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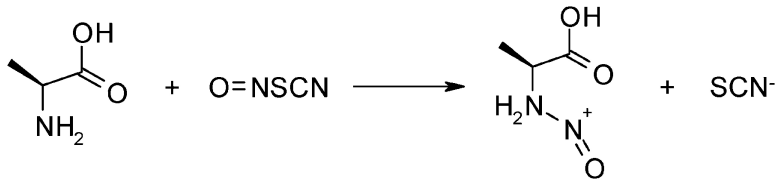

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Effect of Added Nucleophilic Species on the Rate of Primary Amino Acid Nitrosation

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Nitrosation reactions are of considerable biological significance, because primary amines can undergo *N*-nitrosation (diazotization) to form highly carcinogenic *N*-nitrosamines.[1] The nitrosation of amino acids is of particular importance because these compounds are found in the human body and are therefore capable of being nitrosated in vivo. Amino acid nitrosation in the acidic environment of the human stomach can lead to *N*-nitroso products or further decomposition products such as alkylating agents.[2] Furthermore, the amino acid groups which constitute DNA can undergo nitrosative deamination (or reaction with electrophilic alkylating agents)[3] with subsequent mutagenic effects. Accordingly, considerable work has been undertaken on amino acid nitrosation, particularly investigating the effect of different substrates on reactivity when undergoing reaction with the nitrosating agent dinitrogen trioxide (N_2O_3).[2a,4] While nitrosation reactions have long been known to be accelerated by strong nucleophilic species (the so-called nucleophile-catalyzed mechanism) through elevated equilibrium formation of the effective nitrosating agent,[5] to our knowledge the nucleophile-accelerated nitrosation of amino acids has not been investigated. This is even though certain nucleophilic species known to accelerate the rate of nitrosation, such as chloride, bromide, and iodide ions, are naturally present in the human body. Furthermore, the potent catalyst thiocyanate is known to exist at elevated concentrations in the saliva and stomachs of smokers[6] (up to 1×10^{-3} M).[7] We therefore propose that the rate of in vivo amino acid nitrosation may be considerably greater than currently thought, due to the presence of nucleophilic species.

We have studied the reactions of the amino acids alanine, glycine, and valine with the nitrosating agent N_2O_3, as well as the nitrosating agents generated by the addition of the nucleophilic species Cl^-, Br^-, SCN^-, and thiourea (i.e., ONCl, ONBr, ONSCN, and ON^+-$SC(NH_2)_2$ or ONTU), using stopped-flow UV/visible spectrophotometry. Kinetic experiments were conducted at 25 °C using an Applied Photophysics RX 2000 stopped flow unit interfaced with a Pharmacia LKB Ultrospec III UV/visible spectrophotometer. Experiments were initiated by rapidly mixing a sodium nitrite solution with an equal volume of an acidified solution containing both an amino acid and a nucleophilic catalyst. The rate of reaction was followed by observing the decreasing absorbance of nitrous acid with time at a wavelength of 371 nm,[8] as the rate of both nitrous acid and nitrosating agent consumption are equivalent. In separate experiments, we measured the absorption spectra of nitrous acid to exhibit a peak at around 371 nm with an extinction coefficient of 43.9 M^{-1} cm^{-1}, which agrees well with previously published measurements.[9] All experiments were conducted at high acidities (greater than 0.25 M acid), so that essentially all the sodium nitrite present would exist as nitrous acid, thus eliminating any interference from the overlapping nitrite ion spectra. Furthermore, the high acidity meant that the amino acids all existed primarily with a protonated carboxyl group, simplifying the kinetic analysis. Sodium nitrite was the limiting reagent, with the amino acid concentration 20 times in excess. Typically, the sodium nitrite concentration was ca. 0.0125 M, with the amino acid added at ca. 0.25 M. For chloride ion catalysis, hydrochloric acid was used as a source of chloride ions, at concentrations of around 0.75 M. For all other nucleophiles, sulfuric acid was used at ca. 0.25 M, with the required nucleophilic species added at ca. 0.5 M for Br^- catalysis, and ca. 0.25 M for the remaining nucleophiles. For nitrosation via N_2O_3, ca. 0.0125 M sodium nitrite was treated with a solution of ca. 0.25 M amino acid and ca. 0.25 M sulfuric acid.

Amino acid nitrosation by nitrosating agents formed from added nucleophiles is known to obey the rate law of eq 1,[10] where k_N is the nitrosation rate constant, K_{ONX} is the equilibrium constant for formation of the nitrosating agent ONX (where X represents the nucleophile) and K_a is the equilibrium constant for deprotonation of the substrate's amino group. Amino acid nitrosation by N_2O_3 follows the rate law of eq 2. According to the rate equations, the product of k_N, K_{ONX}, and K_a provides an indication of the observed reaction rates. By comparison of eq 2 to eq 1, we note that the rate of uncatalyzed nitrosation is inversely proportional to $[H^+]$, while nucleophilic catalyzed nitrosation is independent of $[H^+]$. Accordingly, pH measurements were required for the experiments without added nucleophiles, and in all cases the pH was found to be around 1.1−1.2. All equilibrium constants used in eqs 1 and 2 are provided as Supporting Information. We have analyzed the kinetic stopped-flow data according to the proposed rate equations, and the obtained k_N values are reported in Table 1, while the product of k_N, K_{ONX}, and K_a is given in the Supporting Information. Furthermore, experiments were also performed in the temperature range of 20−45 °C, to determine activation energies (also presented in Table 1). In calculating k_N, initial rate data was typically used so as to minimize errors introduced by fluctuations within the stopped-flow cell, such as those caused by mass transfer effects or by the production of nitrogen gas bubbles from nitrosamine decomposition. Quoted rate constants in Table 1 are the averages of at least four replicated experiments; the average standard deviation for these results was 8%. This error was largest for the slower reactions, i.e. with ONCl and N_2O_3. For activation energy experiments, two repeat runs were typically performed at each temperature. From the experimental results reported in Table 1 we observe that, for each particular nitrosating agent, k_N varies little between each of the substrates tested. García-Santos et al.[2a] also found this to be the case for the nitrosation of nine primary, secondary, and tertiary amino acids by N_2O_3.

$$r = k_N K_{ONX} K_a [AA][X^-][HNO_2] \quad (1)$$

$$r = k_N K_{N_2O_3} K_a [AA][HNO_2]^2/[H^+] \quad (2)$$

In Figure 1, the rate of nitrosation of valine is compared under

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Table 1. Nitrosation Rate Constants at 25 °C (k_N, M^{-1} s^{-1}) and Activation Energies at 20−45 °C (E_a, kJ mol^{-1})

	k_N					E_a				
	ONCl	ONBr	ONSCN	ONTU	N_2O_3	ONCl	ONBr	ONSCN	ONTU	N_2O_3
alanine	$(4.7 \pm 0.7) \times 10^9$	$(2.4 \pm 0.8) \times 10^9$	$(7.4 \pm 0.8) \times 10^7$	$(7.9 \pm 0.1) \times 10^5$	$(8.2 \pm 0.8) \times 10^8$	13	31	31	46	11
glycine	$(1.0 \pm 0.1) \times 10^{10}$	$(2.9 \pm 0.1) \times 10^9$	$(3.71 \pm 0.04) \times 10^8$	$(9.2 \pm 0.3) \times 10^5$	$(2.34 \pm 0.05) \times 10^9$	13	28	42	42	N/A
valine	$(6.7 \pm 0.7) \times 10^9$	$(6.3 \pm 0.2) \times 10^9$	$(3.4 \pm 0.3) \times 10^8$	$(5.99 \pm 0.07) \times 10^5$	$(3.7 \pm 0.3) \times 10^9$	11	21	37	40	N/A

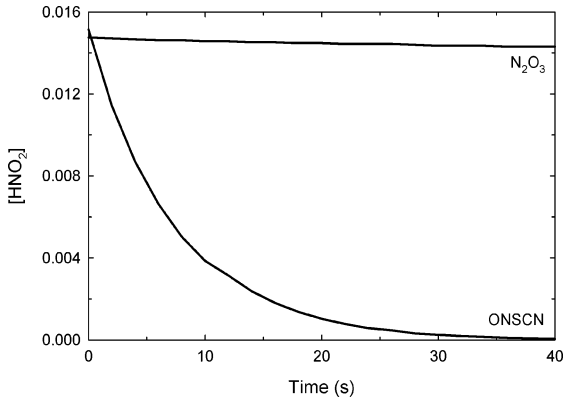

Figure 1. Comparison between the rate of valine nitrosation with and without the addition of 0.25 M NaSCN at 25 °C. $[H_2SO_4] = 0.25$ M.

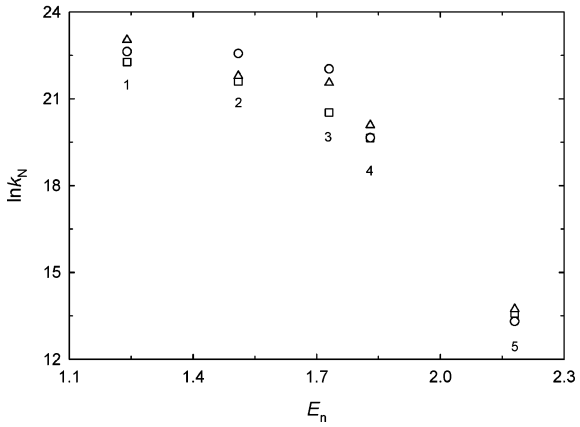

Figure 2. Relationship between ln k_N and E_n for the nitrosation of alanine (open squares), glycine (open triangles), and valine (open circles) by ONCl (1), ONBr (2), N_2O_3 (3), ONSCN (4), and $ON^+SC(NH_2)_2$ (5).

similar conditions with and without the addition of SCN^-. We see that the presence of SCN^- dramatically increases the rate of nitrosation, with the half-life for reaction dropping from over 4000 s for N_2O_3 nitrosation to around 5 s for ONSCN nitrosation. The acceleration observed in Figure 1 is a result of the greater equilibrium formation of ONSCN compared to N_2O_3, as we note from Table 1 that their k_N values are of similar magnitude. A similar degree of acceleration to that for SCN^- was witnessed for nitrosation in the presence of thiourea, while a smaller effect was obtained using Cl^- and Br^-. This order of reactivity is consistent with the calculated values for $k_N K_{ONX} K_a$ presented in the Supporting Information.

From Table 1 we observe that, for the three most reactive nitrosating agents, ONCl, ONBr, and N_2O_3, k_N values level off at or just below 10^{10} M^{-1} s^{-1}, which represents the encounter-controlled limit. This conclusion is supported by the measured activation energies, which for both ONCl and ONBr are in the range expected for encounter-controlled conditions.[11] For nitrosation by N_2O_3, alanine returned an activation energy of the expected magnitude, although glycine and valine demonstrated curved activation energy diagrams, which we believe indicates transition from reaction-controlled to encounter-controlled conditions. For all other nucleophiles, the experimental results yielded Arrhenius and Eyring plots of good linearity (correlation coefficients for the Arrhenius plots are tabulated in the Supporting Information). For nitrosation by ONSCN and ONTU, the measured activation energies are consistent with those expected for reaction-controlled nitrosation.

Previously, a kinetic model was proposed for amine nitrosation where reactivity (ln k_N) is linearly proportional to Edwards' nucleophilic constant (E_n) within the reaction-controlled regime.[11] Here, the slope of this relationship is equal to $-17.9\alpha_{ONX}$, where α_{ONX} is a parameter that varies between 0 and 1 and measures the resemblance of the transition state to the reaction products. The measured k_N values are plotted according to E_n in Figure 2, and we find that each plot approaches a linear relationship as we depart from the encounter-controlled regime. Constructing linear relationships between the kinetic results of the two least reactive nitrosating agents, α_{ONX} values of 0.96, 1.01, and 1.01 were found for the amines alanine, glycine, and valine, respectively, indicating that the reaction transition states would be product-like. Similarly, product-like transition states have been predicted for the nitrosation of aniline and its derivatives (comparatively, an average α_{ONX} value of 0.83 was found for aniline nitrosation).[11]

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Supporting Information Available: Equilibrium constants used to determine k_N values (K_{ONX} and K_a), table of $k_N K_{ONX} K_a$ values, and correlation coefficients for Arrhenius plots. This material is available free of charge via the Internet at http://pubs.acs.org.

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