# The Nitrosation of *N*-Alkylureas: Evidence for a Proton Transfer Mechanism

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## ABSTRACT

The kinetics of the nitrosation of methyl, ethyl, propyl, butyl, and allyl urea were studied by conventional and stopped-flow spectrophotometry in the presence or absence of acetate or mono-, di-, or trichloroacetate anions. In the presence of a large excess of urea, the observed rate equation was

$$\mathbf{v} = \frac{[\text{urea}][\text{nitrite}][\mathrm{H}^+]^2}{K_a + [\mathrm{H}^+]} \left(\vartheta + \xi \frac{K_R[\text{carboxylic acid}]}{K_R + [\mathrm{H}^+]}\right)$$

where  $K_a$  is the acidity constant of nitrous acid and  $K_R$  that of the carboxylic acid. The ureas exhibited the reactivity order methylurea  $\geq$  (ethylurea  $\approx$  propylurea  $\approx$  butylurea)  $\geq$  allylurea. Experiments in D<sub>2</sub>O afforded values of  $k_{H_2O}/k_{D_2O}$  in general agreement with the values 4.1-5.5 predicted by a semiclassical transition state theory of kinetic isotope effects [i.e.,  $k_{H_2O}/k_{D_2O} = \exp(0.130h\overline{\nu}/kT)$ ], where  $\overline{\nu}$  is the frequency of  $R_3N$ —H stretching (2700-2250 cm<sup>-1</sup>) in the protonated urea. This result, the observed catalysis by carboxylate ions and the value of the Brønsted parameter  $\beta(0.45)$  show the rate-controlling step of these reactions to be the transfer of a proton from the protonated *N*-alkyl-*N*-nitrosourea to the solvent or to the organic anion, if present. The observed order of substrate reactivities is explicable in terms of the capacity of the protonated *N*-alkyl-*N*-nitrosourea for forming a hydrogen bond with the water molecule to which the proton will be transferred, and the degree to which the formation of such bonds is hindered by the hydrophobic alkyl chain of the nitrosourea. (© 1996 John Wiley & Sons, Inc.

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# INTRODUCTION

The numerous environmental chemicals threatening the integrity of the DNA of living cells include nitrosamines, which cause adenine  $\cdot$  thymine  $\rightarrow$ guanine  $\cdot$  cytosine and guanine  $\cdot$  cytosine  $\rightarrow$ adenine  $\cdot$  thymine transitions [1], and alkylating agents such as alkylnitrosoureas, which mainly affect guanine [2]. Nitrosation of ureas, guanidines, and carbamates may be important because at least 14 ureas and 8 guanidines are found in nature, where the ureas probably arise by bacteria-catalyzed deimidation of guanidines or by carbamylation of amines [3].

The action of alkylnitrosoureas tends to produce quite definite types of tumour [4, 5]; the key to such organ specificity may be, especially in the case of directly acting carcinogens such as alkylnitrosoureas, their interaction with critical molecules in certain cells. The organ specificity mentioned above is illustrated by reports that allylnitrosourea (ANU) induces tumors in the colon and jejunum of male rats and that butylnitrosourea (BNU) induces colon tumors ([5], p. 357; [6, 7]). Somewhat paradoxically, nitrosoureas have also exhibited a degree of efficacy for the chemotherapy of cancer [8].

In spite of the importance of nitrosoureas, the mechanisms of their formation (first investigated by Mirvish in 1971 for methylnitrosourea [9]) have not previously been studied systematically as has the formation of other nitroso compounds [10]. As part of our on-going research on the formation of nitroso compounds of biochemical interest [11], and prompted by earlier results on the nitrosation of methylurea [12], in this work we studied the mechanisms of the nitrosation of methylurea (MU), ethylurea (EU), propylurea (PU), butylurea (BU), and allylurea (AU).

# **EXPERIMENTAL**

Solutions of MU, EU, BU, and AU were made up by weight from p.a. products from Merck (Darmstadt, Germany) (MU, AU) or Fluka (Buchs, Switzerland) (EU, BU) with nominal purities of 98–99%. Solutions of PU were made up by weight from a product from Alfa (Karlsruhe, Germany) after prior recrystallization from ethanol/water. NaNO<sub>2</sub>, NaClO<sub>4</sub>, CH<sub>3</sub>COOH, ClCH<sub>2</sub>COOH, Cl<sub>2</sub>CHCOOH, Cl<sub>3</sub>CCOOH (all from Merck), and HClO<sub>4</sub> (60% by weight, from Fluka) were all used without previous purification. D<sub>2</sub>O (98% D) was supplied by CIEMAT (Madrid, Spain).

Ionic strength (I) was maintained with Merck p.a. sodium perchlorate monohydrate solutions made up by weight. Acidity was measured with a Radiometer M64 digital pH-meter with a GK2401 combined electrode. Except for the faster reactions, nitrosation kinetics were recorded by monitoring absorbance by nitroso compounds at 249 nm (in perchlorate media) or 260 nm (in the presence of carboxylic acids) in a Shimadzu UV-240 spectrophotometer equipped with a thermoelectric cell holder thermostat maintaining temperature within  $\pm 0.1$  °C. Under these conditions the molar absorptivity of the nitrosoureas studied is much greater (e.g., for MNU  $\varepsilon_{260} = 3405 \text{ M}^{-1} \text{ cm}^{-1}$ ) than that of nitrite ( $\varepsilon_{260} = 6 \text{ M}^{-1} \text{ cm}^{-1}$ ), absorbance by alkylureas is negligible, and absorbance by carboxylic acids is constant. Absorbance-time data were analyzed by the integration or the initial rate method (following the reaction until <5% complete [13, 14]), depending on which was most convenient in each case; similar results were obtained whichever was used.

The faster reactions were studied by means of a modular stopped flow apparatus equipped with a Hewlett-Packard 6267BDD power supply, a Hg/Xe lamp with a Bypass-Starter DIA-RBS, and a Jobin Yvon H20UV monochromator with fiber light guide. Detector signal data were processed on a computer Tandon PCA/12 S140 via an ADAM TC112 analog data acquisitions memory. Software DAGMAR transfer and DAGMAR graphics were used. The flow block was designed at the University of Bielefeld, Germany (Prof. W. Knoche). All kinetic experiments were carried out with a large excess of alkylurea (henceforth called urea) over nitrite.

# **RESULTS AND DISCUSSION**

All kinetic experiments complied with the rate equation

$$\mathbf{v} = k[\text{urea}][\text{nitrite}] \tag{1}$$

with values of k that were reproducible to within  $\pm 3\%$ .

The influence of acidity on k was studied over the proton concentration range 0.005-1.0 M. Within the range of 0.005-0.04 M H<sup>+</sup> the k against [H<sup>+</sup>] data fitted the equation

$$\frac{[\mathrm{H}^+]^2}{k} = b[\mathrm{H}^+] + c \tag{2}$$

(see Fig. 1(a)), which when combined with eq. (1), implies the rate equation

$$v = \frac{[\text{urea}][\text{nitrite}][H^+]^2}{b[H^+] + c}$$
(3)



**Figure 1** Influence of  $[H^+]$  (0.005–0.04 M in Fig. 1(a) and 0.04–0.25 M in Fig. 1(b) on the rate constant k (eq. (1)) of the nitrosation of ( $\bigcirc$ ) MU, ( $\diamondsuit$ ) EU, ( $\square$ ) PU, ( $\triangle$ ) BU, and (+) AU ([urea] = 0.35 M; [nitrite] =  $1.0 \times 10^{-4}$  M; T = 298 K).

In the range 0.04–0.25 M H<sup>+</sup>, the results fitted the equation  $k = b'[H^+]$  (Fig. 1(b)), which implies the rate equation

$$\mathbf{v} = b'[\text{urea}][\text{nitrite}][\mathbf{H}^+]$$
(4)

These rate equations and the values of the rate constants differ from those obtained for the nitrosation reactions of amines, in which the rate-controlling step is the attack on the substrate by the nitrosating agent. Whereas the nitrosation of amines can be of either first- or second-order with respect to nitrous acid (depending on whether the effective nitrosating agent is  $NO_2H_2^+/NO^+$  or  $N_2O_3$ ) [15] and exhibits rate constants close to the diffusioncontrolled limit [16, 17], only first-order [nitrate] dependence has been reported for the nitrosation of ureas and amides [4, 17]. On the basis of the above results and the conclusions of a previous study [12], we hypothesize the following mechanism for the nitrosation of ureas.

Bearing in mind that [nitrite] =  $[HONO] + [NO_2^+]$ ([nitrite] being total nitrite concentration, henceforth [nit]), the above mechanism implies the rate equation

$$\mathbf{v} = -\frac{d[\operatorname{nit}]}{dt} = \left(\frac{1}{\Delta\varepsilon}\right) \left(\frac{d(\operatorname{Abs})}{dt}\right)$$
$$= \frac{[\operatorname{urea}][\operatorname{nit}][\operatorname{H}^{+}]^{2}}{K_{a} + [\operatorname{H}^{+}]} \qquad (5)$$
$$\cdot \left(k_{\operatorname{H_2O}}K_1K_2 + k_{\operatorname{NO_2}}^{-}K_1K_2\frac{K_{a}[\operatorname{nit}]}{K_{a} + [\operatorname{H}^{+}]}\right)$$

where  $\Delta \varepsilon = \varepsilon_{\text{nitrosourea}} - \varepsilon_{\text{nitrite}} \approx \varepsilon_{\text{nitrosourea}}$  (see above).

For the low nitrite concentration used in this work  $(10^{-4} \text{ M})$ , the term in [nit]<sup>2</sup> can be ignored. This has been checked experimentally (for a systematic study of the influence of nitrite concentration in this kind of reaction, see ref. [18]). Equation (5) then reduces to

$$\mathbf{v} = -\frac{d[\text{nit}]}{dt} = \frac{[\text{urea}][\text{nit}][\text{H}^+]^2}{K_a + [\text{H}^+]} k_{\text{H}_2\text{O}} K_1 K_2.$$
(6)

Identification with eq. [1] yields

$$k = \frac{k_{\rm H_2O}K_1K_2[\rm H^+]^2}{K_a + [\rm H^+]}.$$
 (7)

This can be written in the form of eq. (2) with  $b = 1/k_{wexp}$  and  $c = K_a/k_{wexp}$ , where  $k_{wexp} = k_{H_2O}K_1K_2$ 

$$\frac{[{\rm H}^+]^2}{k} = \frac{[{\rm H}^+]}{k_{\rm wexp}} + \frac{K_{\rm a}}{k_{\rm wexp}}$$
(8)

Therefore, the good fit between eq. (2) and the experimental data (Fig. 1(a)) supports the proposed mechanism. The ratio between the observed values of c and b in eq. (2) afford the values of  $pK_a$  (nitrous acid, see Scheme I) that are listed in Table I for the reactions studied. The good agreement between these values and the published [19–21]  $pK_a$  of 3.15 constitutes further support for the proposed mechanism.

At high acidities  $([H^+] \ge K_a)$ , eq. (7) reduces to  $k = k_{wexp}[H^+]$  in good agreement with the experimentally verified eq. (4) (Fig. 1(b)) where  $b' = k_{wexp}$ . Since  $K_1$  and  $K_2$  are equilibrium constants,  $k_{wexp}$  is a measure of the reactivities of the ureas. Table I lists the values of  $k_{wexp}$  observed for the ureas studied.

Support for the hypothesis that the slow step of the reaction is a proton transfer, as postulated in

$$HNO_2 \implies NO_2^- + H^+$$
  $K_a$  fast

$$HNO_2 + H^+ == NO^+ + H_2O \qquad K_1 \text{ fast}$$

$$\begin{array}{c} O & ON O \\ \parallel & & \parallel \\ NO^{+} + RNHC - NH_2 & \underbrace{k_3}_{k_{-3}} & \parallel \\ \hline & & RNHC - NH_2 \end{array} K_2 \text{ fast}$$

ON O ||| ON O |||| RNHC—NH<sub>2</sub> + H<sub>2</sub>O  $\rightarrow$  RN—C—NH<sub>2</sub> + H<sub>3</sub>O<sup>+</sup>  $k_{H_2O}$  slow

 $RNHC - NH_2 + H_2O - RN - C - NH_2 + H_3O^+ \qquad k_{H_2O} \text{ slow}$ 

Scheme I The mechanism of the nitrosation of alkylureas.

Scheme I, was sought by determining the kinetic isotope effect  $k_{\rm H_2O}/k_{\rm D_2O}$ . The results of initial rate experiments carried out in H<sub>2</sub>O and D<sub>2</sub>O for various values of pH or pD (=pH + 0.4 [22]) were analyzed by means of eq. (8), with

$$k = \frac{[d(Abs)/dt]_{t=0}}{\Delta \varepsilon [urea]_0 [nitrite]_0}$$
(9)

This yielded the values of  $K_a$ ,  $pK_a$ , and  $k_{wexp}\Delta\varepsilon$ listed in Table II. Since  $K_1(D_2O)/K_1(H_2O) = 2.7$ [23],  $k_{H_2O}/k_{D_2O}$  (also listed in Table II) was calculated as

$$\frac{k_{\rm H_2O}}{k_{\rm D_2O}} = 2.7 \frac{k_{\rm wexp} \Delta \varepsilon (\rm H_2O)}{k_{\rm wexp} \Delta \varepsilon (\rm D_2O)}$$
(10)

The validity of the  $k_{H_2O}/k_{D_2O}$  data is supported by the agreement between the observed and published values of  $pK_a$  and of the  $pK_a(D_2O) - pK_a(H_2O)$ [19-21, 24]. The hypothesis that the reaction is controlled by the proton transfer steps of Scheme I is therefore supported by the fact that the experimental values of  $k_{H_2O}/k_{D_2O}$  lie in the range of 4.1-5.5 predicted by a semi-classical transition state theory of kinetic isotope effects [25-28]. According to this theory

$$k_{\rm H_2O}/k_{\rm D_2O} = \exp(0.130h\overline{\nu}/kT)$$
 (11)

where  $\overline{\nu}$  is the frequency of R<sub>3</sub>N—H stretching in the protonated *N*-nitrosourea 2700–2250 cm<sup>-1</sup> [29].

Control by a proton transfer step is also supported by the observation of catalysis by  $RCOO^{-}(R = CH_3, CH_2Cl, CHCl_2, and Cl_3)$ . In the presence of these anions, the mechanism shown in Scheme I must also include the step

$$\begin{array}{cccc} ON & O & ON & O \\ | & || \\ RNHC - NH_2 + X_3CCOO^- \longrightarrow RN & C - NH_2 + X_3CCOOH \\ + & + & \end{array}$$

leading to the rate equation

$$\mathbf{v} = -\frac{d[\operatorname{nit}]}{dt} = \frac{[\operatorname{urea}][\operatorname{nit}][\mathrm{H}^+]^2}{K_a + [\mathrm{H}^+]}$$
$$\cdot \left(k_{w \exp} + \frac{K_R k_c [\operatorname{carboxylic acid}]}{K_R + [\mathrm{H}^+]}\right) \quad (12)$$

where  $K_R$  is the acidity constant of the carboxylic acid and  $k_c = K_1 K_2 k_{RCOO}$ . Since for low nitrite concentrations (see above), v = k'[nit],

$$k' = \frac{k_{x} \exp[\text{urea}][\text{H}^{+}]^{2}}{K_{a} + [\text{H}^{+}]}$$
(13)

in which

$$k_{x \exp} = k_{w \exp} + \frac{K_R k_c [\text{carboxylic acid}]}{K_R + [\text{H}^+]} \quad (14)$$

Linear dependence of  $k_{x \exp}$  on carboxylic acid concentration was indeed observed in all cases (Fig. 2 shows the results for catalysis by ClCH<sub>2</sub>COO<sup>-</sup>), and from the slopes of the corresponding plots we obtained the values of  $k_c$  listed in Table III.

Plots like that shown in Figure 3 for MU exhibited linear dependence of log  $k_c$  against pK (carboxylic acid) for the nitrosation of each substrate in the presence of the carboxylic acids, with the Brønsted parameters  $\beta$  listed in Table II. The good correlation between log  $k_c$  and pK, and the values of the Brønsted parameters, are in agreement with (i) the existence of a unitary mechanism controlled by the cleavage of the N—H bond (Scheme I), since the cleavage of any other bond would probably give rise to significantly different values of  $\beta$  for different substrates, and (ii) the occurrence of a transition state that is symmetric with respect to reagents and products (the Hammond postulate [31]).

The proposed mechanism also explains some of the differences observed between the nitrosation of

| Substrate | т.р., °С | $k_{\rm wexp},  {\rm M}^{-2}  {\rm s}^{-1}$ | $\Delta H^{*}$ , kJ/mol | $-\Delta S^{\#}$ , J/mol K | p <i>K</i> <sub>a</sub> * |
|-----------|----------|---|-------------------------|----------------------------|---------------------------|
| MU        | 97-99    | $26.54 \pm 0.8$                             | 36.8 ± 0.6              | 129 ± 2                    | 3.3                       |
| EU        | 92-95    | $7.44 \pm 0.1$                              | $41.0 \pm 1.7$          | $114 \pm 6$                | 3.2                       |
| PU        | 93-96    | $7.03 \pm 0.2$                              | $39.7 \pm 1.0$          | $130 \pm 4$                | 3.2                       |
| BU        | 95-98    | $7.78 \pm 0.3$                              | $40.3 \pm 0.9$          | $126 \pm 3$                | 3.1                       |
| AU        | 83-86    | $4.10 \pm 0.1$                              | $51.5 \pm 0.8$          | 76 ± 3                     | 3.2                       |

Table I Reactivity of Ureas to Nitrosation

\*Nitrous acid, see Scheme I.

| Substrate | Solvent          | $k_{w \exp} \Delta \varepsilon^{a}$ | р <i>К</i> а <sup>а</sup> | $k_{\rm H_2O}/k_{\rm D_2O}^{\rm b}$ | β <sup>ι</sup>  |
|-----------|------------------|-------------------------------------|---------------------------|-------------------------------------|-----------------|
| MÜ        | H <sub>2</sub> O | $(1.11 \pm 0.03) \times 10^5$       | 3.3                       | 2 50 + 0.09                         | 0.41 ± 0.01     |
|           | D <u>2</u> O     | $(8.4 \pm 0.3) \times 10^4$         | 3.9                       | $5.50 \pm 0.08$                     |                 |
| EU        | H <sub>2</sub> O | $(2.05 \pm 0.01) \times 10^4$       | 3.5                       | 16 + 0.2                            | 0.44 ± 0.04     |
|           | $D_2O$           | $(1.2 \pm 0.05) \times 10^4$        | 3.9                       | 4.0 ± 0.2                           |                 |
| PU        | H <sub>2</sub> O | $(1.01 \pm 0.03) \times 10^4$       | 3.3                       | 5 4 × 1 4                           | 0.44 + 0.02     |
|           | $D_2O$           | $(5.0 \pm 0.1) \times 10^{3}$       | 4.3                       | $5.4 \pm 1.4$                       | $0.44 \pm 0.02$ |
| BU        | H <sub>2</sub> O | $(1.03 \pm 0.03) \times 10^4$       | 3.2                       | 47 1 1 2                            | 0.44 + 0.04     |
|           | $D_2O$           | $(5.8 \pm 0.1) \times 10^3$         | 4.2                       | $4.7 \pm 1.2$                       | $0.46 \pm 0.04$ |
| AU        | H <sub>2</sub> O | $(6.17 \pm 0.09) \times 10^3$       | 3.2                       |                                     |                 |
|           | $D_2O$           | $(3.0 \pm 0.1) \times 10^3$         | 3.6                       | $5.1 \pm 0.1$                       | $0.44 \pm 0.03$ |

Table II Primary Kinetic Isotope Effect and Brønsted Parameters for the Nitrosation of Ureas

<sup>a</sup>Equation (8), calculated with 7 values of [H<sup>+</sup>], see Figure 1.

<sup>n</sup>Equation 10.

"See Figure 3.

amines and that of ureas: (i) in the nitrosation of ureas catalysis by halides or pseudo-halides has not been observed [12, 32, 33], and (ii) in the nitrosation of amines catalysis by nitrosyl carboxylates is much weaker than that by nitrosyl halides [34, 35]. There is no reason to think that in the case of ureas the order is inverted to the point of the nitrosyl compounds being effective and the halides having no effect at all. Thus, in the nitrosation of ureas the rate controlling step must not be the reaction between the nitrosating agent and the nitrosatable substrate, as occurs with amines, but the subsequent step in which the intermediate formed loses a proton (see Scheme I).

The series of experiments carried out at 283-303 K afforded (a) the activation parameters



Figure 2 Dependence of  $k_x \exp (\text{eq. (13)})$  on [ClCH<sub>2</sub>COOH] in the nitrosation of (O) MU, ( $\diamond$ ) EU, ( $\Box$ ) PU, ( $\triangle$ ) BU, and (+) AU ([urea] = 0.2 M; [nitrite] = 1.0 × 10<sup>-4</sup> M; I = 0.2 M; pH = 2.2; and T = 298 K).

listed in Table I, which exhibit isokinetic correlation [14, 36] (Fig. 4), and (b) a linear log  $k_1/\log k_2$  plot [37, 38] (Fig. 5). Both findings are in keeping with the assumption that all the studied nitrosation reactions involve the same mechanism.

The results summarized in Tables I-III indicate the reactivity order  $MU \gg (EU \approx PU \approx BU) \gg$ AU. Figure 6 suggests that the greater reactivity of MU can be explained in terms of the extent to which hydrogen bonding between the protonated nitrosourea and the water molecules to which the proton will be transferred is hindered by the alkyl chain of the nitrosourea: the larger, more hydrophobic alkyl chains of EU, PU, and BU must tend to fold back on the rest of the molecules, thereby impeding pretransfer hydrogen bonding. The low reactivity of AU should be associated with its low melting point (Table I), which reflects relatively low capacity for intermolecular hydrogen bonding [39, 40] and hence low capacity for forming hydrogen bonds with water. The high activation enthalpy of AU and the small absolute value of its entropy of activation (Table I) are in keeping with this interpretation.

### CONCLUSION

Kinetic studies of the nitrosation of five alkylureas have shown the following: (1) The rate-controlling step of these reactions is the proton transfer from the protonated alkylnitrosourea to the solvent; (2) The observed order of reactivities  $MU \ge (EU \approx PU \approx$  $BU) \ge AU$  has been rationalized in terms of the capacity of the protonated alkylinitrosourea for forming a hydrogen bond with the water molecule to which the proton will be transferred, and of the degree to which the formation of such bonds is hindered by the

|                        | р <i>К</i> <sup>а</sup> | $k_c,  { m M}^{-3}  { m s}^{-1}{ m b}$ |                |                |                |                |
|------------------------|-------------------------|--|----------------|----------------|----------------|----------------|
| Acid                   | [30]                    | MU                                     | EU             | PU             | BU             | AU             |
| CH <sub>3</sub> COOH   | 4.70                    | $3012 \pm 43$                          | 1176 ± 21      | 1014 ± 51      | 1086 ± 34      | 863 ± 9        |
| CICH <sub>2</sub> COOH | 2.85                    | $465 \pm 4$                            | $276 \pm 3$    | $210 \pm 23$   | 181 ± 6        | $107 \pm 4$    |
| Cl <sub>2</sub> CHCOOH | 1.48                    | $118 \pm 4$                            | 43.5 ± 1.7     | $35.6 \pm 1.3$ | 49.9 ± 2.4     | $24.8 \pm 0.9$ |
| Cl <sub>3</sub> CCOOH  | 0.51                    | $54 \pm 1$                             | $15.9 \pm 1.0$ | $17.8 \pm 1.3$ | $12.4 \pm 0.7$ | $15.9 \pm 1.0$ |
| HClO <sub>4</sub>      |                         | $26.5 \pm 1$                           | $7.4 \pm 0.1$  | $7.5 \pm 0.2$  | $7.7 \pm 0.3$  | $4.5 \pm 0.1$  |

Table III Catalytic Constants for the Catalysis of the Nitrosation of Ureas by Carboxylic Acids

<sup>a</sup>Carboxylic acid.

<sup>b</sup>Equation (14), calculated with 5-6 values of [carboxylic acid], see Figure 2.



Figure 3 Brønsted plot for general base catalysis of the nitrosation of MU by carboxylate ions.

alkyl chain of the alkylnitrosourea; and (3) The results obtained for each nitrosatable substrate as well as the sequence of reactivities found show that it is pointless to try to interpret the nitrosation reactions



Figure 4  $\Delta H^{\#}/\Delta S^{\#}$  plot for the nitrosation of *N*-alkylureas.



**Figure 5**  $\log k_1 / \log k_2$  plot for the nitrosation of *N*-alkylureas.

of amines and amides on the basis of the equality of their mechanisms.



Figure 6 Proposed hydrogen bonding between water and protonated *N*-alkyl-*N*-nitrosoureas.

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