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Kinetics and mechanism of the acid-catalyzed hydrolysis of O^6-benzylguanine

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Summary

A variety of analytical techniques were used to elucidate the kinetics, reaction pathway and mechanism of hydrolysis of O^6-benzylguanine, an O^6-alkylguanine-DNA alkyltransferase depleting agent, as a function of pH, buffer concentration, temperature, substituents and halide nucleophiles. The reaction was also carried out in $H_2{}^{18}O$ in order to determine the site of cleavage. The pH-rate profile indicated that the hydrolysis of O^6-benzylguanine was acid-catalyzed, with the neutral O^6-benzylguanine having greater intrinsic susceptibility to undergo acid catalyzed hydrolysis compared to its protonated form. Alternatively, the kinetics could be described by the kinetically indistinguishable process of spontaneous degradation of fully di- and mono-protonated O^6-benzylguanine. In this case, the di-protonated species is more susceptible than the mono-protonated species. Based on the ^{18}O incorporation data, the site of the bond cleavage for hydrolysis of O^6-benzylguanine was unambiguously assigned to the benzylic carbon-oxygen bond leading to the formation of benzyl alcohol and guanine as the predominant products. Benzyl chloride was also detected as a degradation product when the ionic strength of the solution was adjusted with sodium chloride. The rate of hydrolysis of *p*-substituted O^6-benzylguanines increased with increasing electron donating capability of *p*-substituents, consistent with a mechanism involving positive charge formation on the benzylic carbon in the transition state. An Eyring plot resulted in a value for the observed entropy of activation, $\Delta S^{\neq}$, of -2.4 e.u. which was consistent with a unimolecular, $S_{N}1$, reaction. Although the rate of hydrolysis of O^6-benzylguanine was well correlated with the nucleophilicity of various halide nucleophiles, the magnitude of the catalysis was less than anticipated for $S_{N}2$ type reactions. The results suggested that in the presence of bromide and iodide, the transition state had some $S_{N}2$ character. Based on the above observations, a late transition state for this reaction was suggested, where positive charge development at the benzylic carbon atom was quite advanced.

Introduction

O^6-Benzylguanine (NSC-637037, **1**) (Bowels et al., 1963; Frihart and Leonard, 1973) is an effec-

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tive substrate for depleting the mammalian DNA repair protein O^6-alkylguanine-DNA alkyltransferase (Dolan et al., 1990; Pegg, 1990). Depleting tumor cells of this protein produces a marked enhancement in the cytotoxicity of alkylating antitumor drugs whose mechanism of action involves reaction with the O^6 position of DNA guanine residues (Dolan et al., 1991). Furthermore, it has been shown recently that pretreatment of nude mice bearing human tumor xenografts with O^6-benzylguanine leads to a significant increase in the therapeutic effectiveness of a chloroethylating antitumor agent, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (Mitchell et al., 1992).

During formulation studies (Bindra and Stella, unpublished results), we observed that O^6-benzylguanine was quite unstable under acidic conditions where it decomposed to benzyl alcohol and guanine (Scheme 1). Although similar instability was reported for benzylated guanine nucleosides (Moschel et al., 1984), detailed accounts of the factors contributing to the instability of the related O^6-benzylated bases have not been reported. The results of our studies on the kinetics and mechanism of hydrolysis of O^6-benzylguanine and related derivatives are presented here.

Experimental

Materials

O^6-Benzylguanine was obtained from the National Cancer Institute, Bethesda, MD. O^6-(*p*-Methylbenzyl)guanine (**2**) and O^6-(*p*-chlorobenzyl)guanine (**3**) were synthesized by the methods described by Dolan et al. (1990). All organic solvents were HPLC grade and all other chemicals were ACS grade. The water was deionized and distilled using the Mega-pure System (Model MP-1, Corning). The $H_2{}^{18}O$ (97 atom% ^{18}O) was obtained from Aldrich (Milwaukee, WI).

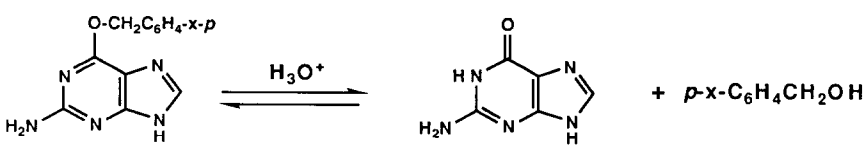
^{18}O-labelling studies

Electron ionization (EI) spectra were obtained on a Nermag (Paris, France) R10-10 quadrupole GC/MS system with a Spectral 30 data system. The quadrupole was scanned from 40 to 650 using a rate of 305 amu/s. Gas chromatographic separations were carried out on a 30 M, 0.25 mm i.d., 0.25 μm film thickness, DB-1 (dimethyl polysiloxane, J&W Scientific, Folsom, CA) capillary column. Helium was used as the carrier gas at a linear velocity of 25 cm/s and splitless injections were made with 45 s reduced flow delays into an injector maintained at 250°C. Capillary column effluent was introduced directly to the source via a 0.25 M (0.20 mm i.d.) section of deactivated fused silica column (Scientific Glass Engineering, Austin, TX) heated to 220°C.

To monitor the production of benzyl alcohol under acidic conditions, 40 μg of O^6-benzylguanine was dissolved in 100 μl of $H_2{}^{18}O$ containing 10 μl 1 N HCl (the enrichment of the final solution was 88 atom% ^{18}O) and was allowed to degrade completely at the room temperature. An aliquot of this reaction mixture was then injected into the GC-MS to test for the presence of benzyl alcohol.

Analytical procedure

High-performance liquid chromatography (HPLC) was performed using a system consisting of a Shimadzu SPD-6A variable-wavelength de-



1, x = H
2, x = Me
3, x = Cl

Scheme 1.

tector operating at 280 nm, Shimadzu LC-6A pumps, and a Shimadzu CR601 integrator for peak processing. The HPLC studies were conducted using a reversed-phase (C_{18}) analytical column (15 cm × 4.6 mm) with mean particle diameter of 5 μm. All the analyses were performed under isocratic conditions at room temperature. Flow rate was set at 1.5 ml/min. The mobile phase containing 50 parts 50 mM phosphate buffer (pH 7.0), 50 parts methanol and 1 mM *t*-butylammonium dihydrogen phosphate. Retention volumes for **1**, **2**, and **3** were 6.0, 9.8 and 10.8 ml, respectively.

Kinetic procedure

The pH of aqueous buffer solutions was adjusted at the experimental temperatures using a Corning pH meter which was standardized at the experimental temperature with NBS buffer solutions. The ionic strength of the solutions was adjusted to 0.5 with KCl, unless stated otherwise. The following buffer systems were employed: HCl (pH 1–2.3), formate (pH 2.4–3.5), and acetate (pH 3.7–5.3).

Stock solutions of the O^6-benzylated guanines **1–3** were prepared in dimethyl sulfoxide. 50 μl aliquots of the stock solution were used to prepare 10 ml dilute solutions of O^6-benzylated guanines **1–3** ($1–2 \times 10^{-4}$ M) in the pH-adjusted buffer solutions. At appropriate time intervals, samples were withdrawn and analyzed. Preliminary data indicated that the degradation of **1** was not subjected to any catalysis by formate and acetate buffers and, therefore, the observed rate constants in 0.08 M buffer were used to construct the pH-rate profile. Pseudo-first order rate constants for the apparent degradation of the O^6-benzylated guanines **1–3** were obtained by following the disappearance of the appropriate O^6-benzylated guanine for at least three half-lives.

Results and Discussion

The kinetics of the degradation of O^6-benzylguanine was studied in dilute solutions as a function of pH, buffer concentration, temperature, and added nucleophiles. The reaction was also carried out in $H_2{}^{18}O$ in order to determine the site of cleavage.

pH-rate profile

The degradation of O^6-benzylguanine in aqueous solutions at 50.0 ± 0.2 and 25.0 ± 0.2°C obeyed pseudo-first-order kinetics for at least three half-lives over the pH region between 1.0 and 5.2. Furthermore, the data suggested that the degradation of O^6-benzylguanine was not buffer catalyzed in the pH range studied. Fig. 1 represents a partial pH-rate profile for the hydrolysis of O^6-benzylguanine at 25 and 50°C. The pH-rate profile shows two linear segments with slopes approximately equal to unity with a break occurring between pH 2 and 4, indicating that the neutral specie and the protonated form of the O^6-benzylguanine undergo acid catalyzed hydrolysis at different rates. The hydrolysis can be adequately described in terms of apparent hydro-

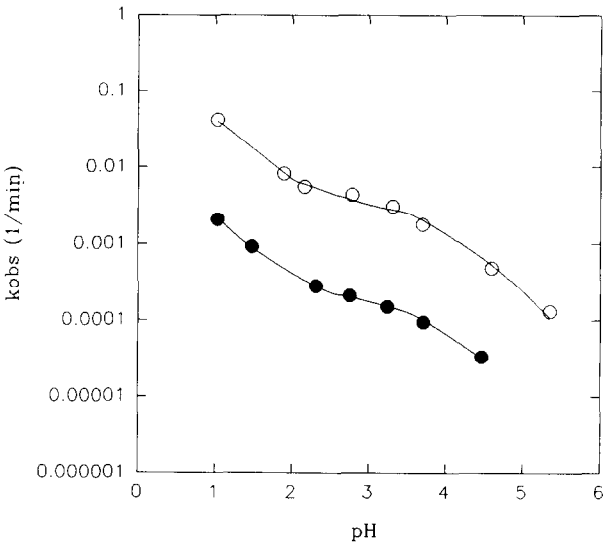


Fig. 1. Partial pH-rate profile for the hydrolysis of O^6-benzylguanine at 50°C (○) and 25°C (●), ($\mu = 0.5$ with KCl). The solid line represents the theoretical profile based on Eqn 1 and values of 0.40 min^{-1} M^{-1} for k_H, 24.9 min^{-1} M^{-1} for k'_H and 1.4×10^{-4} for K_a ($pK_a = 3.9$) at 50°C or values of 0.022 min^{-1} M^{-1} for k_H, 1.1 min^{-1} M^{-1} for k'_H and 1.6×10^{-4} for K_a ($pK_a = 3.9$) at 25°C.

nium ion catalyzed reactions of these species (Eqn 1):

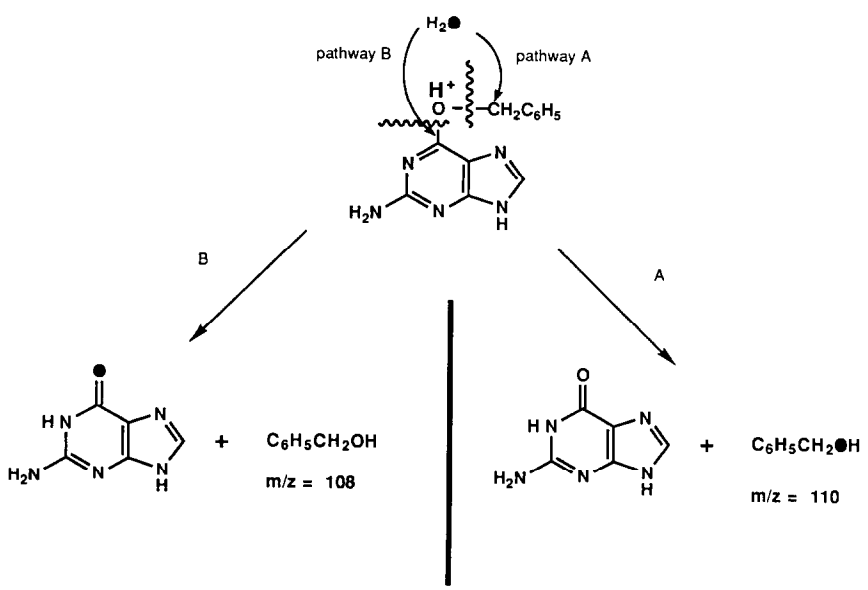
$$k_{obs} = k_H[H^+]\frac{[H^+]}{K_a + [H^+]} + k'_H[H^+]\frac{K_a}{K_a + [H^+]} \quad (1)$$

where $[H^+]/(K_a + [H^+])$ and $K_a/(K_a + [H^+])$ are the fractions of the total O^6-benzylguanine in the protonated and free base form, respectively, k_H and k'_H denote the macroscopic, second-order rate constants for hydronium-ion-catalyzed hydrolysis of the protonated and the neutral O^6-benzylguanine species, respectively, and K_a is the apparent ionization constant of the protonated form of O^6-benzylguanine. Alternatively, the profile is also consistent with the spontaneous degradation of a fully di- and mono-protonated form of **1**. This kinetically indistinguishable process would be described by a similar rate expression over the pH range studied providing the pK_a for the second protonation was $\ll 1$.

The solid lines in Fig. 1 were generated by the non-linear least-squares fit of k_{obs} as a function of hydrogen ion concentration to Eqn 1. The isolated kinetic parameters at 50°C and $\mu = 0.5$ with the associated standard errors were as follows: $k_H = 0.40\ (\pm 0.05)\ M^{-1}\ min^{-1}$, $k'_H = 24.9\ (\pm 2.9)\ M^{-1}\ min^{-1}$, $K_a = 1.4\ (\pm 0.3) \times 10^{-4}$ ($pK_a = 3.9$). The kinetic parameters at 25°C and $\mu = 0.5$ were: $k_H = 0.022\ (\pm 0.001)\ M^{-1}\ min^{-1}$, $k'_H = 1.1\ (\pm 0.075)\ M^{-1}\ min^{-1}$ and $K_a = 1.6\ (\pm 0.17) \times 10^{-4}$ ($pK_a = 3.8$). Spectroscopically, the pK_a of O^6-benzylguanine at 25°C and $\mu = 0.01$ was determined to be 4.2 which compared well with the kinetically obtained value of 3.8 at 25°C and $\mu = 0.5$.

It appears from the values of k_H and k'_H at 50°C that the neutral benzylguanine moiety possesses an almost 62-fold greater intrinsic susceptibility to acid catalyzed hydrolysis as compared to the protonated form. The relatively decreased rate of acid catalyzed reaction of the protonated species may be due to the already existing positive charge on the molecule which is situated in such manner that the electrostatic repulsion diminishes the extent of protonation on the benzylic oxygen.

If the alternative mechanism is assumed, namely, spontaneous degradation of the fully di- and mono-protonated O^6-benzylguanine, the data suggests that the fully di-protonated species is more reactive than the mono-protonated species.



Scheme 2.

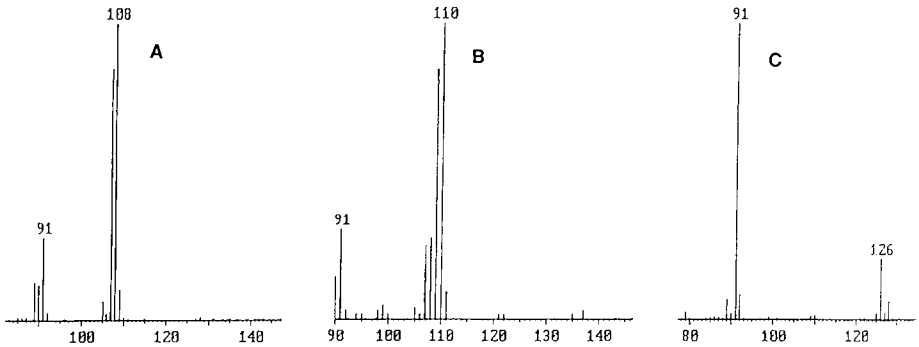
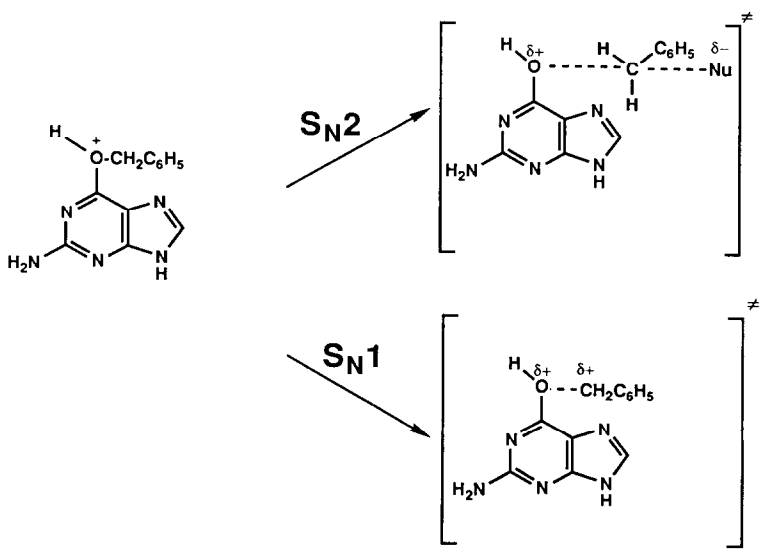


Fig. 2. Mass spectra of a peak corresponding to benzyl alcohol generated from the hydrolysis of O^6-benzylguanine at low pH (HCl) in H_2O (A) and in $H_2{}^{18}O$ (B). The mass spectra of a peak corresponding to benzyl chloride generated from the hydrolysis of O^6-benzylguanine at low pH (HCl) in $H_2{}^{18}O$ (C).

The benzylguanine was found to be extremely stable under basic conditions ($t_{1/2} > 270$ days at pH 7.8 and $T = 50$°C). The drug is currently being formulated in our laboratory as a ready-for-use solution in 40% PEG 400/60% 0.05 M phosphate buffer, pH 8.0 (v/v) and also as a freeze-dried product with mannitol as a bulking agent.

Cleavage site

Two plausible sites may be considered for the cleavage of the ether linkage in O^6-benzylguanine as illustrated in Scheme 2. As shown in this Scheme, both pathways will lead to identical products, namely, benzyl alcohol and guanine. Pathway A involved nucleophilic attack of the water molecule on the benzylic carbon leading to the incorporation of water oxygen into benzyl alcohol. In contrast, pathway B results in the incorporation of water oxygen into the guanine moiety. In order to distinguish between these two possible pathways, GC-MS and reaction in $H_2{}^{18}O$ were utilized to determine the site of the incorporation of the water oxygen. During the acid-catalyzed hydrolysis of O^6-benzylguanine, a molecular ion ($m/z = 108$) which is characteristic of benzyl alcohol was detected in the reaction mix-



Scheme 3.

ture (Fig. 2A). When the same experiment was conducted in ^{18}O-enriched water, the molecular ion was shifted by two mass units ($m/z = 110$) (Fig. 2B). This is consistent with the incorporation of the labeled oxygen into benzyl alcohol. The amount of ^{18}O incorporation ($80 \pm 4\%$) was consistent with theoretically calculated value of 88% for complete incorporation. This suggests that O^6-benzylguanine degrades in acidic media via cleavage of benzylic carbon-oxygen bond (pathway A) and not via oxygen-ring carbon bond cleavage (pathway B). Control experiments demonstrated that the oxygen in benzyl alcohol is not subject to solvent exchange. The absence of solvent exchange was supported by the lack of ^{18}O incorporation into benzyl alcohol when it was stored under identical acidic conditions and the same level of ^{18}O enrichment, as above.

A molecular ion ($m/z = 126$) with a 33% $m/z = 128$ was also detected under the experimental conditions (Fig. 2C), which indicates the formation of benzyl chloride. In a control experiment, when benzyl alcohol itself was reacted in the acidic $H_2{}^{18}O$ solution (same chloride concentration), no benzyl chloride was detected. This indicates that chloride anion was either able to compete with water and trap some of the benzyl carbocation ($S_{N}1$) or chloride was able to displace the benzyl group through a $S_{N}2$ mechanism (Scheme 3). The formation of benzyl chloride further supports pathways A as the likely route for the cleavage of the ether linkage of O^6-benzylguanine.

As implied by Scheme 3, the acidic hydrolysis of O^6-benzylguanine can take place by either an $S_{N}1$ pathway or an $S_{N}2$ pathway. Various probes can be used to distinguish between these two mechanisms. These include studing the effects of temperature, halide nucleophiles and substituents in the *p*-position of the benzyl group on the reactivity of benzylguanine towards hydrolysis.

Temperature effect

The effect of temperature on the rate of hydrolysis of O^6-benzylguanine was studied at pH 1.0 in HCl-KCl buffer over the temperature range 25–60°C. The observed entropy of activation ($\Delta S^{\neq} = -2.4$ e.u.) was obtained from an Eyring plot (Fig. 3), where the apparent second-order rate constant (uncorrected for pK_a of O^6-benzylguanine, however, from the fit of the kinetic data, the pK_a did not appear to be overly sensitive to temperature) was used to correct for the choice of standard state required by the Eyring equation. The value of the observed entropy of activation is more indicative of an unimolecular $S_{N}1$ reaction than a bimolecular reaction. The slightly negative entropy change probably reflects the involvement of an equilibrium proton transfer prior to the rate-determining step, so that the observed

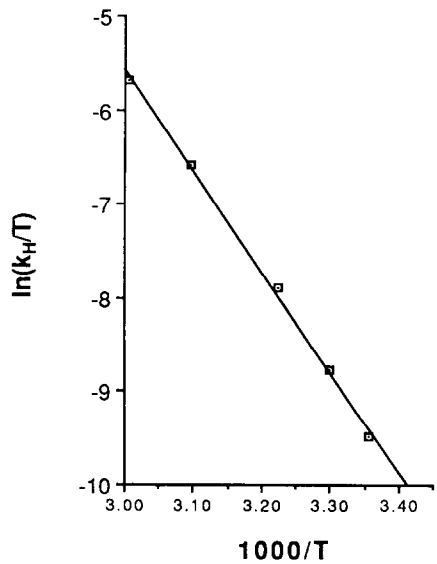
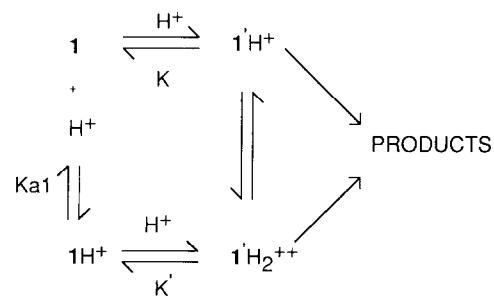


Fig. 3. An Eyring plot for the hydrolysis of O^6-benzylguanine at pH 1.0 (HCl-KCl, $\mu = 0.5$) at 25, 30, 37, 50 and 60°C.



Scheme 4. Decomposition mechanism for O^6-benzylguanine, where $1'H^+$ and $1'H_2^{++}$ are the protonated intermediates with protonation at benzylic oxygen, and K and K' are the equilibrium constants for the protonation of benzylic oxygen of the neutral and the protonated forms of O^6-benzylguanine, respectively.

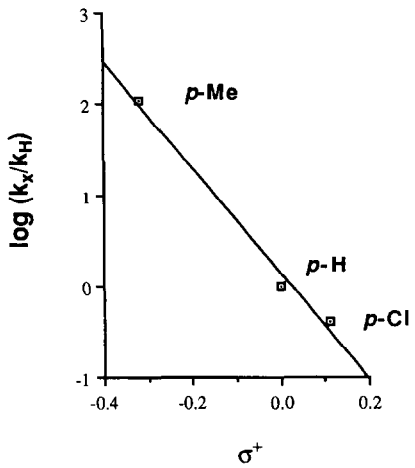


Fig. 4. Hammett plot (log (k_x/k_H) vs σ^+), demonstrating the effect of *p*-substituents on the reactivity of *p*-substituted O^6-benzylguanines **1–3** at 25°C, pH 2.4 (formate buffer, μ = 0.5 with KCl).

entropy of activation includes the entropy for the preliminary proton transfer as well as for the decomposition of the protonated intermediate (Jencks, 1987) (Scheme 4).

Substituent effect

A plot of the log[$(k_{obs})_x/(k_{obs})_H$] vs σ^+ for the relative reactivity of **1–3**, is well correlated with σ^+ values for the substituents (Fig. 4). The ρ value obtained from the slope of this plot is −5.7 (Fig. 4). This suggests that, in the transition state, electron donating groups stabilize the positive charge development at the benzylic carbon, consistent with $S_{N}1$ type mechanism. The σ^+ values were used in this Hammett (Okomoto and Brown, 1957) type plot, since the substituents stabilize the developing positive charge on the benzylic carbon through direct resonance interaction. The magnitude of the ρ value is similar to that obtained for O^6-benzylated guanosines (Moschel et al., 1984).

Halide nucleophile effect

Fig. 5 shows that k_{obs} at pH 2.0 for the disappearance of the O^6-benzylguanine increases upon the addition of bromide and iodide nucleophiles at constant ionic strength (0.5 M), maintained with sodium perchlorate. The substitution of sodium chloride for sodium perchlorate resulted

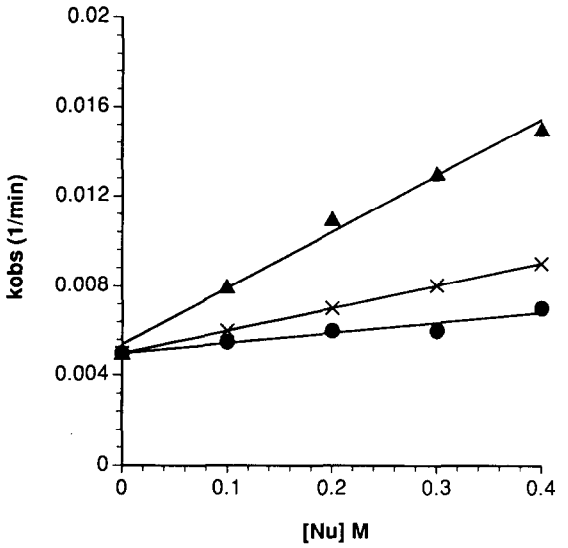


Fig. 5. The dependence of k_{obs} on the concentration of anionic nucleophiles for the reaction of O^6-benzylguanine with iodide (▲), bromide (×) and chloride (●) ions at 50°C, pH 2.0 (μ = 0.5 adjusted with $NaClO_4$).

in a very small change in k_{obs} for the hydrolysis of O^6-benzylguanine. Second-order rate constants, k_n, were obtained from the slopes of these lines for reactions in the presence of chloride, bromide and iodide ions. These second-order rate

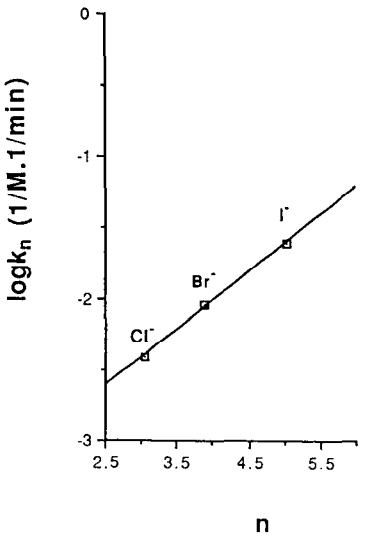


Fig. 6. Swain-Scott plot of the second-order rate constants, log k_n against n, nucleophilic reactivity parameter, for reactions of O^6-benzylguanine, at 50°C, pH 2.0 (μ = 0.5 adjusted with $NaClO_4$) with various halide nucleophiles.

constants appear to follow the Swain-Scott nucleophilic constant, n, for the nucleophilic reactivity of methyl bromide in water, with a slope, s, of 0.4, as shown in Fig. 6. The s value of 0.4 indicates that the sensitivity to the nucleophilicity of the attacking nucleophile is relatively small. It is about half of that seen for the attack of halide and other nucleophilic reagents on methyl bromide ($s = 1.00$) and benzyl chloride ($s = 0.87$) (Swain and Scott, 1953). This also indicates that there is less interaction with the attacking nucleophile in the transition state for the apparent bimolecular reaction of O^6-benzylguanine with the halides than for the similar reactions of methyl bromide or benzyl chloride.

In conclusion, O^6-benzylguanine undergoes an acid-catalyzed hydrolysis. Based on the O^{18} incorporation data the site of the bond cleavage for the hydrolysis was unambiguously assigned to the benzylic carbon-oxygen bond leading to the formation of benzyl alcohol and guanine as the predominant products. Benzyl chloride was detected when the ionic strength of the solution was adjusted with sodium chloride. The rate of hydrolysis of p-substituted O^6-benzylguanines increases with enhancing the electron donating capability of p substituents, consistent with a mechanism involving considerable positive charge formation on the benzylic carbon in the transition state. The value of the observed entropy of activation ($\Delta S^{\neq} = -2.4$ e.u.) is consistent with a unimolecular, S_{N^1}, reaction. Although the rate of hydrolysis of O^6-benzylguanine was well correlated with the nucleophilicity of various added halides, the magnitude of the catalysis was less than anticipated for pure S_{N^2} type reactions. However, the halide experiment results do suggest that in the presence of bromide, iodide and perhaps other nucleophiles, the transition state may have some S_{N^2} character. Similar occurrences have been reported previously by Jencks (1990). A similar scenario has also been proposed by Dolan et al. (1990) for the reactivity of these compounds with the sulfhydryl groups of the cysteine acceptor site on the alkyltransferase protein. Overall, our observations suggest a late transition state with considerable positive charge development at the benzylic carbon atom for reactions of O^6-benzylguanine with water.

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