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Nitrosation of Phenolic Compounds: Effects of Alkyl Substituents and Solvent

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Summary. Nitrosation reactions of phenol, *o*-cresol, 2,6-dimethylphenol, *o-tert*-butylphenol, 2-hydroxyacetophenone, and 2-allylphenol in water and water/acetonitrile were studied. Kinetic monitoring of the reactions was accomplished by spectrophotometric analysis of the nitrosated products at 345 nm. The dominant reaction was C-nitrosation *via* a mechanism consisting of an attack on the nitrosatable substrate by $NO^+/NO_2H_2^+$ followed by a slow proton transfer. The values of the rate constants of phenolic C-nitrosation were increased by electron donating substituents, and a good *Hammett* correlation was observed with $\rho = -6.1$. The results also revealed the strong effect of *pH* and the permitivity of the reaction medium on the rate constant, whose maximum values were observed for $pH \approx 3$, decreasing strongly for higher *pH* values. The study in water/acetonitrile with up to 25% acetonitrile showed that it is possible to inhibit the reaction strongly by increasing the percentage of the organic component. The conclusions drawn show that (*i*) it is possible to predict the rate of nitrosation of phenolics as a function of the *meta*-substituents on the phenol ring and (*ii*) the nitrosation of phenolics can be strongly inhibited by increasing the *pH* of the reaction medium as well as by lowering its dielectric constant.

Keywords. Kinetics; Reaction mechanisms; Nitrosation; Phenolics.

Introduction

Considerable attention has been paid to the possibility of suppressing the formation of nitroso compounds, endogenously and in commercial materials, through the use of inhibitors of nitrosation. Biologists have been mainly interested in the use of these compounds as models for producing a broad range of cancers [1–4], whereas chemists have been more interested first in the mechanisms of formation of nitroso compounds [5–10], and second in blocking or inhibiting the formation of these species [11–14].

Whereas aromatic C-nitration is a well-known reaction and is now well understood [15, 16], aromatic C-nitrosation has been studied mechanistically in relatively few cases, even though certain nitrosatable substrates are of considerable interest due to the pathogenic properties of the reaction products. This is the case

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for phenol and its derivatives [17–20]. Phenolic compounds are commonly used as antioxidants, flavourings, and spices in food. Foods containing significant quantities of phenolics include green tea (>10% of its dry weight are polyphenols) and instant coffee [21].

The relevance of polyphenols in current chemistry and chemical technology should be stressed [22–24]. The biosynthetic pathways leading to tannins, anthocyanins, flavonoids, *etc*., as well as the role of plant polyphenols in flavouring, colouring, leather tanning, and herbal medicines are aspects of the interest in the field of food science from the point of view of determining their potential capacity as nitrosatable substrates. In addition, some polyphenols are able to interact with chlorine or nitrite to yield products of greater mutagenic potential than the original compounds themselves [25].

As part of our ongoing research on the inhibition/blocking of nitrosation reactions, and prompted by earlier results [26], in a recent study on the nitrosation reactions of phenolic compounds [27] the reactivity of these substrates has been found to depend on three main intrinsic factors: (*i*) the preferred *para*-orientation of the hydroxyl group for electrophilic attack by nitrosating agents, (*ii*) the hyperconjugative effect of the methyl substituent, which causes electronic charge to flow into the aromatic nucleus, as well as the opposite electronic withdrawing effect of the halogen substituents, and (*iii*) the steric hindrance of alkyl substituents flanking the site of nitrosation which reduces or even prevents nitrosation.

In order to gain deeper insight into the influence of alkyl substituents in the phenolic ring as well as that of *pH* and the nature of the reaction medium we investigated the nitrosation reactions of phenol (**1**), *o*-cresol (**2**), 2,6-dimethylphenol (**3**), *o-tert*-butylphenol (**4**), 2-hydroxyacetophenone (**5**), and 2-allylphenol (**6**) by sodium nitrite in potassium hydrogen phthalate buffer.

Results and Discussion

Figure 1 offers a schematic representation of the reactions studied, Fig. 2 shows a typical kinetic run. All kinetic runs were performed in triplicate.

On working with water and water/acetonitrile mixtures with up to ~25% of the latter, the rate equation was always first order in nitrite (Fig. 3):

$$rate = k_1[\text{nitrite}] \tag{1}$$

This result suggests that the nitrosating species is NO^+ or $NO_2H_2^+$, which are kinetically indistinguishable.

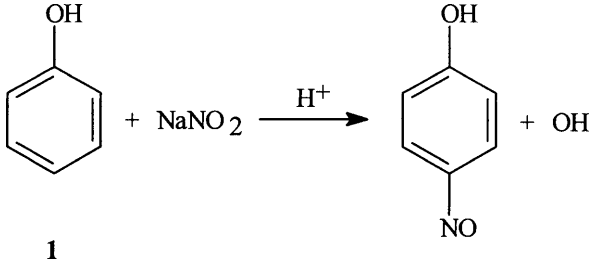

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Fig. 1. Nitrosation reactions of phenolic compounds

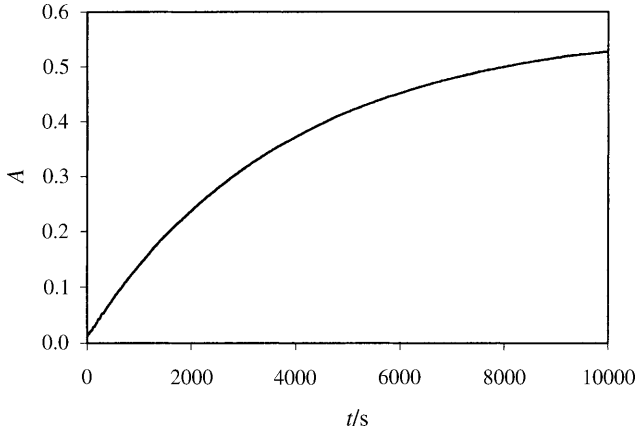


Fig. 2. Variation in the absorbance of the nitrosated product at 345 nm with time; $[\mathbf{6}] = 3.95 \cdot 10^{-2}\,M$; $[NaNO_2] = 1 \cdot 10^{-4}\,M$; $pH = 3.03$; $I = 0.2\,M$; $T = 298\,K$

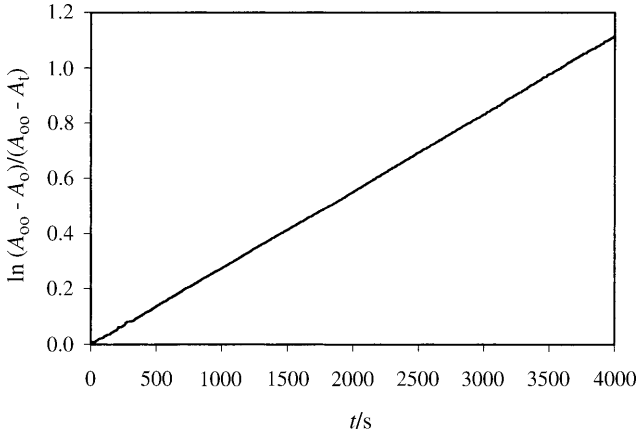


Fig. 3. First-order reaction with respect to the nitrite concentration; $[\mathbf{6}] = 3.95 \cdot 10^{-2}\,M$; $pH = 3.03$; $I = 0.2\,M$; $T = 298\,K$; $[NaNO_2] = 10^{-4}\,M$; A_t, A_o, and A_∞ are the absorbances at any time t, at the beginning ($t = 0$), and upon completion of the reaction, respectively

Experiments carried out with different concentrations of substrate turned out to be first order in substrate (Fig. 4), allowing the second-order rate constant k_2 to be calculated as

$$rate = k_2[\text{nitrite}][\text{substrate}] \tag{2}$$

The true (corrected) value of the rate constant, k_{2corr}, can be expressed as

$$k_{2corr} = k_{2H_2O} + k_B[\text{phthalate}], \tag{3}$$

k_{2H_2O} being the rate constant in the absence of buffer and k_B the rate constant in its presence.

Since Eq. (3) allows one to obtain k_{2H_2O} by extrapolating to [phthalate] = 0 and taking into account the dissociation of nitrous acid ($pK_a = 3.148$ [28]) it is possible to express k_{2corr} as a function of k_2:

$$k_{2corr} = k_{2H_2O} = k_2(1 + K_N/[H^+]) \tag{4}$$

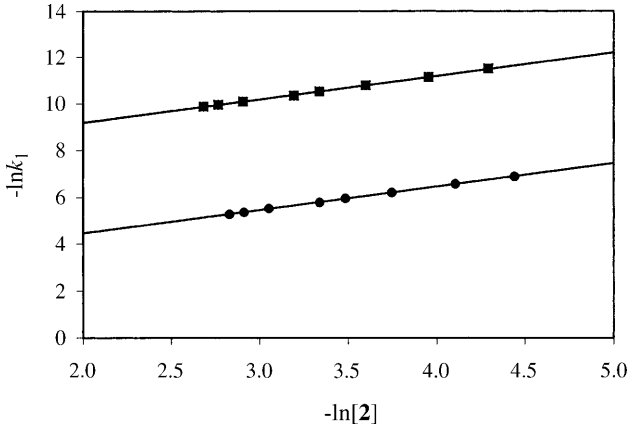
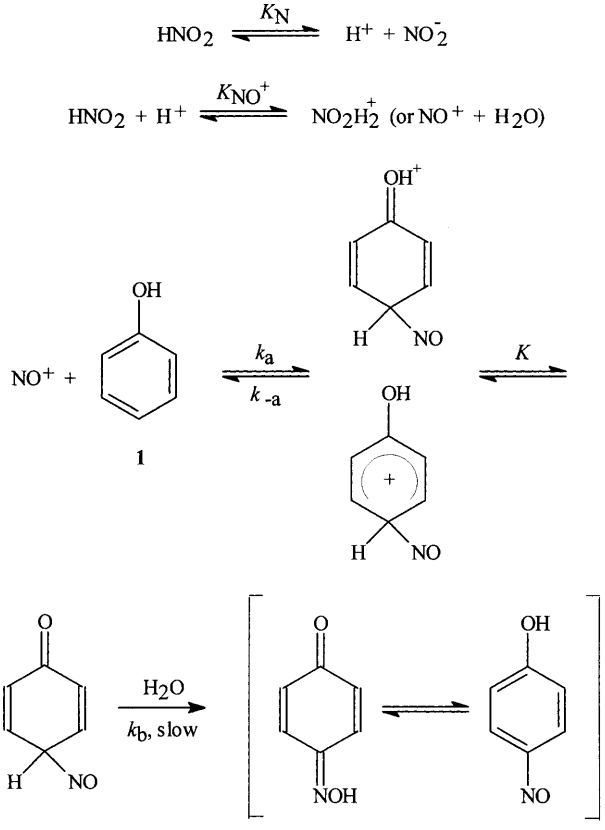


Fig. 4. Effect of the concentration of *o*-cresol on the *pseudo*-first-order rate constant of nitrosation; $[NaNO_2] = 1 \cdot 10^{-4}\,M$; $I = 0.2\,M$; $T = 298\,K$; •: $pH = 2.87$; ■: $pH = 5.10$

The reaction mechanism can be explained in terms of aromatic electrophilic substitution by nitrosonium or nitrous acidium ions (Scheme 1), a mechanism previously suggested in other studies [29, 30] and handled by us earlier [26, 27].



Scheme 1

Since the slow step is a proton transfer (see Scheme 1) and since, under the working conditions, $[\text{nitrite}] = [NO_2^-] + [HNO_2]$, the rate equation is readily achieved:

$$rate = \frac{k_a K_{NO^+}[\text{nitrite}][\text{substrate}][H^+]^2}{(K_N + [H^+])(1 + \frac{k_{-a}}{Kk_b}[H^+])} \tag{5}$$

Comparison of Eqs. (2) (experimental) and (5) (mechanistic) and taking into account Eq. (4) affords

$$k_{2H_2O} = \frac{k_a K_{NO^+}[H^+]}{(1 + \frac{k_{-a}}{Kk_b}[H^+])} \tag{6}$$

Figure 5 shows the variation in k_{2H_2O} with $[H^+]$; Eq. (6) fits the experimental data.

The denominator of Eq. (6) carries a sum of terms which permits the consideration of two limiting cases. When the second term in the denominator is much larger than 1, the rate constant does not depend on the $[H^+]$. This will be most likely true at high $[H^+]$ as can be seen from Fig. 5. When the second term in the denominator is significantly less than one, the rate constant should be first order in $[H^+]$, as also shown in Fig. 5.

This variation in k_{2H_2O} with $[H^+]$ is of biological relevance. As shown in Fig. 5, in more acid media ($pH \leq 3$) the values of k_{2H_2O} vary slowly, falling sharply when the acidity of the medium decreases. This suggests that attention should be focused on the effects of compounds that increase the acidity of the stomach such as those containing hydrochloric acid used to relieve the symptoms of hypochlorhydria.

Equation (6) permits to make some simplistic considerations aimed at rationalizing the experimental values of k_{2H_2O}. It seems logical that any factor that contributes to an increase in negative charge density in the *para*-position with respect to the $-$OH group of the nitrosatable substrate should increase its reactivity (k_{2H_2O}), both by increasing the k_a value and by favouring proton transfer from the solvent (water) to the $-$NO group of the intermediate dienone (Scheme 1).

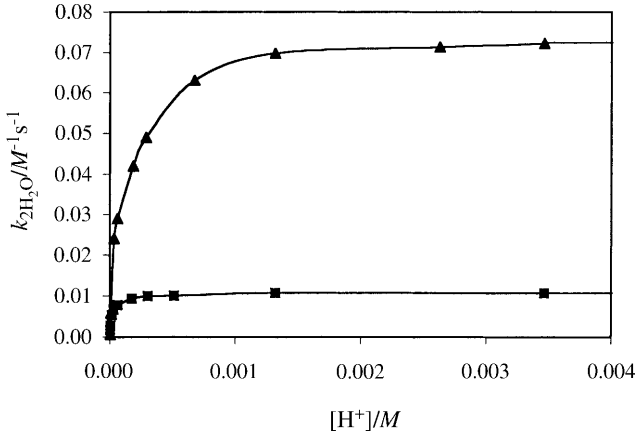


Fig. 5. Effect of $[H^+]$ on the rate constant of nitrosation; ■: [**1**] = 0.200 *M*; $[NaNO_2] = 1 \cdot 10^{-4}$ *M*; *I* = 0.2 M; *T* = 298 *K*; ▲: [**2**] = 0.200 *M*; $[NaNO_2] = 1 \cdot 10^{-4}$ *M*; *I* = 0.2 M; *T* = 298 K

In order to test this, the *Hammett* correlation given below (Eq. (7) [31]) was used:

$$\log (k/k_o) = \sigma_m \rho \quad (7)$$

In Eq. (7), k_o and k denote the kinetic constants for the reference reaction (hydrogen as substituent, **1** in this case) and for reactions with other substituents in *meta*-orientation with respect to the final −NO position (Scheme 1).

The substituent constant σ_m is of general significance, exceeding the range of validity of the *Hammett* equation, *i.e.* derivatives of benzene. If the substituent effects are classified as inductive, mesomeric, and steric, only the first two are involved in σ_m. If the sum is electron withdrawing, the sign of σ_m is positive; if it is electron releasing, it is negative; the corresponding values have been tabulated [32].

In the case of polysubstituted substrates (*e.g.* **3**), the σ values have been shown to be mostly equal to the sum of the values of individual substituents [33, 34]. The slope ρ, termed 'reaction constant', is a function of the reaction series studied. Table 1 lists the values of the substrate reactivities indicated by the values of k_{2H_2O}.

Figure 6 shows the good fitting of the results to the *Hammett* equation ($R = 0.984$). The slope of $\rho = -6.1$ indicates an electrophilic attack of nitrosatable phenolic substrates and is consistent with the positive charge of the nitrosating agents $NO^+/NO_2H_2^+$ (see Scheme 1).

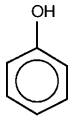
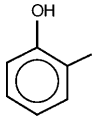
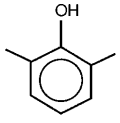
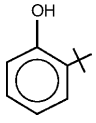
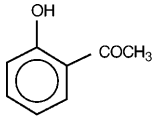
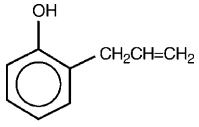
Additionally, on the basis of the proposed reaction mechanism (Scheme 1), with a slow step consisting of proton transfer from water to the −NO group of the intermediate dienone, one might speculate that the progressive substitution of the water of the reaction medium by a solvent with a lower dielectric constant would hinder proton transfer, thus hampering the nitrosation. Figure 7 shows the variation in k_{2H_2O} with the composition of the medium consisting of water/acetonitrile mixtures.

In conclusion, the kinetic study of the nitrosation of phenols reveals that the reactivity of these C-nitrosatable substrates appears to depend on both structural and external factors such as *pH* and the permittivity of the medium. The values of the experimental rate constants fit the *Hammett* correlation. This enables one to make predictions about the reactivity of more complex phenolics.

In view of the foregoing, attention should be paid to the possible enhancement of the nitrosation of certain potentially C-nitrosatable substances as a consequence of the presence of electron releasing substituents. This could be the case for some polyphenols present in tea, which have some strongly nucleophilic carbon atoms, as well as the polyphenol quercetin present in wines (particularly in red varieties) which has been demonstrated to be genotoxic upon nitrosation [35]. Despite this, it should be stressed that even if quercetin and other polyphenols do seem to be major mutagens in wines before or after their nitrosation, it is also known that this flavonol and other flavonoids may display antimutagenic and anticarcinogenic properties [36].

The results also point to the strong influence of *pH* and the permittivity of the reaction medium on the nitrosation rate constant. Maximum values for the

Table 1. Reactivities (k_{2H_2O}, Eq. (3)) of **1** and its derivatives for nitrosation reactions; $pH = 2.9$; $I = 0.2\,M$; $T = 298\,K$

Substrate	Structure	$k_{2H_2O}/M^{-1}s^{-1}$
1		0.0131±0.0001 (0.010±0.001)[a]
2		0.071±0.002 (0.07±0.01)[a]
3		0.37±0.01 (0.53±0.09)[a]
4		0.0915±0.0002
5		0.00014±0.00003
6		0.037±0.002

[a] Results obtained at $pH = 2.5$ [27]

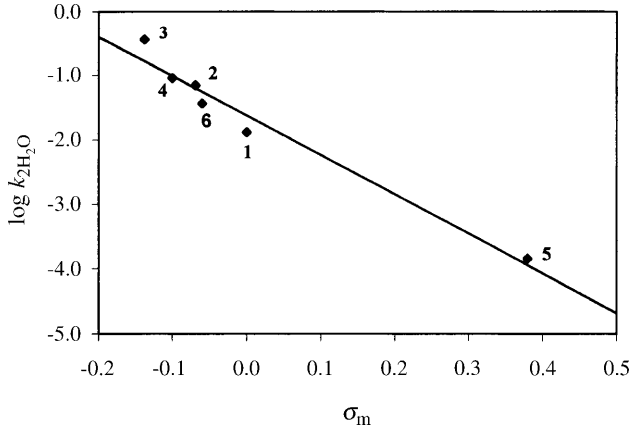


Fig. 6. Fitting of the reaction rate constants of nitrosation to the *Hammett* equation

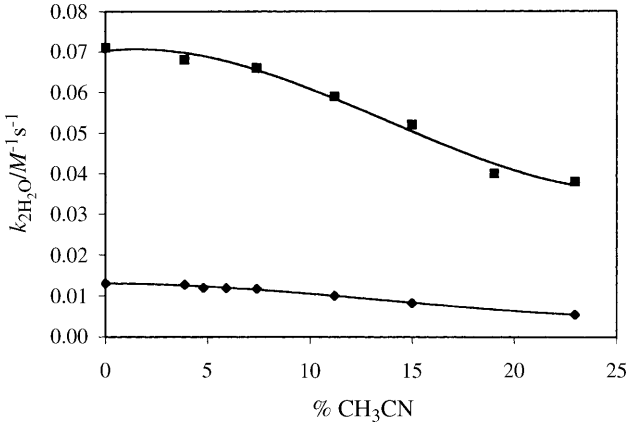


Fig. 7. Variation in the rate constant for the nitrosation of phenolic compounds with the percentage of acetonitrile in the reaction medium; ◆: [**1**] $= 4 \cdot 10^{-2} M$; $[NaNO_2] = 10^{-4} M$; $pH = 2.9$; $I = 0.2 M$; $T = 298$ K; ■: [**2**] $= 4 \cdot 10^{-2} M$; $[NaNO_2] = 10^{-4} M$; $pH = 2.9$; $I = 0.2 M$; $T = 298$ K

rate constant were observed for $pH \approx 3$, decreasing sharply for higher *pH* values. This is of interest in both food science and the medical field, since the intake of acid/antacid compounds may strongly affect the rate of nitrosation in the stomach.

A kinetic study of the nitrosation reactions of phenolic compounds in water/acetonitrile media shows that the reactions can be strongly inhibited by increasing the percentage of the organic component. These results may be significant in cases of the presence of alcoholic spirits in the human stomach. Since the dielectric constant of these mixtures is lower than that of water, a slowing down of the *in vivo* nitrosation of phenolics would be expected in the presence of these substances.

Experimental

Compounds **1**–**6** were purchased from Aldrich (*p.a.*, 98% pure). Solutions of $NaNO_2$, $HClO_4$, $NaClO_4$ (to adjust ionic strength), NaOH (all Merck *p.a.* products), and potassium hydrogen phthalate (Panreac *p.a.*) were made up by weight ($NaNO_2$ after desiccation for 2 h at 110°C). Reaction mixtures were prepared in potassium hydrogen phthalate buffers of $pH = 2.5$–5.5 (this buffer does not generate any potential nitrosating agent); *pH* was measured with a Radiometer M64 *pH*-meter equipped with a GK2401B combined electrode.

Reactions were monitored kinetically by spectrophotometric analysis of the nitrosated products at 345 nm. A Shimadzu 2101PC double-beam apparatus with thermoelectric control was employed to maintain the temperature within 0.1°C. The absorbance *vs.* time data were processed by the integration method [37]. All reactions were followed to at least 70% completion.

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