Nitrosation of Phenolic Compounds: Inhibition and Enhancement

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The nitrosation of phenol, *m*-, *o*-, and *p*-cresol, 2,3-, 3,5-, and 2,6-dimethylphenol, 3,5-di-*tert*-butylphenol, 2,4,6-trimethylphenol, *o*-chlorophenol, and *o*-bromophenol was studied. Kinetic monitoring of the reactions was accomplished by spectrophotometric analysis of the products at 345 nm. At pH > 3, the dominant reaction was *C*-nitrosation through a mechanism that appears to consist of an attack on the nitrosatable substrate by $NO^+/NO_2H_2^+$, followed by a slow proton transfer. The finding of an isokinetic relationship supports the idea that the same mechanism operates throughout the series. The observed sequence of nitrosatable substrate reactivities is explained by (i) the preferred *para*-orientation of the hydroxyl group for the electrophilic attack of nitrosating agents, (ii) steric hindrance of alkyl substituents, which reduces or prevents attack by nitrosating agents, and (iii) 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 induced by halogen substituents. The results show that potential nitrosation of widespread environmental species such as chlorophenols is negligible, but more attention should be paid to polyphenols with strongly nucleophilic carbon atoms.

Keywords: *Nitrosation; phenolic compounds*

INTRODUCTION

The chemistry of nitroso compounds has attracted considerable research owing to their proven toxic, carcinogenic, mutagenic, and teratogenic effects (Casado, 1994; Lijinsky, 1992; Loeppky and Michejda, 1994a; Mirvish, 1995).

While aromatic nitration is a very well-known reaction and is now well-understood (Al-Obaidi and Moodie, 1985; Loeppky et al., 1998; Schofield, 1980), aromatic *C*-nitrosation has been studied mechanistically in relatively few cases although certain nitrosatable substrates are of considerable interest due to the proven pathogenic properties of the products. This is the case of phenol and its derivatives (Kato et al., 1992; Kikugawa and Kato, 1988; Ohshima et al., 1989; Rosenkranz et al., 1990). The phenol derivative tyramine, which occurs in cheese, meat extracts, beer, and soybean products, has been identified as one of the precursors largely responsible for the mutagenic activity of certain Japanese soy sauces treated with nitrite (Wakabayashi et al., 1984). Bamethan [1-(4-hydroxyphenyl)-2-butylaminoethanol], a phenolic drug used for long-term oral treatment of cardiovascular disease, is both nitrosatable and a direct-acting mutagen (Kikugawa et al., 1987).

The relevance of polyphenols in current chemistry and chemical technology should be stressed (Haslam, 1998; Siebert et al., 1996a,b). The biosynthetic pathways leading to tannins, anthocyanins, flavonoids, etc., as well as the role of plant polyphenols in flavoring, coloring, leather tanning, and herbal medicines are aspects supporting the interest in food science in knowing their potential capacity as nitrosatable substrates. In addition, some polyphenols are able to interact with chlorine or nitrite to yield products with greater mutagenic potential than their original compounds (Lin and Lee, 1992).

Moreover, chlorophenols are widespread in the environment. Indeed, these pervasive compounds have been used for domestic, agricultural, and industrial purposes for more than 50 years. In addition to industrial production and usage, chlorophenols are produced from naturally occurring phenols as the result of chlorine bleaching of wood pulp in the paper industry and through the chlorination of domestic water supplies and swimming pools (Jensen, 1996).

In kinetic terms, an important contribution to the understanding of aromatic *C*-nitrosation comes from the work of Challis and co-workers in the 1970s (Challis, 1973; Challis and Higgins, 1973; Challis and Lawson, 1971). It is also worth noting that phenols and phenolic compounds are known to modify the nitrosation of amines through their action either as catalysts or inhibitors, depending on their structure, reaction conditions, and pH and the nitrite concentration (Bartsch et al., 1988; Davies et al., 1978; Loeppky et al., 1994b; Pignatelli et al., 1984, 1985; Shenoy and Choughuley, 1989; Wilcox et al., 1991). Under acidic conditions, phenolics usually react with nitrite more rapidly than most amino compounds. 1,2- and 1,4-Dihydroxyphenols, such as catechol, hydroquinone, 1,2,3-trihydroxyphenols, and many naturally occurring polyphenols, inhibit *N*-nitroso compound formation. Although this has been questioned on the grounds that *N*-nitrosation is catalyzed by monohydroxyphenols (Mende et al., 1993) but not by all polyhydroxyphenols (Loeppky et al., 1994b), it should be borne in mind that catalytic activity is only observed when the concentration of the nitrosating agent significantly exceeds the concentration of phenol, which rarely occurs in vivo or in environmentally

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significant situations. As a result, many attempts have been made, particularly in the field of food science, to block or inhibit the formation of these pathogenic species (Archer, 1984; Bao and Loeppky, 1991a,b; Wilcox et al., 1991).

As part of our ongoing research on inhibiting/blocking nitrosation reactions (García-Prieto et al., 1998; González-Mancebo et al., 1997) and prompted by earlier results (Fernández-Liencres et al., 1997), in this study, we attempt to investigate possible ways of inhibiting nitrosation reactions in general and, in particular, the nitrosation of phenolic compounds. The objectives were (i) to study possible inhibition/blocking through steric hindrance in the *C*-nitrosation of aromatic substrates and (ii) to investigate changes in the reactivity of the phenol molecule caused by different substituents. Because of the rigidity of aromatic rings, the influence of steric hindrance should be higher than that observed in the case of nitrosation of nonaromatic substrates (González-Mancebo et al., 1997). We studied the influence of electron-withdrawing substituents (halogen atoms), as well as of weak electron-releasing substituents, such as the methyl group.

We investigated the nitrosation of phenol (FEN), *m*-cresol (MC), *o*-cresol (OC), *p*-cresol (PC), 2,3-dimethylphenol (D23F), 2,6-dimethylphenol (D26F), 3,5-dimethylphenol (D35F), 2,4,6-trimethylphenol (T246F), *o*-chlorophenol (OCF), *o*-bromophenol (OBF), and 3,5-di-*tert*-butylphenol (DT35F) by sodium nitrite in potassium hydrogen phthalate buffer. This buffer was chosen because it does not form any potential nitrosating agents.

EXPERIMENTAL PROCEDURES

The substrates FEN, OC, MC, PC, D23F, D26F, D35F, T246F, OCF, OBF, and DT35F were obtained from Aldrich p.a. and were 98% pure (Steinheim, Germany). Solutions of $NaNO_2$, $HClO_4$, and NaOH (all Merck p.a. products, Darmstadt, Germany) and potassium hydrogen phthalate (Panreac p.a., Barcelona, Spain) were made up by weight ($NaNO_2$ solutions after desiccation for 2 h at 110 °C). Reaction mixtures were made up in potassium hydrogen phthalate buffers at pH 2.5−5.5. 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. Because of the instability of its nitroso derivative (Maruyama et al., 1967), *p*-cresol was complexed with copper(II) sulfate. A stable red complex in aqueous solution was formed (Maruyama et al., 1967) whose absorbance at 336 nm was measured. We employed a Shimadzu 2101PC double-beam spectrophotometer with thermoelectric control to maintain temperature within 0.1 °C. Figure 1 shows some typical kinetic runs. All kinetic runs were performed in triplicate.

The absorbance−time data were processed by the integration method. All reactions were followed to at least 70% completion.

RESULTS AND DISCUSSION

A first-order reaction with respect to nitrite concentration was observed (Figure 2) so that

$$\text{rate} = k_1[\text{NaNO}_2] \quad (1)$$

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

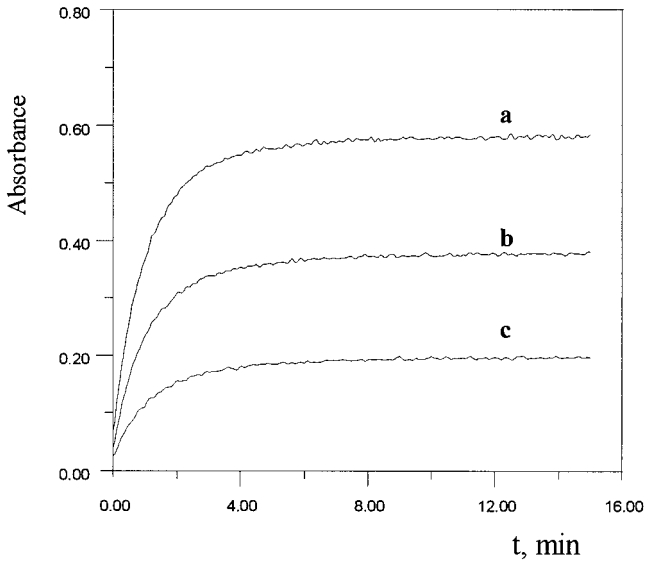


Figure 1. Variation in the absorbance (345 nm) of the nitrosated products with time: [2,6-dimethylphenol] = 3.95×10^{-2} M; pH = 3.03; I = 0.2 M; T = 298 K; (a) [$NaNO_2$] = 1.01×10^{-4} M; (b) [$NaNO_2$] = 6.70×10^{-5} M; (c) [$NaNO_2$] = 3.35×10^{-5} M.

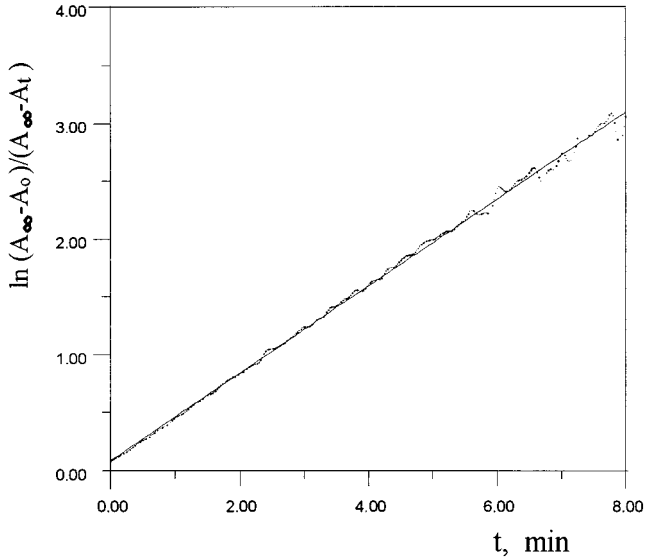


Figure 2. First-order with respect to the nitrite concentration: [*m*-cresol] = 9.35×10^{-2} M; pH = 3.00; I = 0.2 M; T = 298 K; [$NaNO_2$] = 6.70×10^{-5} M.

Experiments carried out with different concentrations of substrate showed a first-order reaction with respect to substrate concentration (Figure 3), allowing the second-order rate constant k_2 to be calculated.

$$\text{rate} = k_2[\text{NaNO}_2][\text{substrate}] \quad (2)$$

Taking into account the dissociation of nitrous acid, pK_a = 3.148 (Tummavuori and Lumme, 1968), we can express the true (corrected) value of the rate constant, $k_{2\,\text{corr}}$ as a function of k_2.

$$k_{2\,\text{corr}} = k_{2\,\text{H}_2\text{O}} + k_B[\text{phtalate}] = k_2(1 + K_{\text{HNO}_2}/[\text{H}^+]) \quad (3)$$

$k_{2\,\text{H}_2\text{O}}$ being the rate constant in the absence of buffer and k_B the rate constant in its presence.

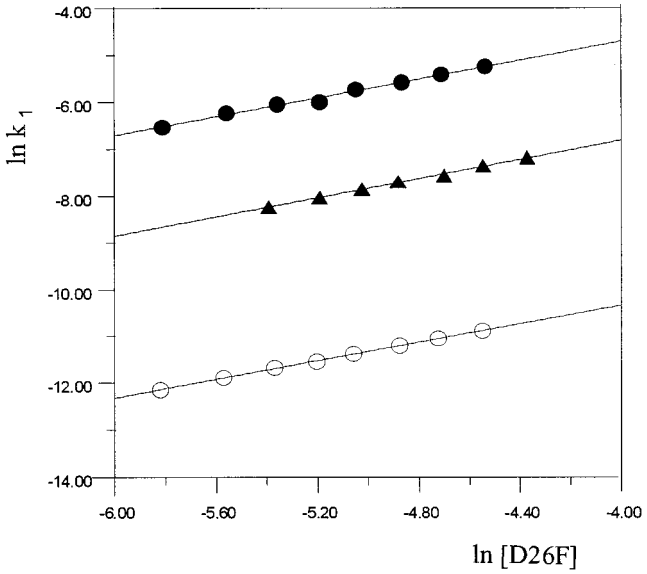


Figure 3. Effect of the concentration of 2,6-dimethylphenol on the pseudo-first-order rate constant of its nitrosation: $[NaNO_2] = 10^{-4}$ M; $I = 0.2$ M; $T = 298$ K; (●) pH = 2.87; (○) pH = 4.06; (▲) pH = 5.11.

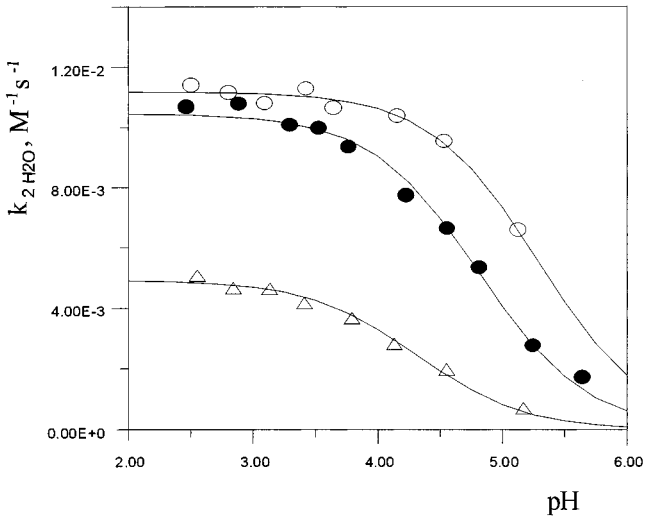


Figure 4. Effect of $[H^+]$ on the $k_{2\,H_2O}$: (●) [phenol] = 0.197 M; $[NaNO_2] = 10^{-4}$ M; $I = 0.2$ M; $T = 298$ K; (○) [3,5-dimethylphenol] = 1.44×10^{-2} M; $[NaNO_2] = 10^{-4}$ M; $I = 0.2$ M; $T = 298$ K; (△) [*o*-chlorophenol] = 5.83×10^{-2} M; $[NaNO_2] = 10^{-4}$ M; $I = 0.2$ M; $T = 298$ K.

On studying the variation in $k_{2\,H_2O}$ with pH, we obtained the results such as those shown in Figure 4. These results afford eq 3.

The reaction mechanism can be explained in terms of aromatic electrophilic substitution by nitrosonium or nitrous acidium ions (Scheme 1), a mechanism previously suggested by other studies (Challis and Lawson, 1971; Ibne-Rasa, 1962) and formerly handled by us (Fernández-Liencres et al., 1997).

From this mechanism the rate equation is readily achieved.

$$\text{rate} = \frac{k_a K_{NO^+}[\text{nitrite}][\text{substrate}][H^+]^2}{(K_N + [H^+])(1 + (k_{-a}/Kk_b)[H^+])} \qquad (4)$$

Comparison of the experimental (eq 2) and mechanism-deduced (eq 4) rate equations, and taking into account eq 3, implies

$$k_{2\,H_2O} = \frac{k_a K_{NO^+}[H^+]}{(1 + (k_{-a}/Kk_b)[H^+])} \qquad (5)$$

Fitting eq 5 to the experimental data by means of an optimization algorithm (Casado et al., 1981a,b) affords the values of $k_a K_{NO^+}$ and k_{-a}/Kk_b. Given that $K_{NO^+} = 3 \times 10^{-7}$ M^{-1} (Turney and Wright, 1958), the value of k_a is easily calculated. Table 1 shows the results for each substrate.

We investigated the primary kinetic isotope effect in all cases, the corresponding expression for $k_{2\,corr}$ in deuterated water being

$$k_{2\,corr} = k_{2\,D_2O} + k_B[\text{phtalate}] \qquad (6)$$

As is well-known, a primary isotope effect can be detected by kinetic measurement only if the bond to the isotopically labeled atom is broken during or before the rate-determining step (Bell, 1980; Carpenter, 1984; Connors, 1990). This means that the C−H proton transfer involved in the slow kinetic step (k_b in Scheme 1) should show a primary isotope effect $k_{b\,H_2O}/k_{b\,D_2O} > 1$. To confirm the existence of this isotopic effect, eq 5 was used. Taking into account that, under pH adequate

Scheme 1. Reaction Mechanism for Nitrosation of Phenolic Compounds

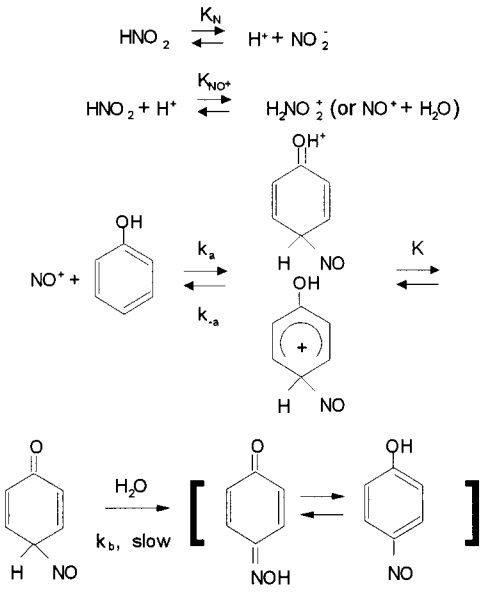


Table 1. Values of $k_a K_{NO^+}$ and k_{-a}/Kk_b

substrate	$k_a K_{NO^+}$, 10^2 M^{-2} s^{-1}	k_a, 10^9 M^{-1} s^{-1}	k_{-a}/Kk_b, 10^4 M^{-1}
phenol	6.6 ± 0.5	2.2 ± 0.2	6.3 ± 0.5
m-cresol	8.1 ± 0.8	2.7 ± 0.3	1.3 ± 0.1
o-cresol	7.1 ± 1.0	2.4 ± 0.3	0.97 ± 0.15
p-cresol	0.83 ± 0.12	0.28 ± 0.04	1.9 ± 0.3
2,3-dimethylphenol	10.7 ± 1.5	3.6 ± 0.2	1.1 ± 0.2
3,5-dimethylphenol	21.3 ± 1.6	7.1 ± 0.5	19.0 ± 1.5
2,6-dimethylphenol	6.9 ± 0.5	2.3 ± 0.2	0.13 ± 0.02
2,4,6-trimethylphenol	no reaction	no reaction	no reaction
3,5-di-*tert*-butylphenol	no reaction	no reaction	no reaction
o-chlorophenol	1.0 ± 0.1	0.33 ± 0.03	2.0 ± 0.2
o-bromophenol	0.80 ± 0.19	0.27 ± 0.06	1.6 ± 0.4

Table 2. Primary Kinetic Isotope Effect in Nitrosation of Phenol and Its Derivatives

substrate	$k_{b\ H_2O}/k_{b\ D_2O}$	substrate	$k_{b\ H_2O}/k_{b\ D_2O}$
phenol	3.5 ± 0.3	3,5-dimethylphenol	4.3 ± 0.7
m-cresol	4.4 ± 0.4	2,6-dimethylphenol	3.0 ± 0.4
o-cresol	3.2 ± 0.3	*o*-chlorophenol	2.2 ± 0.2
p-cresol	3.0 ± 0.4	*o*-bromophenol	1.9 ± 0.2
2,3-dimethyl-phenol	3.0 ± 0.3		

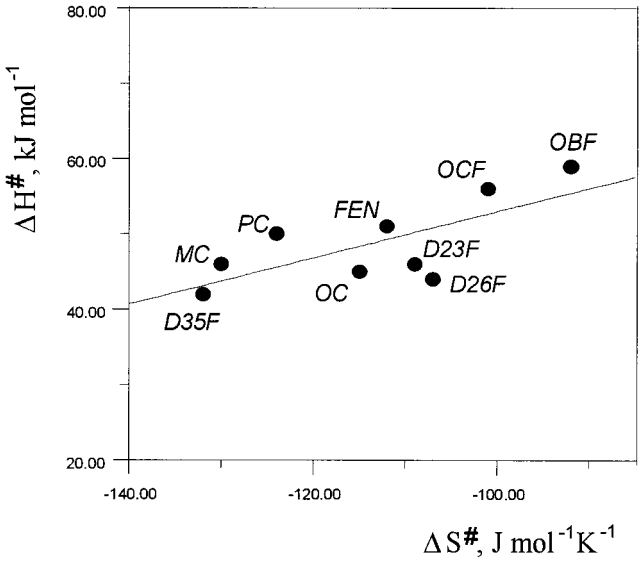


Figure 5. $\Delta H^{\ddagger}/\Delta S^{\ddagger}$ plot for nitrosation of phenol and its derivatives.

conditions $(k_{-a}/Kk_b)[H^+] \gg 1$, eq 5 leads to the expression

$$\frac{k_{2\ H_2O}}{k_{2\ D_2O}} = \frac{K_{NO^+}^{H_2O}}{K_{NO^+}^{D_2O}} \frac{k_{b\ H_2O}}{k_{b\ D_2O}} \qquad (7)$$

Since the average value of $K_{NO^+}^{D_2O}/K_{NO^+}^{H_2O} \cong 2.7$ is known (Casado et al.,1986), knowledge of $k_{b\ H_2O}/k_{b\ D_2O}$ is immediate. Table 2 shows the results, which support the hypothesis that C−H proton transfer is the slow kinetic step.

On the other hand, the existence of an isokinetic relationship can be used to support the argument that the reactions of a series share a common mechanism (Exner, 1988; Leffler and Grunwald, 1989; Senent, 1986). To test this possibility in our work, the $\Delta H^{\ddagger}/\Delta S^{\ddagger}$ plot (Figure 5) and an Exner plot (Exner, 1988) (Figure 6) were drawn. The results confirm the idea of a common mechanism and render the former discussion about a possible singularity of *p*-cresol irrelevant (Fernández-Liencres et al., 1997).

Taking into consideration proton transfer from the solvent water to the −NO group of the intermediate dienone (Scheme 1), the factors that contribute to an increase in electronic density in the −NO group in the *para*-position should increase the reactivity of the corresponding substrate. As a consequence, the higher reactivity of the methylated phenols could be attributed to the hyperconjugative effect of the methyl substituent. Methyl substitution should cause electronic charge to flow into the benzene nucleus and increase the electronic density in the −NO group. Many experimental arguments favor this hypothesis. For example, in methylated benzene, charge appears to flow from the methyl

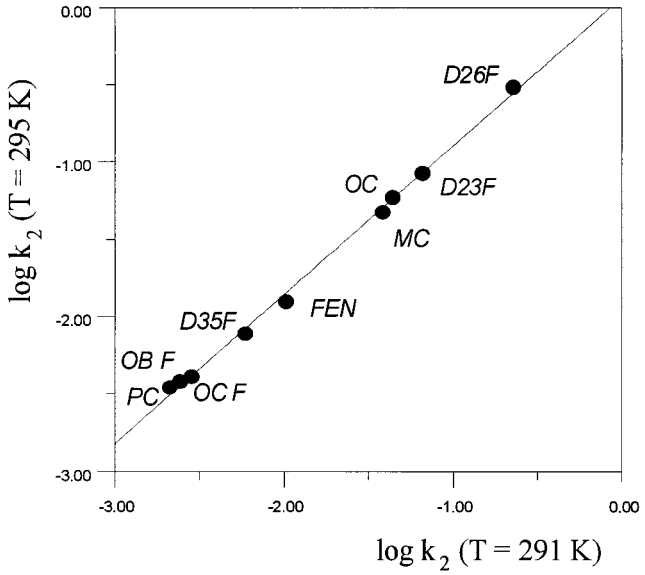


Figure 6. Exner plot for nitrosation of phenol and its derivatives.

group into the π-electron system of the ring. As a result, the group has *ortho*-/*para*-directing effects similar to those of other substituents and may also give rise to appreciable dipole moments in which the methyl group is positive with respect to the benzene ring. Methylation, by feeding electrons into an alternant hydrocarbon, also reduces π ionization potentials and excitation energies, and the resulting red shift in the electronic absorption bands is of some importance in the design of dye molecules. Another effect of this hyperconjugation is a small but measurable reduction in $C-CH_3$ bond length below the value 0.154 nm for a C−C single bond (McWeeny, 1979). Table 3 lists the values of substrate reactivities indicated by the values of $k_{2\ H_2O}$.

On the basis of the π electronic structure of the nitrosatable substrate, we would predict a higher reactivity first for 2,6-dimethylphenol, 3,5-dimethylphenol, and 2,3-dimethylphenol. The monomethylated species *o*-, *m*-, and *p*-cresol would come next, followed by phenol, and finally by bromophenol and chlorophenol owing to the electron-withdrawing effect of the halogen atoms.

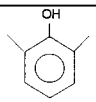
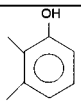
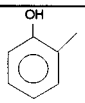
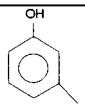
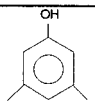
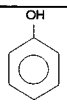
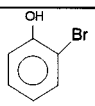
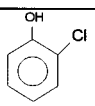
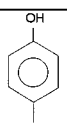
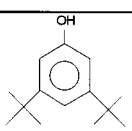
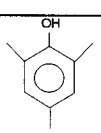
Nevertheless, the picture is not as simple as at first sight owing to the simultaneous influence of the steric factor. The low reactivity of 3,5-dimethylphenol can be explained in terms of the steric hindrance of the two methyl groups flanking the *para*-position, where proton transfer takes place. A similar argument can be used to account for the lower reactivity of 2,3-dimethylphenol when compared with 2,6-dimethylphenol, as well as the reactivity of *m*-cresol with respect to that of *o*-cresol.

On the other hand, the very low reactivity of *p*-cresol must be due to the occupation of the *para*-preferred position by the methyl group. Since 1930 it has been known that the nitrosation of phenol primarily gives (about 90%) *p*-nitrosophenol (Veibel, 1930). This type of occupation obliges nitrosation to occur in the *ortho*-position, which is much less reactive.

It is also easy to understand why no reaction was observed in the case of substrate 2,4,6-trimethylphenol; the lack of reaction must be due to the fact that the *para*- and *ortho*-positions (oriented by the −OH group in mechanisms of electrophilic substitution) are both occupied by methyl groups.

Finally, the lack of reactivity observed in the nitrosation of 3,5-di-*tert*-butylphenol must be caused by the

Table 3. Reactivities ($k_{2\,H_2O}$) of Phenol and Its Derivatives in Nitrosation Reactions (pH = 2.5, I = 0.2 M, T = 298 K)

Substrate	Structure	k_{2H_2O}, $M^{-1}s^{-1}$
2,6-dimethylphenol		0.53 ± 0.09
2,3-dimethylphenol		0.10 ± 0.02
o-cresol		0.07 ± 0.01
m-cresol		0.062 ± 0.008
3,5-dimethylphenol		0.011 ± 0.001
phenol		0.010 ± 0.001
o-bromophenol		0.005 ± 0.001
o-chlorophenol		0.0050 ±0.0007
p-cresol		0.0044 ±0.0009
3,5-di-*tert*-butylphenol		no reaction
2,4,6-trimethylphenol		no reaction

strong steric hindrance of the *tert*-butyl groups, which block the attack of the −NO group in the *para*- and *ortho*-positions with respect to the hydroxy group.

CONCLUSION

Kinetic study of the nitrosation reactions of phenol and phenolic compounds shows that the reactivity of these nitrosatable substrates appears to depend on three main structural factors: (i) the preferred *para*-orientation of the hydroxy group of phenol for the electrophilic attack of $NO^+/NO_2H_2^+$ 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 electron-withdrawing effect of the halogen substituents, and (iii) the steric hindrance of alkyl substituents flanking the active site of nitrosation, which reduces or even prevents nitrosation.

These results could be of interest for food science as well as for environmental chemistry. Among others, two points on which attention should be focused are (i) the very low capacity of halophenols to lead to reactions with nitrite and (ii) in the case of some potentially *C*-nitrosatable substances, the possible enhancement of their nitrosation capacity as a consequence of the presence of some electron-releasing substituents. This could be the case of some polyphenols present in tea leaves, 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. In any case, it should be stressed that, even if quercetin and other polyphenols would 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.

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