Published in final edited form as:

Chem Res Toxicol. 2015 April 20; 28(4): 691–701. doi:10.1021/tx500459t.

Carcinogenesis of Urethane: Simulation versus Experiment

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Abstract

The carcinogenesis of urethane (ethyl carbamate), a by-product of fermentation that is consistently found in various food products, was investigated with a combination of kinetic experiments and quantum chemical calculations. The main objective of the study was to find $G^\ddagger$, the activation free energy for the rate-limiting step of the S_N2 reaction between the ultimate carcinogen of urethane, vinyl carbamate epoxide (VCE), and different nucleobases of the DNA. In the experimental part, the second-order reaction rate constants for the formation of the main 7-(2-oxoethyl)guanine adduct in aqueous solution of deoxyguanosine and in DNA were determined. A series of *ab initio*, density functional theory (DFT) and semiempirical molecular orbital (MO) calculations was then performed to determine the activation barriers for the reaction between VCE and nucleobases methylguanine, methyladenine, and methylcytosine. Effects of hydration were incorporated with the use of the solvent reaction field method of Tomasi and co-workers and the Langevine dipoles model of Florian and Warshel. The computational results for the main adduct were found to be in good agreement with the experiment, thus presenting a strong evidence for the validity of the proposed S_N2 mechanism. This allowed us to predict the activation barriers of reactions leading to side products for which kinetic experiments have not yet been performed. Our calculations have shown that the main 7-(2-oxoethyl)deoxyguanosine adduct indeed forms preferentially because the emergence of other adducts either proceeds across a significantly higher activation barrier or the geometry of the reaction requires the Watson–Crick pairs of the DNA to be broken. The computational study also considered the questions of stereoselectivity, the ease of the elimination of the leaving group, and the relative contributions of the two possible reaction paths for the formation of the 1, N^2 -ethenoguanosine adduct.

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Supporting Information Available

Tables with computational results are available for the following S_N2 reactions: formation of $N^2,3$ -ethenoguanine (Tables S1–S4), formation of 3, N^4 -ethenocytosine (Tables S5–S8), formation of 1, N^6 -ethenoadenine (Tables S9–S12), formation of 1, N^2 -ethenoguanine via an attack by the endocyclic amide nitrogen N^1 (Tables S13–S16), and formation of 1, N^2 -ethenoguanine via an attack by the exocyclic amino nitrogen N^2 (Tables S17–S20). This material is available free of charge via the Internet at <http://pubs.acs.org/>.

TOC image

Keywords

chemical carcinogenicity; urethane; vinyl carbamate epoxide; DNA alkylation; activation free energy; quantum chemical calculations; solvation effects; kinetic studies

Introduction

Urethane (ethyl carbamate) is of great biological importance due to its role in the etiology of cancer. Formerly, it was manufactured in large scale for production of crosslinking agents in permanent press textile treatments and for various medicinal purposes, but this was curtailed when urethane was reported to cause tumors in the skin, liver, lungs, mammary glands, and lymphoid tissues of laboratory animals.^{1–3} Despite being nowadays largely banished from industrial processes, urethane is nevertheless consistently found in virtually all alcoholic beverages and in a variety of food products including yogurt, bread, soy sauce, and cheese, as well as in tobacco. This pervasive presence is a consequence of urethane appearing as a by-product of fermentation.⁴ Due to the ubiquity of the fermentation processes, it is easy to envision the widespread presence of urethane in common food products.

After intake, urethane is in the presence of oxygen and NADPH metabolized by cytochrome P450 2E1 into its ultimate carcinogen, the highly reactive vinyl carbamate epoxide (VCE).⁵ This electrophilic species then reacts with DNA, usually by alkylating guanine at the N⁷ position, leading to the main adduct 7-(2-oxoethyl)deoxyguanosine. Other minor adducts are also formed: N²,3-ethenodeoxyguanosine, 1N²-ethenodeoxyguanosine, 1,N⁶-ethenodeoxyadenosine, and 3,N⁴-ethenodeoxycytidine.⁵ Alkylation is followed by other reactions, typically depurination, thus introducing abasic sites in the genetic material and, together with primary lesions, leading to gene mutations and chromosomal aberrations.

The proposed mechanism for the formation of 7-(2-oxoethyl)deoxyguanosine adduct is depicted in Scheme 1. This adduct is formed through S_N2 substitution – a nucleophilic attack of the bare endocyclic nitrogen of guanine on the terminal epoxide carbon of VCE leading to the formation of an unstable zwitterionic intermediate followed by elimination of a good leaving group. The S_N2 substitution represents the rate-limiting step of the reaction. Other minor adducts are initially formed in an analogous way (Schemes 2, 3, and 4); in the case of an adjacent amino group, cyclization follows via Schiff base formation.⁶ Since no bare nitrogen can be involved in the formation of 1,N²-ethenoguanosine (Scheme 5), a question arises whether the endocyclic amido or exocyclic amino nitrogen initiates the nucleophilic attack.⁶

This study addresses the kinetics of VCE-DNA adduct formation by focusing on $G^\ddagger$, the activation free energy of the rate-limiting step of the reaction. This quantity is directly related to the overall reaction rate and thus to the carcinogenicity of vinyl carbamate epoxide.⁷⁻⁹ In the computational part of the study, $G^\ddagger$ for the reaction between vinyl carbamate epoxide and methylated nucleobases methylguanine, methyladenine and methylcytosine was calculated at several *ab initio*, density functional theory (DFT) and semiempirical molecular orbitals (MO) levels. Solvation effects were incorporated with the solvent reaction field of Tomasi and co-workers¹⁰ and with the Langevin dipole method of Florian and Warshel.¹¹ Hydration effects in conjunction with the semiempirical MO methods were studied at the AM1-SM1 and PM3-SM3 levels.¹² In order to evaluate the computational results, a kinetic experiment was conducted in which the reaction rate constant for the main 7-(2-oxoethyl)deoxyguanosine adduct formation, k , was determined. The transition state theory of Eyring

$$k = \frac{k_B T}{h} \exp \left(-\frac{\Delta G^\ddagger}{k_B T} \right) \quad (1)$$

was used to convert this quantity into $G^\ddagger$ (with k_B representing the Boltzmann constant, h the Planck constant, and T the absolute temperature). The validity of the transition state theory in biocatalysis was proven experimentally by the development of catalytic antibodies and theoretically by the success of the empirical valence bond (EVB) method.¹³

Experimental Procedures

Chemicals and Reagents

Calf thymus DNA and 2'-deoxyguanosine were purchased from Sigma Aldrich (St. Louis, MO). Dry acetone was prepared by treating with anhydrous K_2CO_3 , distilling from P_2O_5 , and storing over heated (200 °C) 4 Å molecular sieves. Authentic 7-(2-oxoethyl)Gua was a gift of the late James A. Miller (Univ. Wisconsin).

Synthesis of Vinyl Carbamate Epoxide

The procedure was based on that developed in the Miller laboratory.^{14,15} Vinyl chloroformate (5 g, 55 mmol) was sparged with NH_3 gas for 5 min in 30 mL of a CH_2Cl_2 :cyclohexane mixture (2:1, v-v) at 0 °C to yield vinyl carbamate: 1H NMR ($CDCl_3$) δ 4.47 (dd, 1H, $J = 1.3, 6.2$ Hz, vinyl), 4.77 (dd, 1H, $J = 1.3, 14$ Hz), 4.91 (s, broad, 2H, $-NH_2$), 7.15 (dd, 1H, $J = 6.3, 14$ Hz, vinyl); ^{13}C NMR 96.1 ($H_2C=$), 142.0 ($=CH-$), 164.0 ($-OCONH_2$) (confirmed with 2-dimensional correlated (COSY) NMR spectroscopy).

Vinyl carbamate (7.8 mg, 25 mmol) was stirred with 30 mL of 0.11 M freshly prepared dimethyldioxirane^{16,17} in 30 mL of dry acetone for 30 min at 23 °C to yield vinyl carbamate epoxide, which was concentrated under a stream of N_2 (56% yield, determined by NMR): 1H -NMR ($CDCl_3$) δ 2.75 (dd, 1H, $J = 1.8, 6.2$ Hz, CH_2 -epoxide), 2.80 (dd, 1H, $J = 1.8, 6.2$ Hz, CH_2 -epoxide), 5.45 (t, 1H, $J = 1.8$ Hz, epoxide C-H), 6.22 (s, broad, 2H, 600 Hz) (residual vinyl carbamate as impurity).

Reaction of Vinyl Carbamate Epoxide

The $t_{1/2}$ of vinyl carbamate was measured in 100 mM potassium 4-(2-hydroxyl)-1-piperazineethansulfonate (HEPES) buffer, pH 7.6, at 25 °C. At varying times 50- μ L aliquots (of a 20 mM solution of vinyl carbamate epoxide) were withdrawn and added to 0.5 mL of 50 mM *p*-nitrobenzylpyridine in mixture of 100 mM potassium HEPES (pH 7.4)/ethylene glycol/acetone (4:2:1, w-v-v). After 5 min at 23 °C, 0.5 mL of a mixture of triethylamine-acetone (1/1, v-v) was added and the tubes were mixed with a vortex device. A_{560} was measured¹⁸ and the $t_{1/2}$ was estimated from a plot of A_{560} vs. time.

In incubations to measure guanyl adducts, 25 μ L of 10 mM freshly prepared vinyl carbamate epoxide (in dry acetone) was added to 1.0 mL of 0–20 mM dGuo or 0–10 mg mL⁻¹ DNA in 5 mM potassium phosphate buffer (pH 7.4), to give a final concentration of 0.25 mM. Reactions proceeded at 25 °C overnight.

In the case of dGuo, 0.15 mL of 1 M HCl was added (to the 1.025 mL reactions) and the samples were heated at 80 °C for 30 min. Particulate matter was removed by centrifugation at $3 \times 10^3 \times g$ for 10 min.

In the case of the DNA samples, 5 mL of cold (4 °C) C₂H₅OH was added to precipitate the DNA. DNA was pelleted by centrifugation ($3 \times 10^3 \times g$ for 10 min), and the pellets were dried in a desiccator *in vacuo*. Each sample was mixed with 0.5 mL of 0.1 M HCl and heated at 75 °C for 45 min. Insoluble material was removed by centrifugation at $3 \times 10^3 \times g$ for 10 min.

In both cases of the dGuo and DNA samples, aliquots (5 μ L) were analyzed for 7-(2-oxoethyl)Gua using LC-FLR, utilizing an external standard curve. Chromatography was performed on a Luna SCX column (Phenomenex, 250 mm $\times$ 4.6 mm, 5 μ m) with a flow rate of 0.6 mL/min using a linear gradient program from 0–50% mobile phase B (v-v) over 20 min (A, 75 mM ammonium formate (pH 2.8)/10% acetonitrile, v-v, and B, 250 mM ammonium formate (pH 2.8)/10% acetonitrile, v-v). The product 7-(2-oxoethyl)Gua had a retention time of 14.2 min and was quantified by fluorescence detection with excitation at 290 nm and emission at 370 nm, with calibration against an external standard curve.

Computational Methods

All calculations were performed at the National Institute of Chemistry in Ljubljana on the CROW cluster.^{19,20} To obtain the Born–Oppenheimer hypersurface for the reaction between vinyl carbamate epoxide and the selected nucleobase, a series of *ab initio*, DFT, and semiempirical MO simulations was performed using the Gaussian 09 suite of programs.²¹

The *ab initio* calculations were performed on the Hartree–Fock (HF) level and on the MP2 (Møller–Plesset perturbation theory of the second order) level of theory, in both cases combined with flexible 6-31G(d), 6-31+G(d,p) and 6-311++G(d,p) basis sets. Additionally, we considered the DFT method B3LYP incorporating the exchange functional by Becke²² combined with the correlation functional of Lee, Yang, and Parr.²³ Again, flexible basis sets 6-31G(d), 6-31+G(d,p) and 6-311++G(d,p) were used. Additionally, two methods with a low computing cost were applied, namely the semiempirical MO methods AM1 and PM3.

Although more empirical in character than the others, these two methods were deemed interesting from the standpoint of QM/MM methods due to their low computational requirements.

To calculate the energy of activation, we first had to find an approximate structure of the transition state using a relaxed potential surface scan: a range of distances between the two reaction centers (r_{cc}) was sampled, each time freezing the internal coordinate corresponding to a given r_{cc} and subjecting the remaining degrees of freedom to optimization. The structure with the highest energy was selected as the starting point for the Berny algorithm,²¹ which in turn produced the optimized structure of the transition state. Vibrational analysis in the harmonic approximation was subsequently performed to ensure that only one imaginary frequency was present (thus confirming that the found structure was indeed a first-order saddle point of the energy surface) and that the vibrational mode corresponding to this single imaginary frequency did in fact represent the cleavage of the existing epoxidic C–O bond coupled with the establishment of the new N–C bond between the reacting molecules. The reactant structure was derived from the transition state structure by displacing one of the molecules so that the distance between the corresponding reactive centers increased by approximately 0.2 Å (thus placing the system on the down-slope of the potential surface) and using the obtained configuration as the input for the energy minimization procedure. At the end of the optimization, vibrational analysis was again performed, this time ascertaining that only real frequencies were present (indicating that a minimum was indeed located). When all of the above steps were completed, the activation energy for the S_N2 reaction *in vacuo* was obtained as the energy difference between the transition state and the reactant state. Vibrational analysis provided us with its zero-point correction.

Hydration free energies for the reactants and the transition state were calculated with the solvent reaction field (SCRF) method of Tomasi and co-workers¹⁰ and with the Langevin dipoles (LD) model of Florian and Warshel.¹¹ For AM1 and PM3 methods, the AM1-SM1 and PM3-SM3 hydration models were used.¹² The SCRF code present in the Gaussian 09 suite was applied at all *ab initio* and DFT methods. The Merz–Kollman partial atomic charges obtained in these calculations served as the input to the LD model provided by the ChemSol program.²⁴ AM1-SM1 and PM3-SM3 calculation were performed with the AMSOL-5.4.1 program of Truhlar and co-workers.¹²

Results and Discussion

Hydrolysis of Vinyl Carbamate Epoxide

The measured half time $t_{1/2}$ of vinyl carbamate epoxide was 13.2 ± 1.5 min (Figure 1). This is a somewhat shorter $t_{1/2}$ than the one reported by Park et al. (32 min), obtained using a similar assay.²⁵ The corresponding pseudofirst-order reaction rate constant, k_a' is 8×10^{-4} s⁻¹.

Reaction of vinyl carbamate epoxide with dGuo

Experimental data was analyzed according to the kinetic model proposed by Brown *et al.*²⁶ In the reaction mixture, a competition between two reactions takes place: the hydrolysis of vinyl carbamate epoxide (VCE)

and the reaction between VCE and deoxyguanosine (dGuo) that yields the main 7-(2-oxoethyl)deoxyguanosine (VC-dGuo) adduct:

The dependence of the main adduct concentration on the initial dGuo concentration was modeled using the equation

$$[\text{VC} - \text{dGuo}] = \frac{[\text{VCE}]_0}{1 + \frac{k_a'}{k_{\text{non}}[\text{dGuo}]}} \quad (2)$$

where [VC-dGuo] represents the final concentration of the main VC-dGuo adduct, [VCE]₀ represents the initial concentration of VCE, k_a' represents the pseudofirst-order reaction rate constant of the hydrolysis of VCE, k_{non} denotes the second-order rate constant for the dGuo alkylation, and [dGuo] denotes the initial concentration of dGuo. In addition, a linearized form of the above equation was considered:

$$[\text{VC} - \text{dGuo}] = \frac{k_{\text{non}}[\text{VCE}]_0}{k_a'} [\text{dGuo}] \quad (3)$$

Such an approximation is valid when $k_{\text{non}}[\text{dGuo}] \ll k_a'$. In our case, the term $k_{\text{non}}[\text{dGuo}]$ turns out to be at least two orders of magnitude smaller than k_a' , so the linearized form of the equation gives results that are almost indistinguishable from those of the original non-linear equation.

For Equations 2 and 3, Levenberg–Marquardt optimization algorithm²⁷ was used to find the value of k_{non} that gives the best fit (in the least-squares sense) to the experimental data points (Figure 2). As expected, due to the equation 2 being practically linear in this regime, there was a high level of agreement between the two results: the difference between k_{non} obtained with equation 2 and k_{non} obtained with equation 3 was two orders of magnitude below the estimated uncertainty of k_{non} . The second-order reaction rate constant of the dGuo alkylation was determined to be $k_{\text{non}} = (2.2 \pm 0.3) \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$. Equation 1 was finally used to convert this value into the activation free energy, giving the result $G^\ddagger = 22.4 \pm 0.1 \text{ kcal/mol}$.

Reaction of vinyl carbamate epoxide with DNA

Experimental data on the reaction between vinyl carbamate epoxide and DNA were treated in an analogous way to the data on deoxyguanosine. The DNA concentrations were converted to guanine concentrations based on the assumption that on average, guanine accounts for one fourth of all nucleobases in DNA. A diagram of the measurements, together with the least-squares fit of Equation 2, is displayed in Figure 3. The second-order reaction rate constant of the guanine alkylation was determined to be $k_{\text{non}} = (2.2 \pm 0.3) \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$ and the corresponding activation free energy is $G^\ddagger = 22.2 \pm 0.1 \text{ kcal/mol}$.

In principle, the $G^\ddagger$ for the reaction of VCE with DNA could be different from $G^\ddagger$ for the reaction of VCE with pure deoxyguanosine due to a number of factors, the main two being steric hindrance of the DNA (causing an increase in activation energy) and the recently discovered DNA catalysis (resulting in a decrease of activation energy).²⁶ In our case, the activation barriers are virtually identical: 22.2 kcal/mol (for DNA) vs. 22.4 kcal/mol (for guanine). Evidently, the two effects are either both insignificant or they cancel each other out. Since VCE does not seem to have a high potential for intercalation with DNA and an appreciable catalytic effect is therefore improbable, we can conclude that neither DNA catalysis nor steric effects play a significant role in the reaction.

Computational results: formation of 7-(2-oxoethyl)guanine

The calculated activation energies, zero-point energies, lowest vibrational frequencies of reactant states, imaginary frequencies of transition states and corresponding distances between the reactive centers are collected in Table 1. Using methods MP2/6-31+G(d,p) and MP2/6-311++G(d,p), the reactant optimization procedure described in the section *Computational methods* failed to produce a reasonable geometry. In these two cases, single-point calculations were performed instead, with the optimized reactant geometry from the MP2/6-31G(d) level serving as the input. These circumstances precluded the use of vibrational analysis, and a best-effort approach to incorporate the zero-point energy correction into further results was made by taking the zero-point energy from the MP2/6-31G(d) level of theory.

The structures of the reactants and the transition state are shown in Figure 4. Considering the gas-phase activation energies recorded in Table 1, we can conclude that the convergence in terms of basis set size was reached at all levels of theory. Very similar geometries were also predicted, as can be observed from the distances between the reactive centres (N^7 of methylguanine and nonchiral epoxidic carbon of vinyl carbamate epoxide) in Table 1. The spread of d^R is slightly larger than the spread of d^{TS} ; however, this is to be expected, since the intermolecular forces in the reactant state are much weaker and the potential hypersurface consequently much shallower. The gas-phase reaction barrier predicted at the Hartree–Fock level lies between 28 and 30 kcal/mol; the predictions of the MP2 level are significantly ($\sim 8 \text{ kcal/mol}$) lower, and DFT methods predict values that are further $\sim 4 \text{ kcal/mol}$ lower. Conversely, the semiempirical MO methods AM1 and PM3 predict values that are much higher, lying in the range between 35–41 kcal/mol. The absolute values of the differences between the zero-point energies of the transition state and the reactant state are on the order of 1 kcal/mol and thus do not contribute significantly to the calculated

activation barriers. In this respect, the above mentioned approximation taken at the MP2/6-31+G(d,p) and MP2/6-311++G(d,p) levels seems to have a negligible effect on the final results.

Because the reaction takes place in a solution, the values of $E^\ddagger$, which hold for *vacuo*, can not be readily compared to the experimental result of our kinetic study. Another series of calculations was thus performed to incorporate the solvation effects. Hydration free energies obtained by the solvent reaction field method are presented in Table 2; solvation free energies calculated by the LD model are collected in Table 3; the hydration free energies according to the AM1-SM1 and PM3-SM3 methods are shown in Table 4. Tables 2–4 also include the activation free energies $G^\ddagger$ calculated as

$$\Delta G^\ddagger = \Delta E^\ddagger + \Delta ZPE + \Delta \Delta G_{\text{hydr}} \quad (4)$$

where $E^\ddagger$ represents the activation free energy in the gas phase, ZPE denotes the difference in zero-point energy between the transition state and the reactant state, and

G_{hydr} stands for the corresponding difference in the hydration free energy. It must be noted that for reactions in solution, the entropy contribution can not be simply calculated as the sum of translational, rotational, and vibrational entropies obtained by the ideal gas, rigid rotor, and harmonic oscillator approximations,²⁸ because this could lead to a dramatic overestimation of the entropic term, particularly due to the neglect of low-frequency vibrational modes that are abundant in larger solutes.²⁹ It is rather the solvation entropies included in the relative solvation free energies that present the correct treatment of the entropic term for reactions in solution.³⁰

G_{hydr} is negative for all theory levels and implicit solvation models used: this means that the transition state is better solvated than the reactant state, with the solvent thus lowering the activation barrier and accelerating the reaction. The observed solvent acceleration is in line with the more polar nature of the transition state close to one of the unstable zwitterionic intermediates. The reduction of the activation barrier predicted by the SCRF method (results in Table 2) is relatively consistent across all considered theory levels, amounting to $G_{\text{hydr}} \approx -5$ kcal/mol. The final values of $G^\ddagger$ given by the Hartree–Fock method exhibit very good agreement with the experimental value (22.4 kcal/mol). MP2 activation barriers are, depending on the basis set used, moderately to significantly underestimated, and the values given by the DFT methods are all significantly underestimated.

Similar conclusions can be drawn for the activation barriers calculated with the LD model of hydration (Table 3). The values of G_{hydr} given by the LD model are about 3 kcal/mol lower than those obtained for the SCRF method. This, in turn, translates into slightly lower activation barriers than with the SCRF method. Again, the results at the HF level can be considered relatively close to the experimental value. On the other hand, both MP2 and DFT methods give barriers that are significantly too low.

Table 4 presents the results obtained by the semiempirical MO methods in conjunction with solvation models of Cramer and Truhlar:^{31,32} AM1-SM1 and PM3-SM3. With these models,

the reduction of the reaction barrier is much less pronounced than with the SCRF and LD models, resulting in significantly overestimated values for $G^\ddagger$.

All in all, the Hartree–Fock method tends to offer results that compare favorably with the experimental data, particularly in combination with the SCRF solvation model. Both the DFT functional and the MP2 level exhibit moderate to significant underestimation of the calculated $G^\ddagger$ regardless of the applied solvation model, while the AM1-SM1 and PM3-SM3 semiempirical MO methods produce barriers that are considerably too high.

Activation barrier for the elimination of the leaving group

In the Introduction, the assertion was made that the S_N2 substitution represents the rate-limiting step of the formation of the major 7-(2-oxoethyl)deoxyguanosine adduct between VCE and guanine and that the subsequent elimination of the leaving group is, in comparison, a fast process. We tested this assertion by calculating the activation barrier for this elimination in an analogous way as for the initial S_N2 substitution: the zwitterionic VC-dGuo intermediate (lying 31.5 kcal/mol below the initial transition state) was used as the starting configuration and the energy difference to the transition state for the elimination of the $^-OCONH_2$ group was determined. We decided to use the HF/6-311++G(d,p) level of theory only, as it gave the best agreement with the experiment for the initial S_N2 substitution.

The calculated gas-phase activation energy was $E^\ddagger = 0.20$ kcal/mol, the zero-point energy difference $ZPE = -0.59$ kcal/mol, the relative hydration free energy calculated with the SCRF model $\Delta\Delta G_{\text{hydr}}^{\text{SCRF}} = 0.90$ kcal/mol, and the relative hydration free energy calculated with the LD method $\Delta\Delta G_{\text{hydr}}^{\text{LD}} = 2.29$ kcal/mol. Activation barriers in solution were thus estimated to be $\Delta G_{\text{SCRF}}^\ddagger = 0.51$ kcal/mol (with the SCRF method) and $\Delta G_{\text{LD}}^\ddagger = 1.90$ kcal/mol (with the LD model). With these barriers being only on the order of the thermal noise, it is clear that the assertion about the fast elimination of the leaving group is indeed justified.

Stereoselectivity of the reaction

Up to this point, all described calculations were carried out with the (*R*)-stereoisomer of VCE. But the chirality of the VCE molecule causes that the reaction of the (*S*)-stereoisomer in principle proceeds with a different geometry, which could—although this seems unlikely—correspond to a significantly different reaction barrier. This possibility was examined by repeating the activation free energy calculation between VCE and methylguanine, this time with the (*S*)-stereoisomer of VCE. Only the HF/6-311++G(d,p) level was, again, used.

Activation barriers in solution were estimated to be $\Delta G_{\text{SCRF}}^\ddagger = 21.24$ kcal/mol (with the SCRF solvation) and $\Delta G_{\text{LD}}^\ddagger = 18.05$ kcal/mol (with the LD model). Comparing this with the values for the (*R*)-stereoisomer, 22.15 kcal/mol (with the SCRF method) and 19.13 kcal/mol (with the LD model), it can be concluded that the stereoselectivity of the reaction is indeed very low and can be dismissed completely for the purpose of the present study.

Computational results: formation of $N^2,3$ -ethenoguanine

The structures of the reactants and the transition state are shown in Figure 5; the Tables with the computational results can be found in Supporting Information (Tables S1–S4). At all levels of theory, the activation barrier for this reaction is consistently higher than the activation barrier for the main attack of N^7 . Based on the calculations at the HF/6-311++G(d,p) level of theory in combination with the SCRF solvation model, the activation barrier is predicted as $G^\ddagger = 27.12$ kcal/mol (attack of the main N^7 -site: 22.15 kcal/mol). This nicely explains why $N^2,3$ -ethenoguanine is observed as a minor adduct only.^{33,34}

Computational results: formation of $3,N^4$ -ethenocytosine

The structures of the reactants and the transition state are shown in Figure 6; the Tables with the computational results can be found in Supporting Information (Tables S5–S8). On all levels of theory, the activation barrier for this reaction is consistently slightly lower than the activation barrier for the formation of the main 7-(2-oxoethyl)guanine adduct. Based on the calculations at the HF/6-311++G(d,p) level in combination with the SCRF solvation model, the activation barrier is predicted to be $G^\ddagger = 21.59$ kcal/mol. Comparing this with the calculated activation barrier for the formation of 7-(2-oxoethyl)guanine (22.15 kcal/mol) and judging only on the basis of $G^\ddagger$, the conclusion could be drawn that these two adducts should appear in roughly similar quantities. But this conclusion is strongly countered by the experiments where $3,N^4$ -ethenocytosine appears as a side adduct only. This apparent discrepancy can be resolved by realizing that in living organisms, VCE does not react with bare nucleobases, but rather with nucleobases included in the DNA. Looking again at the geometry in Figure 6, it becomes clear that VCE approaches cytosine from the side that is directly involved in the Watson–Crick pairing. The contribution that is therefore not counted in the calculated $G^\ddagger$ is the additional energy needed to break the Watson–Crick pair and displace the DNA strands so that VCE can assume the geometry needed for the reaction. This sterical penalty is expected to easily offset the 1 kcal/mol chemical preference for cytosine, making the $3,N^4$ -ethenocytosine a minor adduct only.

Computational results: formation of $1,N^6$ -ethenoadenine

The structures of the reactants and the transition state are shown in Figure 7; the Tables with the computational results can be found in Supporting Information (Tables S9–S12). At all levels of theory, the activation barrier for this reaction is consistently somewhat higher than the activation barrier for the formation of the main 7-(2-oxoethyl)guanine adduct. Based on the calculations at the HF/6-311++G(d,p) level in combination with the SCRF solvation model, the activation barrier is predicted as $G^\ddagger = 24.22$ kcal/mol. Similarly to the case of cysteine, judging solely by $G^\ddagger$, the N^6 -ethenoadenine should be observed much more abundant than the experiments actually show it to be. Again, VCE is observed to react in a geometry that requires the Watson–Crick pairs of the DNA to be broken. The associated increase in the activation free energy slows the reaction down to such a degree that the $1,N^6$ -ethenoadenine adduct is indeed found in small amounts only.

Computational results: formation of 1,*N*²-ethenoguanine

The case of the 1,*N*²-ethenoguanine adduct differs from the other adducts in that it is not clear whether the first nucleophilic attack happens by the endocyclic amide nitrogen N¹ or by the exocyclic amino nitrogen N². To try to resolve this ambiguity, the activation barriers for both mechanisms were determined. The resulting structures of the reactant states and transition states are shown in Figure 8 (attack by the endocyclic amide nitrogen N¹) and Figure 9 (attack by the exocyclic amino nitrogen N²). The Tables with the computational results can be found in Supporting Information (Tables S13–S20).

At all levels of theory, the activation barrier for the attack of N² is significantly lower than the activation barrier for the attack of N¹. Based on the calculations at the HF/6-311++G(d,p) level in combination with the SCRF solvation model, the activation barrier for the attack of N¹ is predicted as $G^\ddagger = 51.34$ kcal/mol and the activation barrier for the attack of N² as $G^\ddagger = 42.67$ kcal/mol. Disregarding other effects (e.g., the need to break the Watson–Crick pairs in the DNA in order to establish the required reaction geometry; this requirement affects both reaction routes), the reaction at the exocyclic amino-nitrogen (N²) seems to be favored. In any case, the reaction barrier height alone also nicely explains the fact that 1,*N*²-ethenoguanosine is experimentally found as a minor adduct only.

Conclusions

In this study, we focused our efforts on characterizing the reactions of vinyl carbamate epoxide with the basic building blocks of the genetic material: nucleobases and DNA. We approached this task from the kinetic standpoint by assessing the activation free energies and the associated reaction rate constants, both experimentally and with quantum chemical simulations. In the experimental part, we have determined the reaction rate constant for the reaction between VCE and deoxyguanosine and between VCE and DNA. Both reactions were found to have a very similar activation free energy, so no large sterical or catalysis effects seem to take place in the DNA complex. In the computational part, the Hartree–Fock level of theory, combined with flexible basis sets and SCRF solvation model, was found to give a very good agreement with experimental activation free energy, which represents a strong argument in favor of the proposed S_N2 mechanism and also a good confirmation of the applicability of quantum chemical simulations to reactions of carcinogenesis. This success allowed us to employ the computational methods to predict the activation barriers of reactions involving other guanine sites and other nucleobases for which kinetic experiments have not yet been performed. The alternative reactive sites that we have considered (nucleophilic attacks of the guanine-N³, guanine-N¹, guanine-N², cytosine-N³, and adenine-N¹ atoms) either have significantly higher activation barriers than the nucleophilic attack of guanine-N⁷ or their transition states happen to lie in the way of Watson–Crick pairs in the DNA, thus making the reactions highly sterically hindered. With the help of quantum chemical simulations, we have also investigated the elimination of the leaving group that follows the initial S_N2 reaction. It has been demonstrated that this detachment passes across an activation barrier that is only on the order of the thermal noise and that it is therefore a very fast process. Moreover, a consideration of the influence of stereoselectivity has shown that stereochemistry is practically negligible for this class of reactions. Finally, our study

addressed the open question on the formation mechanism of the minor 1,*N*²-ethenoguanosine adduct, which seems to be a result of a nucleophilic attack initiated by the exocyclic N² amino nitrogen.^{33,34}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Thanks to L. Li for synthesizing dimethyldioxirane, F. K. Yoshimoto for recording the NMR spectra, J. A. Miller for the 7-(2-oxoethyl)Gua standard, and K. Trisler for assistance in preparation of the manuscript.

Grant support: National Institutes of Health R01 ES010546, Slovenian research agency ARRS grants (P1-0002, J1-5448, and J1-6736).

Abbreviations Used

DFT	density functional theory
dGuo	deoxyguanosine
Gua	guanine
MO	molecular orbital
LD	Langevin dipoles
SCRf	solvent reaction field
EVb	empirical valence bond
HF	Hartree-Fock
MP2	Møller-Plesset perturbation of the second order
VC	vinyl carbamate
VCE	vinyl carbamate epoxide
ZPE	zero-point energy

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Figure 1.

Hydrolysis of vinyl carbamate epoxide (in 100 mM potassium phosphate buffer, pH 7.4).

The initial concentration was 20 mM and the line shows a fit to $t_{1/2}$ 13.2 (± 1.5) min

$$(k'_a = (8 \pm 1) \times 10^{-4} \text{ s}^{-1}).$$

Figure 2.

Experimental data on the reaction between VCE and deoxyguanosine (circles) and a matching least-squares fit of Equation 2 (line). The fit of the linearized form of the equation (Eq. 3) is not displayed as it matches the shown curve so closely that the difference would be impossible to see in print.

Figure 3.

Experimental data on the reaction between VCE and DNA (circles) and a matching least-squares fit of Equation 2 (line). The fit of the linearized form of the equation (Eq. 3) is not displayed as it matches the shown curve so closely that the difference would be impossible to see in print.

Figure 4. Structure of the reactant state (A) and the transition state (B) for the nucleophilic attack of the N⁷ atom of methylguanidine as predicted by the HF/6-311++G(d,p) level of theory. Carbon is depicted in orange, oxygen in red, nitrogen in cyan, and hydrogen in white.

Figure 5. Structure of the reactant state (A) and the transition state (B) for the nucleophilic attack of the N³-site of methylguanine as predicted by the HF/6-311++G(d,p) level of theory. Carbon is depicted in orange, oxygen in red, nitrogen in cyan, and hydrogen in white.

Figure 6. Structure of the reactant state (A) and the transition state (B) for the nucleophilic attack of the N³-site of methylcytosine as predicted by the HF/6-311++G(d,p) level of theory. Carbon is depicted in orange, oxygen in red, nitrogen in cyan, and hydrogen in white.

Figure 7. Structure of the reactant state (A) and the transition state (B) for the nucleophilic attack of the N¹-site of methyladenine as predicted by the HF/6-311++G(d,p) level of theory. Carbon is depicted in orange, oxygen in red, nitrogen in cyan, and hydrogen in white.

Figure 8. Structure of the reactant state (A) and the transition state (B) for the nucleophilic attack of the N¹-site of methylguanine as predicted by the HF/6-311++G(d,p) level of theory. Carbon is depicted in orange, oxygen in red, nitrogen in cyan, and hydrogen in white.

Figure 9. Structure of the reactant state (A) and the transition state (B) for the nucleophilic attack of the N²-site of methylguanine as predicted by the HF/6-311++G(d,p) level of theory. Carbon is depicted in orange, oxygen in red, nitrogen in cyan, and hydrogen in white.

Scheme 1.

Proposed mechanism of the reaction between vinyl carbamate epoxide and guanine giving rise to the 7-(2-oxoethyl)deoxyguanosine adduct.

Scheme 2.

Proposed mechanism of the reaction between vinyl carbamate epoxide and guanine giving rise to the $N^2,3$ -ethenodeoxyguanosine adduct.

Scheme 3.

Proposed mechanism of the reaction between vinyl carbamate epoxide and adenine giving rise to the 1,*N*⁶-ethenodeoxyadenosine adduct.

Scheme 4.

Proposed mechanism of the reaction between vinyl carbamate epoxide and cytosine giving rise to the 3,N⁴-ethenodeoxycytidine adduct.

1,N²-ethenoguanine

Scheme 5.
Structure of the 1,N²-ethenodeoxyguanosine adduct.

Activation energies for the formation of the major 7-(2-oxoethyl)deoxyguanosine adduct calculated with different methods

Table 1

method	$E^{\ddagger a}$ (kcal/mol)	$ZPE^R b$ (kcal/mol)	$ZPE^{TS c}$ (kcal/mol)	ZPE^d (kcal/mol)	$\omega^R e$ (cm ⁻¹)	$\omega^{TS f}$ (i cm ⁻¹)	$d^R g$ (Å)	$d^{TS h}$ (Å)
HF/6-31G(d)	29.84	160.69	160.22	-0.48	12.9	567	3.54	2.02
HF/6-31+G(d,p)	28.62	159.65	159.01	-0.64	14.4	553	3.54	2.05
HF/6-311++G(d,p)	29.20	158.97	158.35	-0.63	13.5	554	3.56	2.06
B3LYP/6-31G(d)	19.48	148.41	147.94	-0.47	12.2	429	3.41	2.02
B3LYP/6-31+G(d,p)	18.08	147.49	146.97	-0.52	11.1	427	3.44	2.05
B3LYP/6-311++G(d,p)	17.95	146.97	146.43	-0.54	12.6	422	3.42	2.06
MP2/6-31G(d)	22.45	150.16	149.80	-0.36	7.6	575	3.35	1.94
MP2/6-31+G(d,p) ⁱ	20.30	150.16	149.80	-0.36	7.6	567	3.35	1.97
MP2/6-311++G(d,p) ^j	21.88	150.16	149.80	-0.36	7.6	588	3.35	1.96
AM1	41.24	150.20	148.86	-1.34	10.6	721	4.00	1.98
PM3	35.79	142.92	142.85	-0.07	8.4	752	3.74	1.95

^a Gas-phase activation energy.^b Zero-point vibrational energy for the reactants.^c Zero-point vibrational energy for the transition state.^d Zero-point energy of the transition state minus zero-point energy of the reactants.^e The lowest frequency value corresponding to the reactant state.^f The imaginary frequency corresponding to the transition state.^g The distance between the reacting N atom of nucleobase and the nonchiral epoxidic carbon of vinyl carbamate epoxide for the reactant state.^h The distance between the reacting N atom of nucleobase and the nonchiral epoxidic carbon of vinyl carbamate epoxide for the transition state.ⁱ Single point reactant state calculation; atom positions and vibrational data taken from MP2/6-31G(d) level.^j Single point reactant state calculation; atom positions and vibrational data taken from MP2/6-31G(d) level.

Table 2

Activation free energies for the formation of the major 7-(2-oxoethyl)deoxyguanosine adduct calculated with the solvent reaction field (SCRF) method (experimental value: $G^\ddagger = 22.4 \pm 0.1$ kcal/mol).

method	$\Delta G_{\text{hydr}}^{\text{SCRF}}(\text{R})^a$ (kcal/mol)	$\Delta G_{\text{hydr}}^{\text{SCRF}}(\text{TS})^b$ (kcal/mol)	$\Delta\Delta G_{\text{hydr}}^{\text{SCRF}}^c$ (kcal/mol)	$\Delta G_{\text{SCRF}}^\ddagger^d$ (kcal/mol)
HF/6-31G(d)	-3.42	-9.68	-6.26	23.10
HF/6-31+G(d,p)	-4.94	-11.37	-6.43	21.55
HF/6-311++G(d,p)	-4.32	-10.74	-6.42	22.15
B3LYP/6-31G(d)	-0.13	-5.42	-5.29	13.72
B3LYP/6-31+G(d,p)	-2.40	-7.69	-5.29	12.26
B3LYP/6-311++G(d,p)	-2.04	-7.17	-5.13	12.28
MP2/6-31G(d)	2.62	-2.25	-4.87	17.23
MP2/6-31+G(d,p) ^e	1.57	-3.92	-5.49	14.45
MP2/6-311++G(d,p) ^f	2.42	-2.80	-5.22	16.30

^a Hydration free energy for the reactant state, obtained by the SCRF method.

^b Hydration free energy for the transition state, obtained by the SCRF method.

^c Hydration free energy of the transition state minus hydration free energy of the reactant state.

^d Activation free energy.

^e Zero-point energy taken from MP2/6-31G(d) level.

^f Zero-point energy taken from MP2/6-31G(d) level.

Table 3

Activation free energies for the formation of the major 7-(2-oxoethyl)deoxyguanosine adduct calculated with the Langevin dipoles (LD) method (experimental value: $G^\ddagger = 22.4 \pm 0.1$ kcal/mol).

method	$\Delta G_{\text{hydr}}^{\text{LD}}(\text{R})^a$ (kcal/mol)	$\Delta G_{\text{hydr}}^{\text{LD}}(\text{TS})^b$ (kcal/mol)	$\Delta\Delta G_{\text{hydr}}^{\text{LD}}^c$ (kcal/mol)	$\Delta G_{\text{LD}}^\ddagger^d$ (kcal/mol)
HF/6-31G(d)	-29.45	-38.96	-9.51	19.85
HF/6-31+G(d,p)	-32.35	-41.73	-9.38	18.60
HF/6-311++G(d,p)	-31.82	-41.26	-9.44	19.13
B3LYP/6-31G(d)	-26.35	-35.14	-8.79	10.22
B3LYP/6-31+G(d,p)	-30.69	-39.36	-8.67	8.88
B3LYP/6-311++G(d,p)	-30.42	-38.59	-8.17	9.24
MP2/6-31G(d)	-25.86	-35.65	-9.79	12.30
MP2/6-31+G(d,p) ^e	-28.08	-39.08	-11.00	8.94
MP2/6-311++G(d,p) ^f	-27.40	-37.75	-10.35	11.17

^a Hydration free energy for the reactant state, obtained by the LD method.

^b Hydration free energy for the transition state, obtained by the LD method.

^c Hydration free energy of the transition state minus hydration free energy of the reactant state.

^d Activation free energy.

^e Zero-point energy taken from MP2/6-31G(d) level.

^f Zero-point energy taken from MP2/6-31G(d) level.

Table 4

Activation free energies for the formation of the major 7-(2-oxoethyl)deoxyguanosine adduct calculated with the AM1-SM1 and PM3-SM3 methods (experimental value: $G^\ddagger = 22.4 \pm 0.1$ kcal/mol).

method	$G_{\text{hydr}}(\text{R})^a$ (kcal/mol)	$G_{\text{hydr}}(\text{TS})^b$ (kcal/mol)	G_{hydr}^c (kcal/mol)	$G^\ddagger^d$ (kcal/mol)
AM1	-30.09	-32.89	-2.81	37.09
PM3	-41.28	-41.52	-0.23	35.48

^a Hydration free energy for the reactant state, obtained by the AM1-SM1 and PM3-SM3 methods.

^b Hydration free energy for the transition state, obtained by the AM1-SM1 and PM3-SM3 methods.

^c Hydration free energy of the transition state minus hydration free energy of the reactant state.

^d Activation free energy.