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The Alkylating Properties of Chlorambucil

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KUNDU, G. C., J. R. SCHULLEK AND I. B. WILSON. *The alkylating properties of chlorambucil.* PHARMACOL BIOCHEM BEHAV **49**(3) 621-624, 1994.—Previous work has indicated an aziridinium ion mechanism in the hydrolysis of chlorambucil, and the present work on the alkylation of nucleophiles fully supports this mechanism. This mechanism forms the basis for understanding the kinetics of alkylation reactions because their rates are limited by the rate of formation of the aziridinium ion and the alkylation reaction competes with the hydrolytic reaction. We have measured α_N, where $\alpha_N(N)$ is the rate of reaction of the aziridinium ion with a nucleophile N relative to its reaction with water for several nucleophiles that are related to those found in proteins. The α values for hydroxide ion and some other bases are greater than 10^3, but the effective values at pH 7.5 are much smaller because there is little base present. The kinetic equations show that it is very difficult to alkylate a nucleophile extensively at pH 7.5 before chlorambucil has hydrolyzed. Therefore, it is not clear why angiotensin-converting enzyme is completely inhibited by low concentrations of chlorambucil. On the other hand, damage to DNA is easily understood.

Chlorambucil Alkylation

WE INVESTIGATED the alkylating characteristics of the antitumor drug chlorambucil (*p*-[*N*,*N*-bis(chloroethyl)amino] phenyl butyric acid) using nucleophiles related to those found in proteins. These nucleophiles were studied because chlorambucil inhibits angiotensin-converting enzyme (4,5,11) and is a potential inhibitor of other enzymes. Despite the very weak basicity of the amine function, an aziridinium ion mechanism is attractive because it would explain the fact that the rate of hydrolysis of chlorambucil is a million times faster than the rate of hydrolysis of primary alkyl chlorides.

The hydrolysis of chlorambucil (Fig. 1) and the alkylation of haemoglobin and serum albumin by chlorambucil have been studied (1-3,6-10), and good evidence has been obtained for the aziridinium ion mechanism (Fig. 2). Because only the unprotonated amine can form the aziridinium ion, the rate of hydrolysis should, as is observed, increase with pH in accordance with the fraction of unprotonated amine (pK_a = 2.5) and level off at pH > 4 (1-3,8,10). The aziridinium ion reacts rapidly with water and other nucleophiles. In this reaction chloride is a special nucleophile because the reaction reforms chlorambucil and therefore should decrease k_1. Chloride does decrease k_1 (1). The reactions with other nucleophiles should compete with the reaction of the aziridinium ion with water and should decrease the amount of hydrolysis but should not change k_1. If, on the other hand, the reactions should not involve the rate-determining formation of a reactive intermediate, chloride should not affect k_1 and other nucleophiles should increase k_1.

The kinetic equations derived from the aziridinium ion mechanism form the basis for the study of the alkylation reactions. When chloride and other nucleophiles, N, are present, k_1 (and k_2) are changed by the factor:

$$\left[1 + \frac{\alpha_{Cl^-}(Cl^-)}{1 + \Sigma\alpha_N(N)}\right]^{-1}$$

Thus, the presence of other nucleophiles should decrease the chloride effect.

METHOD

Chlorambucil (Sigma Chemical Co., >98% pure) and its hydrolytic products were detected photometrically (λ = 255 nm) and quantitated by high performance liquid chromatography (HPLC) (Shimadzu) on a μ-Bondapak C_{18} column (Waters Associates) connected online with a Spectra-Physics Model SP 4290 integrator using a linear acetonitrile gradient from 0% to 80% in 10 mM phosphate buffer, pH 6.0, over a period of 20 min at a flow rate of 0.7 ml/min.

Chlorambucil was used from a stock solution in methanol. For kinetic runs, the chlorambucil stock was diluted 100 times to 0.190 mM in the appropriate aqueous solvent at 25°C or 37°C. Aliquots of 65 μl were used for injection in HPLC

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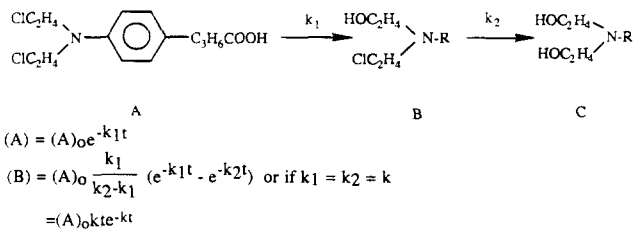

FIG. 1. Hydrolysis of chlorambucil. (A) Chlorambucil, (B) chlorohydroxy compound, and (C) di-hydroxy compound.

(alkaline or acidic solutions were first roughly neutralized to not damage the column). In measuring (A), (B), and (C) the relative peak areas were converted to relative concentrations. The conversion factors were obtained by comparing peak areas at zero, intermediate, and "infinite" times. For example, after 24 h (pH 7.5) with the initial peak area of A set at 1.00, the peak area of C, the only peak, was 0.92. The value for B was 0.95. Other peak areas were not accurately evaluated but none were very different from these values except for a compound formed in 1 M NaOH that is probably a morpholine. This compound had a relative peak area of about 0.39.

RESULTS AND DISCUSSION

We had to repeat the hydrolytic measurements and chloride effect to have our own basis for studying the alkylation of nucleophiles, but our results were not very different from those previously obtained by others (1,3,10). The concentrations of A, B, and C closely followed the kinetic equations with k_2 (0.0086 min^{-1}) slightly smaller than k_1 (0.0090 min^{-1}) (Table 1). The concentration of B rose to a broad maximum of 39% of A_0 at about 110 min and declined to zero. The temperature coefficients of k_1 and k_2 are moderately high, indicating a greater than average energy of formation for the aziridinium ion compared to reactions in general. The chloride effect is less at 37°C than at 25°C, indicating that the energy of activation of the reaction of the aziridinium ion with chloride is less than for water, as expected for a better nucleophile.

Compound B is expected to be more basic than A because the basicity of amines is lowered more by chloroethyl groups than by hydroxyethyl groups (12). The near equality of k_1 and k_2 probably arises from two offsetting effects. The statistical effect should decrease k_2 relative to k_1 by a factor of two, but the greater basicity of B compared to A should facilitate the formation of an aziridinium ion.

The greater basicity of B suggests that in acid solution its hydrolysis will be slowed more than the hydrolysis of A and its maximum concentration will be much greater than at pH

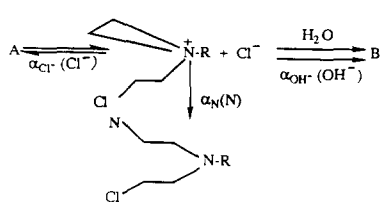

FIG. 2. Scheme for aziridinium ion mechanism. $\alpha_N(N)$ is the rate of reaction of the aziridinium ion with a nucleophile relative to its rate of reaction with water.

7.5. This proved to be the case; in phosphoric acid solution at pH 2.20 both k_1 (0.00239 min^{-1}) and k_2 (0.000232 min^{-1}) were decreased sharply, k_2 more than k_1, and the concentration of B rose to a maximum of 80% of A_0. The pK_a values calculated from these changes were 2.6 for A and 3.7 for B. The pK_a values measured spectrophotometrically were 2.5 for A and 4.2 for C.

Hydroxide ion should be a very good nucleophile, but the products will be the same as those formed by reaction of A and B with water. However, as already noted, the chloride effect should decrease in strong alkali and α_{OH^-} can be evaluated as indicated above. We found that the chloride effect was completely eliminated in 0.1 M NaOH but only slightly decreased at pH 10. The change in 0.8 mM NaOH was suitable for evaluating α_{OH^-}.

We also ran the chlorambucil in 1 M NaOH because some morpholine should form from B by a ring closure in parallel with the formation of the aziridinium ion. A new compound (presumably morpholine) eluted between B and C and it was 15% more than C and k_2 was more than doubled.

We investigated the ability of the kinds of functional groups found in polypeptides and proteins to serve as nucleophiles in reaction with chlorambucil. Three new compounds should be anticipated in accordance with the scheme (Fig. 3). Compound B can undergo a ring closure in strong alkali and it is possible that the first new compound with some nucleophiles might also undergo a ring closure. The fraction of A that goes to B (and then on to other products) is $[1 + \alpha_N(N)]^{-1}$ and the fraction that goes to the first new product is $\alpha_N(N)/[1 + \alpha_N(N)]$.

Sodium acetate was used to study the reactivity of carboxyl groups. In 100 mM acetate, three new well-separated compounds were observed during the course of the reaction. Compound B and the first new compound, the chloro-acetate derivative, were formed in equal amounts, and the final products, the dihydroxy derivative (C), the hydroxy-acetate, and the diacetate derivatives were formed in the approximate ratio 1 : 2 : 1. Thus, A and B and, in this case, the new compound react with acetate to the same extent; $\alpha = 10$ in all reactions. The value of k_1 did not change.

Because sulfur is a good nucleophile, the methionine side chains are reasonable alkylation sites in proteins. At 10 mM concentration of *N*-acetyl-L-methionine amide, somewhat more alkylation occurred than hydrolysis corresponding to $\alpha = 138$. The value of k_1 was not changed, even at 100 mM concentration where there is very little hydrolysis. The first alkylated product, the chloro-(*N*-acetyl-L-methionine amide) derivative, a sulfonium ion was relatively stable and persisted for a long time, corresponding to $k = 1.3 \times 10^{-4}$ min^{-1}. Two possible reasons for this slow reaction come to mind: one, the positive charge of the sulfonium ion may inhibit the formation of the aziridinium ion because this reaction produces a second positive charge only three bond lengths away; two, there may be steric hindrance arising from the cluster about the sulfur atom.

Sulfonium ions are subject to β-elimination reactions in strong alkali, but the sulfonium ions formed as final products in the reaction of chlorambucil and *N*-acetyl-L-methionine amide were stable in 0.1 M NaOH for at least 20 h at 25°C. By contrast, the chlorambucil derivative of angiotensin-converting enzyme is labile in 0.1 M NaOH.

Trimethylamine reacts with chlorambucil to form a quaternary ammonium ion, the chloro-(trimethylamine) derivative, that reacts only very slowly; $k = 2.8 \times 10^{-4}$ min^{-1}. At 50 mM and pH 9.0, trimethylamine completely eliminates the

TABLE 1

RATE CONSTANTS AND α VALUES OF CHLORAMBUCIL REACTION

Nucleophile	pK_a[1]	α[2]	Effective α at pH 7.5
Water	−1.70	0.018	0.018
Chloride		15	15 (9.2 at 37°C)
Hydroxide	15.7	1.6×10^3	0
Acetyl methionine amide		138	138
Trimethylamine	9.80	2.6×10^3	13
Ammonia	9.26	108	1.8
Imidazole	7.00	130	99
Glycylglycine	8.26	1.5×10^3	300
Acetate	4.70	10	10
α-Acetyl lysine methyl amide	10.4	8.7×10^3	11
N-acetyl cysteine	9.5	2×10^4	190

[1]The values for k_1 and k_2 are 0.0090 min^{-1} and 0.0086 min^{-1} at 25°C and 0.036 min^{-1} and 0.034 min^{-1} at 37°C respectively. k_1 was measured in all experiments but did not differ significantly except when chloride was the nucleophile.

[2]The values of α for the reactions of A are given but the values for the reactions of B are the same for chloride, hydroxide, and acetate. With other nucleophiles the reactions of B were more difficult to follow but the α values were not very different.

water reaction and k_1 is unchanged. Also under these circumstances, there is no chloride effect. Even at pH 7.5, trimethylamine is an effective competitor with water. The effective value of α is 13. The value of α for the free base is 2.6×10^3. Ammonia is a much poorer nucleophile. The high nucleophilicity of trimethylamine probably arises from its polarizability.

The effective value of α for ammonia at pH 8.5 is 16. The calculated value for the free base is $\alpha = 108$ and the calculated effective value at pH 7.5 is $\alpha = 1.8$.

A six-membered ring probably forms when glycylglycine is the nucleophile. After 0.5 h of reaction with 15 mM glycylglycine at pH 7.5, there is no C and very little B. There is a new compound in an amount equal to B and another new compound formed in a much larger amount, 3.5 times larger. The new compound formed in the smaller amount behaves like the chloro-(glycylglycine) derivative; the amount of this compound goes through a maximum and falls to zero as the reaction progresses. The other new compound grows as the reaction progresses and is a final product. These observations can be explained if the chloro-(glycylglycine) derivative undergoes a fairly rapid ring closure. Glycylglycine is a good nucleophile; the effective value of α at pH 7.5 is 300 and for the free base, $\alpha = 1.5 \times 10^3$.

The effective value of α for α-acetyl-L-lysine methyl amide measured at pH 7.5 is 11. For the free base $\alpha = 8.7 \times 10^3$.

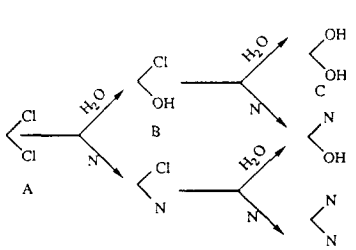

FIG. 3. Possible reaction pathways of chlorambucil with water and an added nucleophile.

At pH 7.5, imidazole is mostly free base. For the free base, $\alpha = 130$.

Even at pH 7.5, *N*-acetyl-L-cysteine is a good nucleophile with an effective $\alpha = 190$. This compound contains a free carboxyl group, but this function is expected to be a poorer nucleophile than the carboxyl group of acetate because it is much less basic. There was no indication that the carboxyl group reacted at the very low concentrations that we used, 5–10 mM to evaluate α. The α value for the sulfydryl anion is $= 2 \times 10^4$ taking the pK_a as 9.5.

In all the reactions, chlorambucil and the chloro-hydroxy derivative reacted quantitatively about the same, but the first derivative formed with an added nucleophile in some cases had a very different rate constant and a different α.

In our experiments we have been concerned with the fraction of chlorambucil that reacted with a nucleophile, but when chlorambucil is used as an enzyme inhibitor we are concerned with the fraction of the nucleophile that is alkylated. Usually the concentration of chlorambucil will be much greater than the concentration of enzyme, even when fairly low concentrations of chlorambucil are used. Even so, it turns out to be very difficult to alkylate a nucleophile extensively with low concentrations of chlorambucil and, therefore, it is surprizing that angiotensin-converting enzyme is so readily inhibited. Consider the reactions of chlorambucil and B derived from chlorambucil with a particular nucleophile with $k_1 = k_2 = k$ and α_N the same for both steps:

$$-\frac{1}{(N)}\frac{d(N)}{dt} = \frac{k[(A) + (B)]\alpha_N}{1 + \alpha_N(N)} \xrightarrow{\alpha_N(N) < 0.05} k\alpha_N[(A) + (B)]$$

$$= k\alpha_N(A)_0(e^{-kt} + kte^{-kt})$$

$$\ln\frac{(N)}{(N)_0} = \alpha_N(A)_0(2e^{-kt} + kte^{-kt} - 2) \xrightarrow[t\to\infty]{} -2\alpha_N(A)_0$$

The fraction of the nucleophile that is alkylated after the reaction is over (i.e., at infinite time) may be small. If $\alpha_N(A)_0 = 1$, about 87% alkylation will be obtained, but if $\alpha_N(A)_0 = 0.1$ only 18% will be obtained. When $\alpha_N(A)_0 = 1$, about 60% alkylation will have occurred when $kt = 1$ (110 min at 25°C), mostly by reaction with A; 44% by reaction

with A and 15% by reaction with B. As time goes on another 27% alkylation will occur, now somewhat more by reaction with B; 17% by reaction with B and 10% by reaction with A. Overall, 54% is alkylated by A and 32% by B. However, the products will be the same with a macromolecule, if a ring closure or cross-linking reaction does not occur.

From the above equations, it is apparent that the speed and the extent of alkylation of a nucleophile will depend upon α_N. For example, if the initial concentration of chlorambucil is 20 μM, α_N must be 10^5 for the alkylation of the nucleophile to approach completion. Angiotensin-converting enzyme is irreversibly inhibited by chlorambucil (4,11). When the nucleophile is a carboxyl group, the reaction is pseudo first order with a second-order rate constant at pH 7.5 and 25°C, about 4×10^3 M^{-1} min^{-1}, and the alkylation is complete with 70 μM chlorambucil (5). The present author measured the rate constant of 2×10^3 M^{-1} min^{-1} and found the extent of inhibition exceeded 95% with as little as 20 μM chlorambucil. This indicates α for angiotensin-converting enzyme is about 10^5 at pH 7.5, 10^4 times higher than for acetate ion and at least 300 times greater than the α values for the nucleophile we have studied. Thus, some explanation for the remarkable reactivity of angiotensin-converting enzyme is required.

Although haemoglobin and serum albumin have been alkylated with chlorambucil, it is difficult to tell from this experiment whether those proteins enhance reactivity or not.

It is easier to understand the damage to DNA caused by chlorambucil because in this case extensive alkylation is not required, the alkylation of one base in 10^5 will probably have serious consequences.

The chloride effect has an interesting pharmacological consequence. The alkylation and hydrolytic reactions are diminished by the high chloride concentration in blood and interstitial fluid, but this inhibition will probably be largely released when chlorambucil enters the cells where the chloride concentration is low.

The alkylation equation looks very different from the equation for a pseudo first-order reaction, yet the deviation of the reaction from a first-order reaction is small for $kt < 1$. The reason for this small deviation is that B is just as good an alkylating agent as A. As a result, a semilog plot of the experimental points will look linear and the value of k will be only about 5% too small for points up to $kt = 1$.

ACKNOWLEDGEMENT

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