated. Aliquots were taken at pre-determined time intervals, and immediately analysed by HPLC (see below). The following buffers were utilised, and prepared by titration of the appropriate solutions to the required pH, using a PHM 83 AUTOCAL pH meter with a PHC 4406 combined electrode (Radiometer).

pH–Rate profile. Buffers within the pH range 1.5–5 were prepared by mixing 0.05 mol dm^{-3} $HClO_4$ and 0.05 mol dm^{-3} $NaClO_4$, and within the pH range 3–8, by mixing 0.1 mol dm^{-3} citric acid and 0.2 mol dm^{-3} Na_2HPO_4.

Rate in different buffers. The following buffer systems were used. (*i*) 0.2 mol dm^{-3} NaH_2PO_4 titrated against 0.2 mol dm^{-3} Na_2HPO_4 at pH 7, 8 and 8.5. (*ii*) 0.1 mol dm^{-3} Citric acid titrated against 0.2 mol dm^{-3} Na_2HPO_4 at pH 5, 6, 7 and 8. (*iii*) A solution 0.1 mol dm^{-3} with respect to both KCl and H_3BO_3 titrated against 0.1 mol dm^{-3} NaOH at pH 8 and 9. (iv) 0.05 mol dm^{-3} TRIS-HCl titrated against 0.05 mol dm^{-3} TRIS-base at pH 6, 7, 8 and 9.

Effects of salts and buffers

The effect of phosphate concentration on the rate of reaction was measured using a NaH_2PO_4–Na_2HPO_4 buffer at pH 7, over the range 0.001 to 0.5 mol dm^{-3}. Similarly, the effect of chloride concentration was studied using 0.025 mol dm^{-3} NaH_2PO_4–Na_2HPO_4 buffer at pH 7, with added sodium chloride from 0 to 0.6 mol dm^{-3}.

Nature of the nucleophile

The effect of imidazole concentration on the rate of reaction was measured in 0.2 mol dm^{-3} NaH_2PO_4–Na_2HPO_4 buffer at pH 7, with added imidazole from 0 to 0.1 mol dm^{-3}. The relative rates of the competing second step were determined in 0.03 mol dm^{-3} NaH_2PO_4–Na_2HPO_4 buffer at pH 8, with added imidazole over the concentration range 0 to 0.05 mol dm^{-3}.

Alkylation of 2-deoxyguanosine (0.01 mol dm^{-3}) by chlorambucil was attempted in buffer made of a mixture of 0.05 mol dm^{-3} $HClO_4$ and 0.05 mol dm^{-3} $NaClO_4$ at pH 5.

The relative rates of the competing second step in the presence of 2-sulfanylethanol were determined in 0.03 mol dm^{-3} NaH_2PO_4–0.03 mol dm^{-3} Na_2HPO_4 buffer at pH 7 and 8 with added thiol from 0 to 0.01 mol dm^{-3}.

Medium effects

The rate of reaction was studied in both 100% buffer and 50% buffer–acetone (v/v). The buffer was made by titration of 0.05 mol dm^{-3} $HClO_4$ against 0.05 mol dm^{-3} $NaClO_4$ to pH 5. The effect of addition of hexadecyltrimethylammonium chloride (CTAC) to the reaction mixture was determined in 0.2 mol dm^{-3} NaH_2PO_4–Na_2HPO_4 buffer at pH 7 with 0.015 mol dm^{-3} CTAC [critical micelle concentration (CMC) of CTAC = 1.4×10^{-3} mol dm^{-3}].

Effect of temperature

The rate of reaction at temperatures of 27, 32, 37, 42 and 47 °C

was studied in 0.025 mol dm^{-3} NaH_2PO_4–Na_2HPO_4 buffer at pH 7 .

HPLC

HPLC was carried out using a Gilson 712 system fitted with 306 pumps, 811C dynamic mixer and a Rainin Dynamax UV-1 detector with detection at 258 nm. The products were separated on a 4.6 × 125 mm i.d. Whatman Partisphere C_{18} reverse phase column, by a gradient elution method at 1 cm^{-3} min. Eluent A consisted of 1 mol dm^{-3} ammonium acetate pH 6–MeCN (9:1), and B consisted of MeOH–MeCN (9:1). The mobile phase used for chlorambucil was a linear gradient from 30% B (0 min) to 60 %B (8 min) and back to 30 %B (12 min). For the conjugate, the mobile phase was again a linear gradient, but from 40 %B (0 min) to 80% B (12 min), to 80% B (13 min) and back to 40 %B (18 min). Samples were injected *via* a Rheodyne injector fitted with a 20 mm^3 loop. Quantitation was by peak area using calibration curve for chlorambucil from 2.5–100 μmol dm^{-3}, which was linear with a correlation coefficient of 0.996 and a response factor of 2.2×10^5. Under the conditions described above, the extinction coefficients of the nitrogen mustards and their products were found to be the same, and were therefore assumed to have the same response factors as chlorambucil.

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