Kinetics and mechanism of the basic hydrolysis of nitrosoureas

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Kinetic experiments on 1,3-dimethyl-1-nitrosourea (DMNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) and 1,3,3-trimethyl-1-nitrosourea (TMNU) in the presence and absence of strong nucleophiles such as HOO^- show that the basic hydrolysis of the nitrosoureas with an acidic hydrogen atom proceeds mainly by abstraction of the proton to afford an anion the subsequent decomposition of which is the rate-controlling step of the overall reaction. TMNU, which has no acidic hydrogen atom, is hydrolysed by nucleophilic attack on the carbonyl group.

Introduction

While nitrosoureas in general are carcinogenic to human beings, some also have useful antitumour properties. However, there is no clear distinction between the origins of these two activities: the strongest antitumour agents are also strong carcinogens.[1] Since the effects of nitrosoureas on cellular DNA appear to be related to their efficiency as alkylating agents[2–4] [or rather, to the alkylating efficiency of the species formed by loss of the –N(NO)R unit through hydrolysis], current research is directed towards species the toxicity of which is limited by their selectivity as alkylating agents.[5] The mechanism of the decomposition of nitrosoureas nevertheless continues to be a topic of considerable biological relevance. The literature on nitrosoureas offers a wide range of possible mechanisms. Most of the recent papers discuss two main possibilities for the mechanism of the hydrolysis of nitrosoureas in neutral or basic media.

(*a*) Addition of HO^- to the urea carbonyl group to form a tetrahedral intermediate, which then undergoes decomposition. This mechanism was originally put forward by Garrett *et al.*[6] and Snyder and Stock[7] for some alkylnitrosoureas, and by Lown and Chauhan[8] for chloroethylnitrosoureas.

(*b*) Alternatively, the hydrolysis of nitrosoureas with acidic hydrogen atoms (pK_a 10–13) might occur *via* the corresponding anionic conjugate base of the nitrosourea. This mechanism was first proposed by Hetch and Kozarich[9] to explain the observed kinetics of the decomposition of MNU, and has recently been used by Golding *et al.*[10] to explain the results of a ^{13}C NMR study of the decomposition products of MNU.

For alkylnitrosoureas with acidic hydrogen atoms, the mechanistic problem is thus to determine whether the role of the HO^- group is predominantly deprotonation (to afford an unstable anion) or attack on the carbonyl group (to afford an unstable tetrahedral intermediate). Because of their antitumour activity, particular attention has been paid to haloethylnitrosoureas; their decomposition has been studied kinetically, and quantitative analysis of their decomposition products has been performed,[11–13] but in most cases the results have been inconclusive because neither the final products nor the kinetics differentiate between the two possible mechanisms. *Ab initio* calculations of possible intermediates have also been carried out,[14] likewise inconclusively.

This paper presents new kinetic results on 1,3-dimethyl-1-nitrosourea (DMNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) and 1,3,3-trimethyl-1-nitrosourea (TMNU) that have allowed determination of which of the two possible mechanisms mediates the basic hydrolysis of each.

Experimental

Solutions of DMNU and TMNU were prepared *in situ* from 0.001–0.01 mol dm^{-3} solutions of $NaNO_2$ in acidic media (*ca.* pH 2) and 0.01–0.1 mol dm^{-3} solutions of, respectively, 1,3-dimethylurea (Aldrich) and 1,3,3-trimethylurea (Alfa). CCNU (Aldrich) was used as supplied, without further purification; because of their instability and low solubility in water, CCNU solutions were made up in acetonitrile and reactions were initiated by addition of small volumes of these solutions to the reaction mixtures (the proportion of organic solvent in the final mixture was never greater than 1%). Deuteriated water (99.77% D) was supplied by CIEMAT (Spain). All other reagents were Aldrich or Merck products of the maximum commercially available purity and were used as supplied, without further purification.

Kinetics were recorded on Kontron Uvikon 930 or Spectronic 3000 Diode Array spectrophotometers equipped with multiple thermostatted cell carriers, except that the fastest reactions were monitored with an Applied Photophysics DX.17MV sequential stopped-flow spectrofluorimeter. Acidity was measured with a Radiometer pHM82 pH-meter equipped with a GK2401B combined glass electrode and calibrated with commercial buffer solutions of pH 7.02 (from Crison) and pH 12.45 (from Beckman).

All kinetic experiments were carried out at 25 °C under pseudo-first-order conditions. Acidity was controlled either with buffers of the desired pH (in the range pH 8–11.5) or by addition of appropriate concentrations of NaOH (for pH > 12), except that in experiments carried out in the presence of HOO^-, the buffer was H_2O_2–HOO^-. Reactions were followed by recording the absorbance at 240–260 nm due to the nitrosoureas, except that in the presence of HOO^- the wavelength used was 390 nm to avoid interference at *ca.* 250 nm (in these experiments the concentration of nitrosourea was *ca.* 3×10^{-1} mol dm^{-3}). In all cases the absorbance–time data fit the first-order integrated equation well, affording pseudo-first-order rate constants that were reproducible to within 3%.

Results and discussion

DMNU

At low HO^- concentrations, the observed rate constant for the basic hydrolysis of DMNU was proportional to $[HO^-]$, eqn. (1).

$$v = k[HO^-][DMNU] \quad (1)$$

The observed value of k, 2.1 dm^3 mol^{-1} s^{-1}, agrees fairly well with the value reported by Snyder and Stock.[7] At higher HO^- concentrations, plots of k_0 *versus* $[HO^-]$ are non-linear (Fig. 1), presumably because, like other nitrosoureas,[15] DMNU undergoes ionization.

The absence of NO_2^- in the final reaction mixtures (as shown

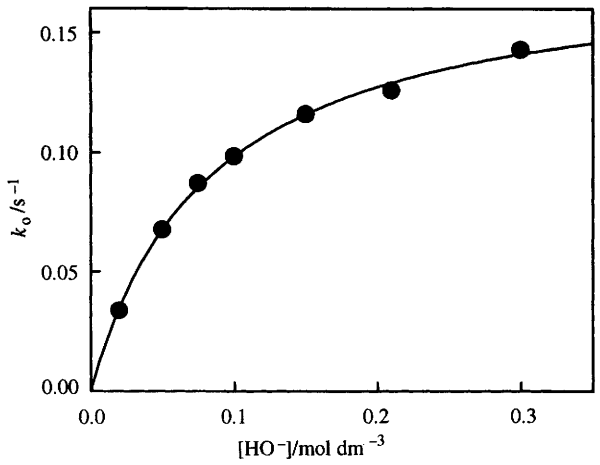
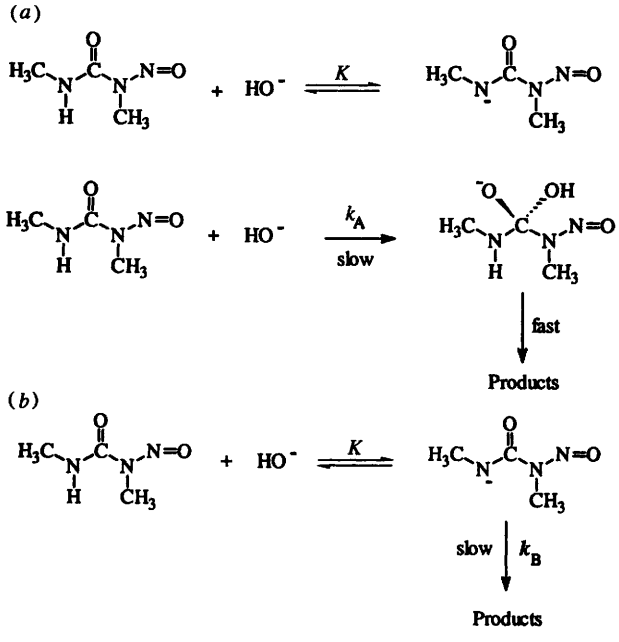


Fig. 1 Influence of HO^- concentration on k_0 in the basic hydrolysis of DMNU

by Shinn's method)[16] implies that there is no nucleophilic attack by HO^- at the nitroso group. However, the above results are compatible with both mechanisms mentioned in the Introduction, *i.e.* with both an attack on the carbonyl group [Scheme 1(a)] and decomposition of the deprotonated



Scheme 1

substrate [Scheme 1(b)]. Both these mechanisms lead to eqn. (2)

$$k_0 = \frac{a[HO^-]}{1 + K[HO^-]} \qquad (2)$$

where a is k_A for the mechanism of Scheme 1(a), and k_BK for that of Scheme 1(b). Fitting eqn. (2) to the experimental data yielded the values $a = 2.16 \pm 0.07$ dm^3 mol^{-1} s^{-1} and $K = 12.0 \pm 0.6$ dm^3 mol^{-1}; the value obtained for K implies a pK_a of 12.9 for DMNU.

Neither of the possible mechanisms was ruled out by the solvent isotope effects calculated for k_A and k_B after fitting eqn. (2) to the results of experiments carried out in deuteriated water. The value of 0.63 obtained for the ratio k_A^H/k_A^D is close to the value of 0.65 reported for the basic hydrolysis of *p*-nitrophenylacetate,[17] the rate-controlling step of which is known to be nucleophilic attack by HO^- on the carbonyl group; and the value of 0.96 obtained for k_B^H/k_B^D is in the expected range for processes in which the transition state involves no proton transfer. Under both hypotheses, the solvent isotope effect on the deprotonation equilibrium, K^H/K^D, is 0.66.

Since direct kinetic discrimination between the two possible mechanisms was not possible, we decided to investigate the general susceptibility of DMNU to nucleophilic attack by reacting it, under conditions in which the basic hydrolysis reaction is negligible, with nucleophiles known for their reactivity with NO-bearing electrophiles.[18] At pH 6.7 (controlled with NaH_2PO_4/Na_2HPO_4 buffer), decomposition of DMNU was insignificant (during the first 12 h of reaction) not only in the absence of nucleophiles, but also in the presence of 5×10^{-2} mol dm^{-3} N_3^- or I^-, showing that neither of these nucleophiles efficiently attacks the DMNU nitroso or carbonyl groups. In 0.1 mol dm^{-3} $Na_2SO_3/NaHSO_3$ buffer of pH 7, the DMNU was consumed with an estimated pseudo-first-order rate constant of 6×10^{-6} s^{-1}, which if entirely due to reaction with the sulfite ion implies a value of only 10^{-4} dm^3 mol^{-1} s^{-1} for the bimolecular rate constant. Finally, the linear dependence of k_0 on nucleophile concentration that was observed in the presence of the nucleophile HOO^- (Fig. 2) was attributed to weak nucleophilic attack on the carbonyl group (the bimolecular rate constant given by the slope of the k_0–$[H_2O_2]$ plot and the calculated concentration of HOO^- is 0.165 dm^3 mol^{-1} s^{-1}, more than 10 times less than the constant for the basic hydrolysis reaction). The possibility that the reaction with HOO^- might proceed *via* nucleophilic attack on the nitroso group was ruled out by the non-formation of the peroxynitrite ion,[18,19] which is stable under the experimental conditions employed; while the possibility of general base catalysis by HOO^- was ruled out by the fact that, in analogous experiments, no catalytic action was exerted by other bases† (Table 1 lists the results obtained with pyrrolidine, which, with a pK_a of 11.3, is of similar basicity to HOO^-).

The above results show (*a*) that the nitroso group of DMNU is much less susceptible to nucleophilic attack than those of *N*-nitroso-*N*-methyl-*p*-toluenesulfonamide (MNTS) or diverse alkyl nitrites;[18] and (*b*) that the reaction with HOO^-, attributed to nucleophilic attack on the carbonyl group, is much slower than the basic hydrolysis reaction. The first of these findings suggests that the –NO group is only electrophilic when bound to an efficient charge-withdrawing group [$ArSO_2$–$N(CH_3)$– in MNTS, an alkoxy group in alkyl nitrites] or a strongly electronegative atom (as in NOCl, NOBr, ONSCN, *etc.*). In the latter case, in particular, attack by typical nucleophiles is very fast, often approaching the diffusion-controlled limit.[20] The $(CH_3)HN$–C(O)–$N(CH_3)$– group of DMNU appears to be too weak a charge-withdrawing group for efficient facilitation of nucleophilic attack on the –NO group, a conclusion that is in keeping with the observed non-production of NO_2^- in the basic hydrolysis of DMNU.

The second finding noted at the beginning of the previous paragraph, that the reaction of DMNU with HOO^- is much slower than its basic hydrolysis, makes it extremely unlikely that the latter reaction can occur to any significant extent through nucleophilic attack on the carbonyl group (the mechanism of the reaction with HOO^-), since HOO^- is usually very much more reactive than HO^- (*ca.* 200 times in the reaction with the sulfonyl group of MNTS,[18] *ca.* 300 times in reactions with ester carbonyl groups,[21] and *ca.* 3000 times in reactions with the –NO groups of alkyl nitrites[18]). It may be concluded that the basic hydrolysis of DMNU takes place

† In our work, experiments have been carried out using a wide range of buffers and if Snyder and Stock find buffer catalysis at pH 9.7 we should observe this same effect at lower pH even using lower concentrations of buffer. Calculations in their conditions show that in some of their experiments pH should probably not be constant as they expected.

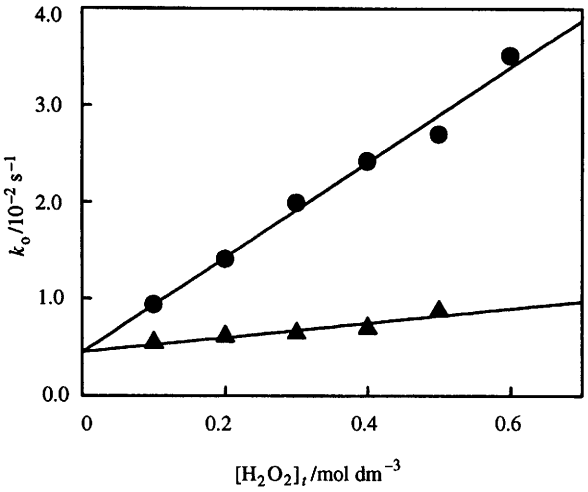


Fig. 2 Influence of total H_2O_2 concentration on the pseudo-first-order rate constant k_0 in the reaction of (●) DMNU with HOO^- ($[HOO^-]/[H_2O_2] = 0.43$); (▲) CCNU with HOO^- ($[HOO^-]/[H_2O_2] = 0.11$)

Table 1 Influence of the concentration of pyrrolidine buffer of pH 11.4 on the pseudo-first-order rate constant of the basic hydrolysis of DMNU

$[Pyrrolidine]_t$/mol dm^{-3}	$k_0/10^{-3}$ s^{-1}
0.04	5.70
0.06	5.73
0.08	5.83
0.10	5.83
0.14	5.94
0.18	5.86
0.20	5.95

through the mechanism of Scheme 1(b), in which the role of HO^- is abstraction of the –NH proton. A similar reasoning was used by Hecht and Kozarich [9] in the study of the decomposition of MNU using phenoxide and thiophenoxide in 1,2-dimethoxyethane discarding possible nucleophilic attack on the carbonyl group.

Incidentally, the estimated value of the bimolecular rate constant for the reaction of DMNU with HOO^-, 0.165 dm^3 mol^{-1} s^{-1}, shows the DMNU carbonyl group to be some 100 times less reactive than the MNTS sulfonyl group; this lower electrophilicity is in part related to the carbon atom being bound to just one oxygen atom, as opposed to the two to which the MNTS sulfur atom is bound and to the intrinsic electrophilicity of carbon and sulfur. It may also be pointed out that the basic hydrolysis of DMNU is faster than that of MNTS (in which HO^- attacks the $-SO_2-$ group) or that of 2-ethoxyethyl nitrite (in which HO^- attacks the –NO group).

CCNU

At low and moderate HO^- concentrations the observed kinetics of the basic hydrolysis of CCNU were similar to those of DMNU. At low HO^- concentration, the reaction rate was affected by neither the identity nor the concentration of the buffer in which the reaction was carried out (Table 2), and the observed pseudoconstant depended linearly on $[HO^-]$ in accordance with eqn. (1), with $k = 18.3$ dm^3 mol^{-1} s^{-1} (Fig. 3). The curvature of k_0–$[HO^-]$ plots at higher HO^- concentrations (Fig. 4) is attributable to the deprotonation of CCNU, in keeping with the fact that the initial absorbance of reaction mixtures with equal total CCNU concentrations decreased with increasing $[HO^-]$ (allowing a value to be roughly estimated for the pK_a of CCNU: 12.5 ± 0.3). At still higher pHs, however, the observed pseudo-first-order rate constant of CCNU did not tend to a limit (like that of DMNU), but again increased

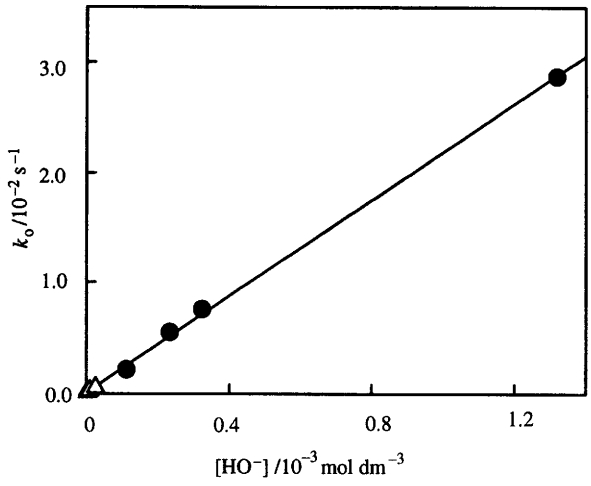


Fig. 3 Influence of low HO^- concentrations on k_0 in the basic hydrolysis of CCNU. (△) in morpholine buffer; (●) in dimethylamine buffer

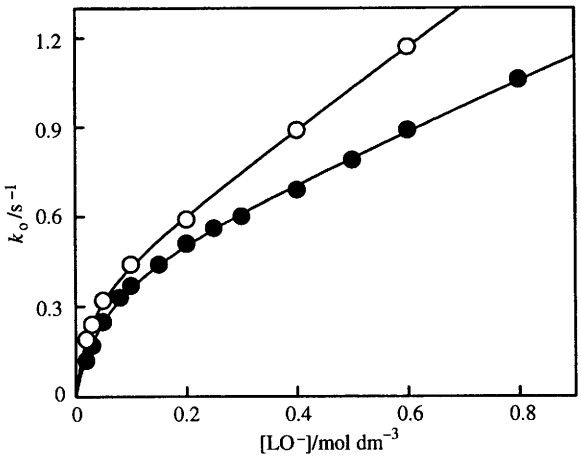


Fig. 4 Influence of LO^- concentration on k_0 in the basic hydrolysis of CCNU at high concentrations of base ($\mu = 1.0$ mol dm^{-3}, controlled with $NaClO_4$); (●) reaction in H_2O; (○) reaction in D_2O

linearly with $[HO^-]$ (Fig. 4), implying that the expression for k_0 must contain a quadratic term in $[HO^-]$, eqn. (3).

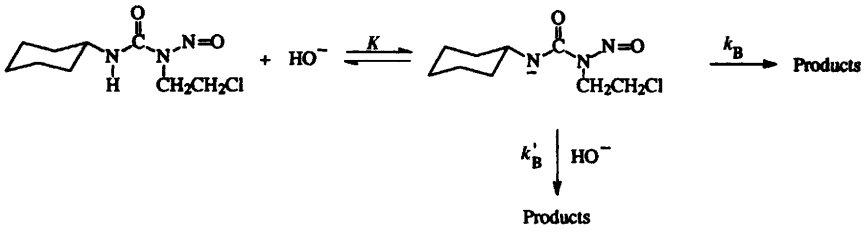
$$k_0 = \frac{a[HO^-] + b[HO^-]^2}{1 + c[HO^-]} \quad (3)$$

The similarity of CCNU to DMNU is sufficient to rule out the possibility that its basic hydrolysis proceeds by attack on the –NO group (at least under the working conditions used), but eqn. (3) is compatible with both the mechanisms illustrated for DMNU in Schemes 1(*a*) and 1(*b*). The decomposition of CCNU was not catalysed by HOO^- when the reaction was followed in H_2O_2–HOO^- buffer of pH 10.6 (see Fig. 2) which means that at least nucleophilic attack by HOO^- is 200 times slower than reaction with HO^-. The possibility that the basic hydrolysis reaction involves attack by HO^- on the CCNU carbonyl group appears to be ruled out by the same reasoning as was used above for DMNU (namely, that the stronger nucleophile would have catalysed decomposition if the mechanism of decomposition were nucleophilic attack). This might appear to leave the mechanism put forward for DMNU [that of Scheme 1(*b*)] as the only alternative. Indeed, the quantitative results obtained by interpreting eqn. (3) in accordance with Scheme 1(*b*) are not unsatisfactory. Fitting eqn. (3) to the data of Fig. 4 affords values of 12.8 dm^3 mol^{-1} s^{-1} for *a*, 19 dm^6 mol^{-2} s^{-1} for *b*, and 24 dm^3 mol^{-1} for *c*. The value for *c* (= *K*) implies a pK_a of 12.6 for CCNU (which is similar to

Table 2 Influence of the concentrations of various buffers on the pseudo-first-order rate constant of the basic hydrolysis of CCNU. Ionic strength was controlled with NaCl; μ = 0.5 mol dm^{-3} for dimethylamine buffer, μ = 1.0 mol dm^{-3} otherwise

	k_0/s^{-1}					
[Buffer]/mol dm^{-3}	pH = 7.18 (phosphate)	pH = 8.60 (boric acid)	pH = 8.63 (morpholine)	pH = 9.42 (morpholine)	pH = 10.51[a] (dimethylamine)	pH = 11.12[a] (dimethylamine)
0.04	2.62 × 10^{-5}	1.52 × 10^{-4}	1.47 × 10^{-4}	5.16 × 10^{-4}	—	—
0.08	2.44 × 10^{-5}	1.44 × 10^{-4}	1.57 × 10^{-4}	5.18 × 10^{-4}	6.98 × 10^{-3}	2.45 × 10^{-2}
0.12	2.68 × 10^{-5}	1.43 × 10^{-4}	1.51 × 10^{-4}	5.82 × 10^{-4}	7.29 × 10^{-3}	2.26 × 10^{-2}
0.16	2.82 × 10^{-5}	1.42 × 10^{-4}	1.41 × 10^{-4}	5.79 × 10^{-4}	7.56 × 10^{-3}	2.91 × 10^{-2}
0.20	2.91 × 10^{-5}	1.44 × 10^{-4}	1.38 × 10^{-4}	5.39 × 10^{-4}	7.53 × 10^{-3}	2.79 × 10^{-2}
0.25	2.92 × 10^{-5}	1.46 × 10^{-4}	1.57 × 10^{-4}	5.51 × 10^{-4}	7.60 × 10^{-3}	2.92 × 10^{-2}

[a] Ionic strength μ = 0.5 mol dm^{-3} (NaCl).



Scheme 2

the value of 12.5 ± 0.3 obtained spectrophotometrically, to the 12.9 obtained for DMNU and to the pK_a values of other nitrosoureas[7,15]), and the value for a ($=k_BK$) is not only in keeping with that obtained at lower pH, but also implies (with that of c) a value of 0.53 s^{-1} for k_B, which is of the same order as the 0.16 s^{-1} obtained for DMNU. Nevertheless, it still remains to explain the difference between the kinetics of DMNU and CCNU at high [HO$^-$], *i.e.* to explain the quadratic term in the numerator of eqn. (3), which indicates the involvement of a second hydroxy ion in the rate-controlling step of the overall reaction. This term would be explained if, at high HO$^-$ concentration, the deprotonated form of CCNU decomposes by two pathways rather than the single path assumed for DMNU (see Scheme 2). We have no clear evidence for the nature of this second pathway to propose a proper mechanism. It is clear that a second HO$^-$ should be involved in the decomposition of the anionic form of CCNU. Again, in our opinion, the most likely possibilities should be nucleophilic attack at the carbonyl or elimination of another proton. The kinetic solvent isotope effect‡ (see Fig. 4) yielded values of K^H/K^D = 0.59, k_B^H/k_B^D = 1.32 and $k'_B{}^H/k'_B{}^D$ = 0.57 for the constants in Scheme 2. In particular, the value $k'_B{}^H/k'_B{}^D$ = 0.57 is compatible either with nucleophilic attack or proton elimination. Nevertheless, the former possibility seems quite unlikely since we have demonstrated that there is no nucleophilic attack on the neutral form of CCNU.

TMNU

The experiments with TMNU were carried out to support the argument that was applied to DMNU and CCNU to rule out the possibility of attack on the carbonyl group. If attack on the TMNU -NO group is assumed negligible (for the same reasons as in the cases of DMNU and CCNU), the basic hydrolysis of TMNU must proceed by attack on the carbonyl group, since its lack of acidic hydrogen atoms rules out the mechanism of Scheme 1(*b*). If HOO$^-$ behaves in the same way as with DMNU and CCNU, the reactions of TMNU with HO$^-$ and HOO$^-$ therefore have the same mechanism. We reasoned that experimental verification that the reaction with HO$^-$ is the slower (as expected, HOO$^-$ being the stronger nucleophile) would strengthen the argument used previously for DMNU and CCNU (namely, the fact that the hydrolysis reactions of DMNU and CCNU are faster than their reactions with HOO$^-$ shows that the two reactions involve different mechanisms).

The experimental results for the basic hydrolysis of TMNU (Fig. 5) show k_0 to depend linearly on [HO$^-$] up to HO$^-$ concentrations of at least 0.4 mol dm^{-3}, eqn. (4).

$$k_0 = k_A[HO^-] \quad (4)$$

This is the behaviour expected for hydrolysis by attack on the carbonyl group [Scheme 1(*a*) without the deprotonation equilibrium], and the value of k_A calculated from the data of Fig. 5, 2.8 × 10^{-2} dm^3 mol^{-1} s^{-1}, is similar to the value of 2.2 × 10^{-2} dm^3 mol^{-1} s^{-1} reported by Snyder and Stock,[7] who assumed the same mechanism. In the presence of HOO$^-$ at pH 11.6 (the pK_a of H_2O_2), k_o increased linearly with [H_2O_2] (Fig. 6), showing that HOO$^-$ too reacts with the TMNU carbonyl group; and the bimolecular rate constant for this reaction was calculated from the slope of Fig. 6 and the proportion of peroxide in HOO$^-$ form as 2.24 dm^3 mol^{-1} s^{-1}. The rate constant for HOO$^-$ is thus 80 times greater than the rate constant for HO$^-$, in keeping with the usual relative nucleophilicity of these groups. As explained in the previous paragraph, this supports the mechanism put forward for the basic hydrolysis of DMNU and CCNU.

Final remarks

The mechanism hypothesized by Snyder and Stock[7] for the basic hydrolysis of alkylnitrosoureas, nucleophilic attack on the carbonyl group, implies linear dependence of k_0 on [HO$^-$]. Although Snyder and Stock themselves used a deprotonation equilibrium to explain the pH-dependence of the bimolecular rate constant of DMNU (calculated assuming linearity), they did not consider an elimination mechanism, as is shown in Scheme 1(*b*), as an explanation for their experimental results. Table 3 lists both the values of K, pK_a, k_A and k_B implied, for each mechanistic hypothesis, by the results of fitting eqns. (2) (DMNU), (3) (CCNU) or (4) (TMNU) to the experimental data

‡ In our conditions we always observed very clean first-order plots with no signs of the behaviour reported by Buckley (N. Buckley, *J. Org. Chem.*, 1987, **52**, 484) that was taken as evidence for the formation of an iminourea intermediate.

Table 3 Kinetic implications of the two mechanisms shown in Scheme 1. Values in parentheses were obtained from the data of Snyder and Stock [7] (see text, Final remarks)

Nitrosourea	K/dm^3 mol^{-1}	pK_a	k_A/dm^3 mol^{-1} s^{-1}	k_B/s^{-1}
MNU	(4.6 × 10^3)	(10.3)	(928)	(0.20)
DMNU	12.0 (9.5)	12.9 (13.0)	2.16 (1.21)	0.18 (0.13)
CCNU	24.0	12.6	12.8	0.53
TMNU	—	—	2.8 × 10^{-2} (2.2 × 10^{-2})	—

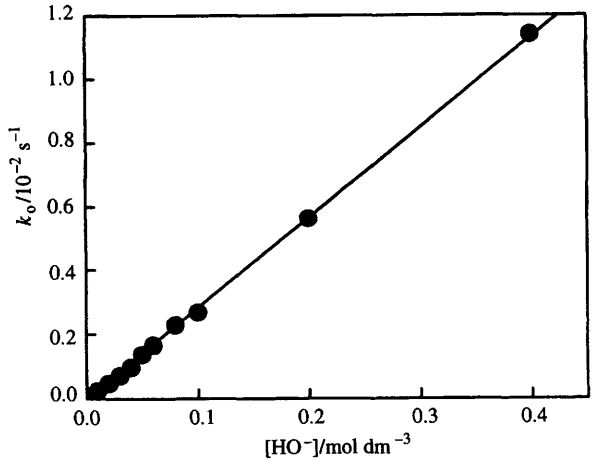


Fig. 5 Influence of HO$^-$ concentration on k_0 in the basic hydrolysis of TMNU

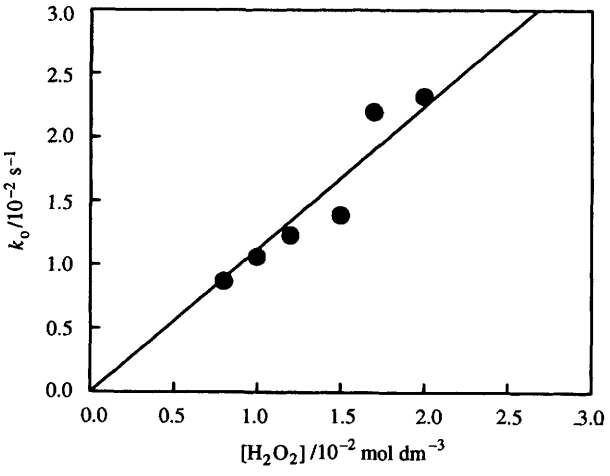


Fig. 6 Influence of total H_2O_2 concentration on the pseudo-first-order rate constant k_0 in the reaction of TMNU with HOO$^-$. [HOO$^-$]/[H_2O_2] = 0.50

obtained in this work, and, in parentheses, those implied by the results of fitting eqns. (2) (MNU and DMNU) or (4) (TMNU) to the data published by Snyder and Stock.

The fact that k_A decreases in the order MNU > DMNU > TMNU was attributed by Snyder and Stock to steric effects. However, the ratio k_A(MNU)/k_A(TMNU), about 4 × 10^4, is too large to be attributed solely to steric hindrance by the pair of 3-methyls in TMNU, especially when it has been found [22] that a series of 1-methyl-3-alkyl-1-nitrosoureas with very diverse alkyl groups (including both –CH_3 and Bu^t) all have quite similar k_A values. In this work, we have shown that the difference between the bimolecular rate constants of TMNU and DMNU is attributable to the two reactions involving different mechanisms.

Snyder and Stock also based their conclusions on ^{18}O labelling studies but, as one of the referees pointed out, in these experiments there was very little ^{18}O incorporation from the solvent. On the other hand, their kinetic solvent isotope effects are compatible with both mechanistic explanations, as has been discussed in this paper.

In conclusion, the results of this study suggest that the basic hydrolysis of nitrosoureas with no acidic hydrogen atoms takes place by nucleophilic attack on the carbonyl group, and that the basic hydrolysis of non-cyclic nitrosoureas with acidic hydrogen atoms takes place by decomposition of the deprotonated substrate. However, the fact that NIM, which also has acidic hydrogen atoms, is hydrolysed by attack on the carbonyl group [15] shows that there still remain questions to be answered in this field.

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