Hydrolysis of Nitrite Esters: Putative Intermediates in the Biotransformation of Organic Nitrates

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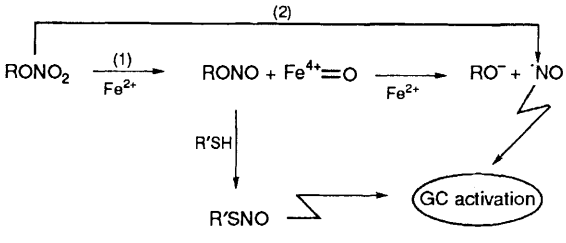
Study and comparison of the pH-independent hydrolysis of eight alkyl nitrites shows 3-nitroso-1,2-glyceryl dinitrate, a putative intermediate in the biotransformation of glyceryl trinitrate, to be unexpectedly reactive and too labile to be detected as a biotransformation intermediate in aqueous solution, suggesting a role for neighbouring group participation by the β-nitrate group.

Organic nitrates, in particular glyceryl trinitrate (GTN), are effective vasodilators of clinical importance. It is believed that GTN undergoes biotransformation to either nitric oxide ('NO) or a nitrosothiol.[1] Transfer of an 'NO equivalent to a heme-site on guanylyl cyclase results in enzyme activation, increased cyclic GMP accumulation and smooth muscle relaxation. Further interest in this biotransformation pathway has been stimulated by the intense, current research activity into nitric oxide biochemistry.[2] At present, the mechanism of biotransformation of GTN is poorly understood, due in part to the relative lack of information on the underlying solution chemistry of organic nitrates and related compounds.

Organic nitrites have vasodilatatory properties themselves.[3] Furthermore, the nitrite ester 3-nitroso-1,2-glyceryl dinitrate (NGDN) is a potential intermediate in the biotransformation of GTN (Scheme 1). Study of the reactivity of nitrite esters has



Scheme 1

focused on reactions in acidic[4] and alkaline[5] media; a recent thorough analysis of acid-dependent hydrolysis suggests a dissociative general acid catalysed mechanism *via* NO^+.[4a] To provide a basis for reactivity under physiological conditions, rate data for hydrolysis of a series of seven alkyl nitrites have been obtained in the pH region 5–10. As anticipated from previous work, an H^+-dependent pathway is evident at pH < 6, but a pH-independent process dominates in the neutral pH region (pH > 6) (Fig. 1). Hammett and Bronsted plots have been obtained by use of Taft parameters, allowing prediction of the reactivity of NGDN. However, its observed reactivity is far greater than predicted, demonstrating that NGDN is too labile to be a persistent biotransformation intermediate. This enhanced reactivity suggests a novel catalytic mechanism, involving neighbouring group participation by the β-nitrate group, in hydrolysis of NGDN.

Two general pathways for organic nitrate biotransformation may be described and characterized as sulfhydryl-dependent and heme-dependent. Recently evidence has been accumulating for the involvement of cytochrome P-450 isozymes. For example, Bennett and co-workers have demonstrated that cytochrome P-450 is responsible for the formation of 1,2-

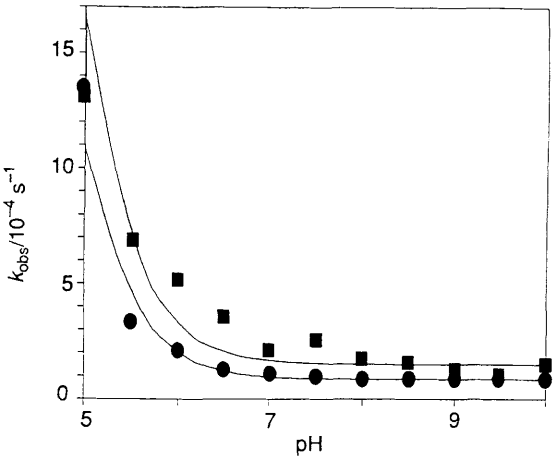


Fig. 1 pH–rate profiles for hydrolysis of chloroethyl nitrite (●) and methoxyethyl nitrite (■) obtained from pH-stat measurements at 24 °C in 40% CH_3CN: 60% aq. 0.1 mol dm^{-3} KCl in the absence of buffer. Products confirmed as alcohol by NMR analysis.

glyceryl dinitrate (GDN) from GTN in rat liver microsomes and have reported the cytochrome P-450 mediated stimulation of guanylyl cyclase by GTN.[6] Two general routes may be postulated for the heme-dependent pathway. Firstly, reduction of GTN and release of NGDN, followed by subsequent reaction to generate nitrosothiol, 'NO or NO^+ [Scheme 1; path (1)]. Secondly, direct *in situ* reduction of GTN to 'NO [Scheme 1; path (2)]. Yeates has proposed a pathway similar to that shown in path (1)[1,7] and indeed suggested that the biological activity of nitrate esters may result from, and require, initial biotransformation to the nitrite ester.[3a]

Buffer-independent rates were obtained at pH 7.6 from extrapolation of the rate data (Table 1). Use of Taft parameters permits estimation of the pK_a for each alcohol associated with the seven alkyl nitrites and NGDN, and construction of Hammett and Bronsted plots, giving ρ and β_{lg} values of 0.15 and −0.11 respectively.[8] These correlations, together with the estimated pK_a for GDN of 12.97, have been used to predict the reactivity of the NGDN nitrite ester and determine its viability as a biotransformation intermediate *in vivo*. The estimated half life for NGDN at neutral pH and room temperature in aqueous solution is 24 min. [*cf.* observed $t_{\frac{1}{2}}(Cl_2CHCH_2ONO) \approx 40$ min]. NGDN could not be isolated from nitrosation of GDN employing $NaNO_2$/acid mixtures, nor by use of alternative NO^+ equivalents. NGDN was prepared in CH_3CN solution, from reaction of GDN and *tert*-butyl nitrite and characterized by ^1H and ^{13}C NMR. This method has been used previously for

Table 1 Estimated pK_a, buffer independent (k_o) and dependent (k_{cat}) rate constants for hydrolysis of primary alkyl nitrites at pH 7.6 with ^1H NMR chemical shifts of diagnostic protons

R-CH_2-ONO R-	pK_a[a]	k_o[b,c]	k_{cat}[b,d]	$\delta_H(CDCl_3)$[e] RCH_2ONO	RCH_2OH
$CH_3[CH_2]_2$	16.07	1.81 (±0.13)	0.7 (±0.2)	4.70	3.62
CH_3	15.90	2.13 (±0.15)	3.5 (±1.5)	4.73	3.70
$Cl[CH_2]_2OCH_2$	14.71	3.3 (±0.5)	3.7 (±0.7)	4.85	3.65
$ClCH_2$	14.52	3.77 (±0.02)	9.5 (±0.2)	5.03	3.85
$PhOCH_2$	14.57	4.13 (±0.02)	6.3 (±0.2)	5.09	3.95
Cl_2CH	13.15	3.75 (±0.15)	13.7 (±0.2)[f]	5.24	3.92
Cl_3C	12.14	5.53 (±0.02)	10.3 (±0.3)	5.49	4.12

[a] Estimated from Taft parameters. [b] Data at 28 °C, in 0.1 mol dm^{-3} aq. KCl (2% dioxane; 0.001 mol dm^{-3} substrate) and phosphate buffer at pH 7.6 (0.016, 0.04, 0.08 mol dm^{-3} in triplicate) from pseudo-first order UV analysis of decay of nitrite ester absorbance at ≈250 nm. [c] 10^{-4} dm^3 mol^{-1} s^{-1}. [d] 10^{-3} dm^3 mol^{-1} s^{-1}. [e] 200 MHz. [f] In phosphate buffer (data collected at 7.6 < pH < 8.2) k_{HA} = 0.02 dm^3 mol^{-1} s^{-1}, k_A − ≈ 0. For Brønsted plots and plots of δ_H(ONO) and δ_H(OH) *versus* pK_a, R^2 values are 0.83, 0.95 and 0.77 respectively.

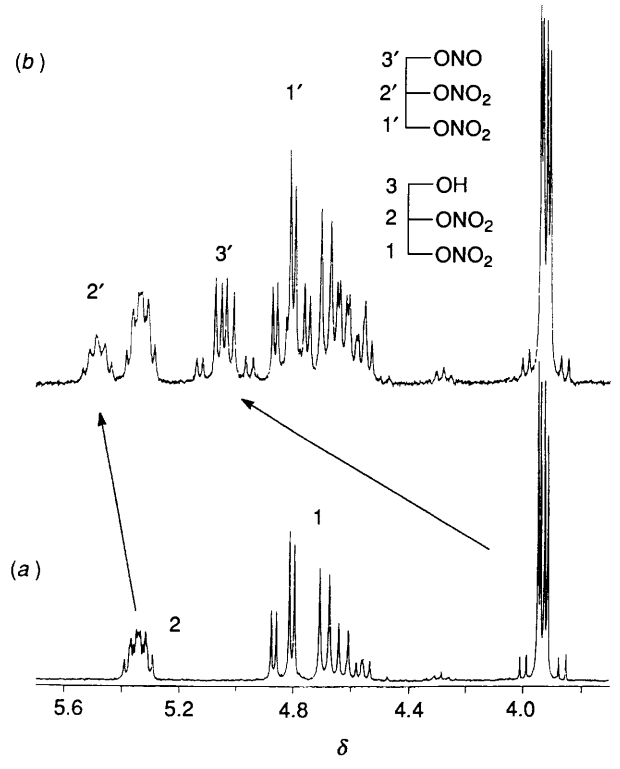
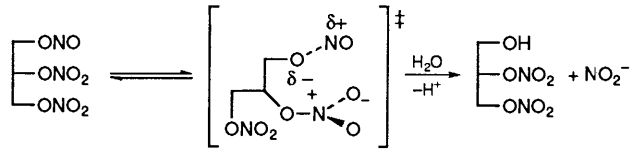


Fig. 2 ^1H NMR (CD_3CN) spectra of: (*a*) GDN, purified by flash chromatography; (*b*) NGDN–GDN mixture after nitrosation employing *tert*-butyl nitrite. Spectrum of hydrolysis product corresponds to GDN. The downfield shift of 3′-H in NGDN is symptomatic of nitrosation (see Table 1). Spectrum (*b*) was reproduced by combination of the experimental spectrum for GDN and a simulated spectrum for NGDN.

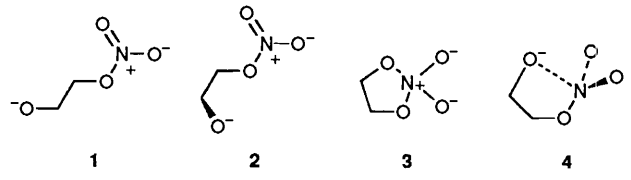
preparation and kinetic assay of labile nitrite esters.[4b] Addition of aqueous phosphate buffer (pH 7.0, 20 mmol dm^{-3}, 10 or 60% v/v D_2O/CD_3CN) to the CD_3CN solution of NGDN, led to complete hydrolysis to GDN within 2 min, as assayed by ^1H NMR (Fig. 2), the rate of reaction being far greater than predicted. A reasonable correlation exists between estimated pK_a(ROH) and ^1H NMR chemical shifts for both parent alcohol (R′CH_2OH) and nitrite esters (R′CH_2ONO) (Table 1), allowing alternative estimates of the pK_a of GDN of 13.6 and 14.3 respectively; again these values are inconsistent with the high observed reactivity of NGDN.

A substantial buffer effect is observed, in particular for activated nitrite esters, which on further examination is shown to be general acid catalysis (Table 1). The importance of general acid catalysis in the acid[4a] and neutral pH regions suggests a role for intramolecular Lewis acid or charge transfer catalysis by the β-nitrate group in the rapid hydrolysis of NGDN, in which the β-nitrate substituent assists departure of the leaving group (Scheme 2). *Ab initio* and semi-empirical molecular



Scheme 2

orbital calculations, with solvent correction, on a model compound for the leaving group ($O_2NO[CH_2]_2O^-$) yield three energy minima, corresponding to the *trans*- (**1**) and *cis*- (**2**) isomers and a cyclic orthonitrate (**3**)[9] (Table 2 and Scheme 3).



Scheme 3

A secondary transition state (**4**) (two imaginary frequencies) is located which from Natural Bond Orbital analysis demonstrates stabilizing interactions between the oxyanion and nitrogen even at the N–O distance of 2.41 Å (Table 2).[10] This data lends credence to the possibility of a mechanism in which charge transfer from the leaving group oxygen to the nitrate group assists N–O bond cleavage, accounting for the rapid hydrolysis of NGDN (Scheme 2).

The implications for the mechanism of biotransformation of GTN are clear. NGDN is too reactive to be detected as a discrete intermediate in the biotransformation of GTN. If released into aqueous solution, NGDN undergoes spontaneous and abortive hydrolysis. For NGDN, and by implication other organic nitrites, to be intermediates in organic nitrate biotransformation, further reduction must occur *in situ* by a heme-dependent pathway to nitric oxide, or by a mixed heme–sulfhydryl pathway by reaction with a thiol group at the reducing site. The extensive work of Williams' group supports both the facile reaction of thiols with alkyl nitrites and the subsequent generation of nitric oxide from the resulting nitrosothiol.[11] Furthermore, evidence for reduction of alkyl nitrites by heme systems has been provided in the literature.[12] The unexpected reactivity of NGDN and the computational location of structures involving interaction of the leaving

Table 2 Relative molecular energy or heat of formation (kcal mol^{-1}) with aqueous solvation energy corrections, for conformers and isomers of ionized hydroxyethyl nitrate[a]

Structure	PM3	3-21G(*)	6-31G*	SM3 + PM3	SM3 + 6-31G*
1	+26.27	0	0[b]	+19.88	0
2	+27.09	—	+2.51	+20.32	—
3	0	+12.67	+18.20[b]	0	+33.4
4	—	—	+18.70[c]	—	—

[a] All structures obtained by full geometry optimization using *ab initio* [HF/3-21G(*)//3-21G(*) (J. S. Binkley and J. A. Pople, *J. Am. Chem. Soc.*, 1980, **102**, 939) and HF/6-31G*//6-31G* (W. J. Hehre, R. Ditchfield and J. A. Pople, *J. Chem. Phys.*, 1972, **56**, 2257)] or semi-empirical [PM3 (J. J. P. Stewart, *J. Comput. Chem.*, 1989, **10**, 209)] calculations. SM3 aqueous solvation energies (C. J. Cramer and D. G. Truhlar, *J. Am. Chem. Soc.*, 1991, **113**, 8305) calculated as SM3/PM3 point calculations and added to 6-31G* and PM3 optimized energies as solvation corrections. [b] Calculation of analytical frequencies confirms structures as energy minima. [c] Two imaginary frequencies obtained from calculation on geometry optimized structure. Calculations performed using SPARTAN 3.0 on an SGI Indigo or GAUSSIAN 92 on an IBM 355.

group oxyanion with the nitrate nitrogen demands further study of neighbouring group participation by nitrate groups in hydrolysis of nitrite esters and other reactions.

Acknowledgements

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