for conversion of AFM_1 to AFM_x may only account for diffusion of molecules in the reaction mixture.

Results of this study lead to the following conclusions:

(a) The pH of the aqueous solution of AFM_1 in the range 3–7 did not have any noticeable effect on the extent of conversion of the toxin by UV irradiation.

(b) When AFM_1 in an aqueous system was irradiated at a low temperature (0 °C), elimination of AFM_1 was accompanied by an accumulation of AFM_x in the reaction mixture. If these results can be extrapolated to milk, concern may arise because of the accumulation of AFM_x and, therefore, its toxicity needs to be assessed.

(c) Irradiation of aqueous solutions of AFM_1 at relatively high temperatures (e.g., at 60 °C) increased elimination of AFM_1, but the extent of elimination was lower than what is usually observed in chemical reactions. At those high temperatures, the reaction product (AFM_x) disappeared faster than it did at the lower temperatures.

If milk is to be irradiated with a UV source with higher intensity than that used in this study, there may be organoleptic changes in milk (Li and Bradley, 1969). Irradiating milk at a high temperature may further aggravate those changes in quality. Hence, in the future, it is necessary to investigate the optimum conditions that maximize elimination of the toxin, with minimum accumulation of reaction products and minimum organoleptic changes in milk.

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N-Nitrosation and N-Nitration of Morpholine by Nitrogen Dioxide in Aqueous Solution: Effects of Vanillin and Related Phenols

Robert V. Cooney* and Philip D. Ross

Micromolar concentrations of vanillin were found to enhance the formation of nitrosomorpholine from nitrogen dioxide (NO_2) and morpholine in neutral aqueous solution. This effect is attributed to the presence of the hydroxyl group in the vanillin molecule since phenol, guaiacol, and resorcinol also enhanced N-nitrosation over the same concentration range. In contrast, hydroquinone and catechol were potent inhibitors of N-nitrosation by NO_2, presumably because these compounds are more easily oxidized by NO_2 to the corresponding quinones and therefore do not form N-nitrosating intermediates. All phenols with the exception of *p*-nitrophenol, which did not affect either N-nitrosation or nitrite formation, were quite efficient at removing bubbled NO_2 from air and converting it to nitrite, approaching yields of 100% with millimolar phenol concentrations. This effect was pH dependent for vanillin, increasing significantly above pH 6. *N*-Nitromorpholine formation was strongly inhibited by all the phenolic compounds tested. It appears that the mechanism by which phenols enhance N-nitrosation by NO_2 is different from that proposed for phenolic catalysis of N-nitrosation by nitrite in acidic media and may involve the formation of an intermediate alkyl nitrite.

The majority of *N*-nitrosamines are potent mutagens, many of which have been shown to cause cancer in a variety of animals (Lijinsky, 1980; Magee and Barnes, 1967). A causative link between human cancer and *N*-nitrosamines has not been conclusively demonstrated (Choi, 1985), possibly because of the difficulty in predicting or measuring the products and extent of endogenous formation of *N*-nitrosamines. A considerable amount of research in the last 20 years has focused on the chemistry of acid-catalyzed N-nitrosation by nitrite (Douglass et al., 1978; Challis and Challis, 1982). Model systems simulating conditions found in the stomach have been used to estimate both *N*-nitrosamine formation in vivo (Walters et al., 1976; Ziebarth and Teichmann, 1980) and the effects of chemical modifiers on N-nitrosation reactions. Gray and Dugan (1975), utilizing such a model, reported that vanillin

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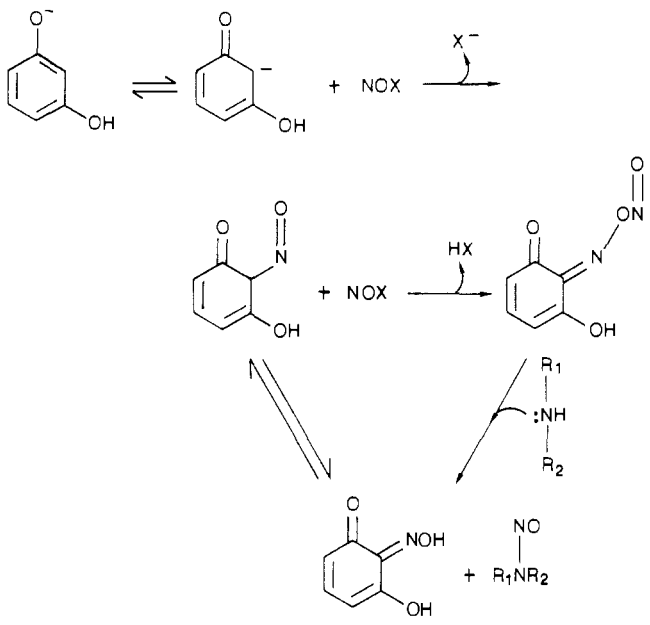
Scheme I[a]



[a] X = ONO, ONO_2.

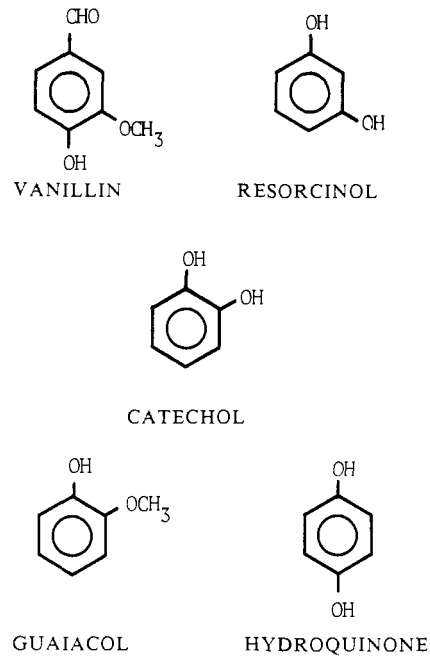


Figure 1. Structures of test compounds.

and hydroquinone inhibited the acid-catalyzed reaction of nitrite with secondary amines to yield *N*-nitrosamines. Others (Davies and McWeeny, 1977; Pignatelli et al., 1982) have reported that certain types of phenols such as resorcinol, catechin, and nitrosophenols catalyze the reaction. Davies et al. (1980) postulated that the mechanism of this catalysis involves a two-step process in which the phenolic compound is first C-nitrosated followed by reaction of the quinone monoxime tautomer with an additional molecule of N_2O_3 to yield an *N*-nitrosating agent (Scheme I).

Whereas N-nitrosation by nitrite occurs only in acidic media, nitrogen dioxide (NO_2) can react rapidly with amines in both organic (White and Feldman, 1957) and neutral aqueous solutions to form carcinogenic *N*-nitrosamines and, in some instances, *N*-nitramines (Challis et al., 1981; Cooney et al., 1987). NO_2 is a common pollutant formed from combustion processes such as gas cooking, cigarette smoking, welding, automobile exhaust, and direct-heat (flame) drying of foods (Wade et al., 1950; Lewis, 1980; National Research Council, 1977). N-Nitrosation in vivo appears to be an important route of human exposure to *N*-nitrosamines. Mergens and Newmark (1981) have estimated that the contribution of potential N-nitrosating agent from inhaled NO_2 may exceed that from ingested nitrite. Furthermore, direct-heat (from combustion) drying or smoking of foods has been shown to be an important source of preformed *N*-nitrosamines to which humans are exposed. Consequently, there is a clear need for information concerning the chemistry of N-nitrosation and N-nitration by NO_2 in both aqueous and lipophilic environments and how various chemicals modify these reactions.

This paper describes the effects of vanillin and related phenolic compounds (Figure 1) on the formation of *N*-nitrosomorpholine (NMOR) and *N*-nitromorpholine (NTMOR) from NO_2 in aqueous solution. Possible reaction mechanisms are discussed, as well as the potential role of dietary phenols in N-nitrosation reactions.

MATERIALS AND METHODS

Chemicals. NO_2 (99 ppm) diluted in air was purchased from Union Carbide Corp. NO_2 and nitrite concentrations were determined by the method of Saltzman (1954). For NO_2 measurements greater than 50 ppm a 2:1 ratio of air sample to Griess reagent was required. Other chemicals (reagent grade) were obtained from Aldrich Chemical Co., Milwaukee, WI (catechol, gallic acid, 2,4,6-trimethylphenol, 2,6-dimethylmorpholine), Sigma Chemical Co., St. Louis, MO (vanillin, guaiacol, sodium ascorbate, morpholine, resorcinol, hydroquinone), and Matheson, Coleman and Bell, Cincinnati, OH (*p*-nitrophenol). *N*-Nitrosamine standards were synthesized in the manner described by Lijinsky et al. (1970) and confirmed by mass spectrometry. NTMOR was synthesized from NMOR by the method described by Emmons (1954), using 30% hydrogen peroxide and trifluoroacetic acid. Since most *N*-nitrosamines are carcinogens, adequate precautions were taken (rubber gloves, fume hoods, etc.) to ensure that human contact would not occur. Operations involving high concentrations of *N*-nitrosamines and *N*-nitramines were conducted in a specially equipped (P-3) facility.

Sample Exposure to NO_2. In a typical experiment, 99 ppm NO_2 (±4 ppm) was bubbled through a glass pipet (1-mm orifice) into 5.0 mL of 10 mM morpholine (pH 7.4) in a 16 × 150 mm test tube at a flow rate of 69 mL/min. At a specified time (generally 15 min), the test tube was removed and 100 μL of 1.0 M sodium ascorbate added to prevent further N-nitrosation during workup as described previously (Cooney et al., 1986). This was followed by the addition of 200 ng of *N*-nitrosopyrrolidine in methanol as an internal standard. Samples were then extracted twice with 4.0 mL of CH_2Cl_2, and the organic extract was dried by filtering through cotton and reduced in volume to 1.0 mL. Nitrosomorpholine (NMOR) and *N*-nitromorpholine (NTMOR) were then quantitated as described below.

Various types of phenols were employed in order to investigate the relationship between phenol structure and effect on the reaction of NO_2 with amine. All experiments were performed at room temperature (approximately 23 °C). Each experiment involving a specific phenol was conducted on a single day and compared to control samples run on that day in order to minimize potential effects of day to day variations. Previous work (Cooney et al., 1986) indicated that ascorbate addition prior to CH_2Cl_2 extraction was essential for preventing N-nitrosation during workup. In order to test for artifactual N-nitrosation and confirm that the observed enhancement of N-nitrosation by vanillin occurred during exposure and not workup,

Table I. Evaluation of Artifactual N-Nitrosation during Sample Workup[a]

vanillin concn, mM	time of DMM addn	yield, ng NMOR	yield, ng NDMM
0	after ascorbate addn	479	7
0.10	after ascorbate addn	3179	9
0.50	after ascorbate addn	4662	23
0	before ascorbate addn	437	44
0.10	before ascorbate addn	3476	53
0.50	before ascorbate addn	5652	72
0	before NO_2 exposure	711	1752

[a] A solution of 200 μL of methanol containing 200 ng of NPYR and 0.1 mmol of 2,6-dimethylmorpholine (DMM) (final concentration 20 mM DMM) was added at various stages of sample preparation as indicated. Other conditions were as described in Materials and Methods. Background levels of NDMM (no NO_2 exposure) were 7 ± 5 ng (n = 4).

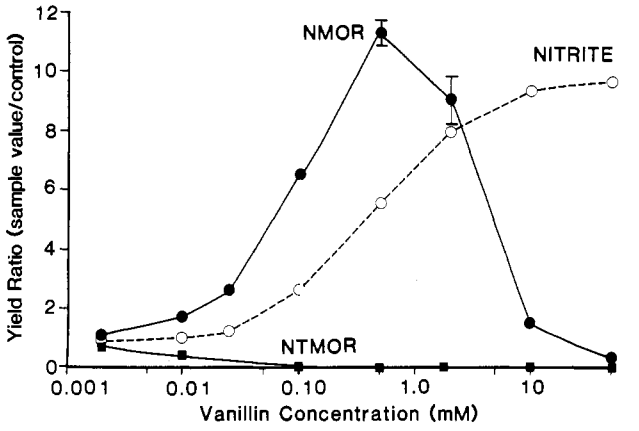


Figure 2. Effect of vanillin on N-nitrosation of morpholine by NO_2. NO_2 (99 ppm in air) was bubbled through 10 mM morpholine containing various concentrations of vanillin as described in Materials and Methods. The yield ratio was calculated by dividing the value in the presence of vanillin by the mean of control values. Duplicate samples were analyzed for NMOR, NTMOR, and nitrite. Control values (duplicate samples, no vanillin) were 560 ± 98 ng, 1098 ± 30 ng, and 13.7 μg for NMOR, NTMOR, and nitrite, respectively. In this and all subsequent figures, the range of values is indicated by a vertical bar. Where no bar is shown, the range was smaller than the symbol size.

2,6-dimethylmorpholine (DMM) was added to samples both before and after exposure to NO_2 and before and after addition of ascorbate (Table I). .

***N*-Nitrosamine and *N*-Nitramine Determination.** *N*-Nitrosamines and *N*-nitramines were quantitated with gas chromatography using 10% Carbowax 20 M on Chromosorb WHP 80–100 mesh (Alltech Associates, Inc., Deerfield, IL) packed in a glass column (6 ft × 2 mm (i.d.)) at a temperature of 120 °C interfaced to a thermal energy analyzer detector (Thermo Electron Corp.) with a pyrolyzer temperature of 475 °C. Helium was used as a carrier gas with a flow rate of 10 mL/min. Retention times (min) were as follows: NPYR, 4.2; NMOR, 5.2; NTMOR, 9.1.

RESULTS AND DISCUSSION

Vanillin enhanced *N*-nitrosamine formation over a broad concentration range (Figure 2). This enhancement was maximal at approximately 0.5 mM vanillin (resulting in an 11-fold increase in NMOR yield). At this concentration, the molar ratio of morpholine to vanillin is 20:1. Only at high concentrations of vanillin (near the limit of solubility) did N-nitrosation drop below control values. Dissolved nitrite levels rose with increasing vanillin concentration. At the highest vanillin concentration (50 mM) approximately 83% of the NO_2 bubbled through the solution was converted to nitrite. NO_2 levels in gas measured downline from the vanillin sample showed >90% reduction, verifying the efficiency of vanillin in absorbing NO_2.

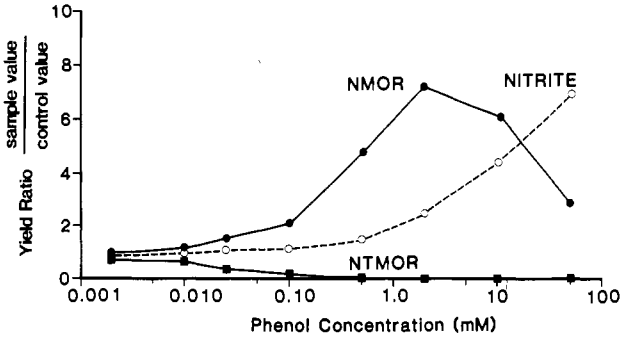


Figure 3. Effect of phenol on N-nitrosation of morpholine by NO_2. Samples were exposed as described in Figure 2. Control values (duplicate samples, no phenol) were 524 ± 3 ng, 1124 ± 30 ng, and 17.3 μg for NMOR, NTMOR and nitrite, respectively.

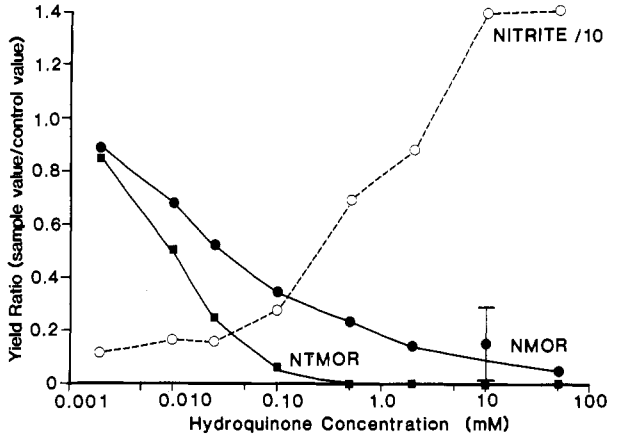


Figure 4. Effect of hydroquinone on N-nitrosation of morpholine by NO_2. Samples were exposed to NO_2 as in Figure 2. Control values (duplicate samples, no hydroquinone) were 490 ± 11 ng for NMOR, 1167 ± 85 ng for NTMOR, and 16.7 μg for NO_2^-.

The data in Table I confirm that the NMOR is formed during exposure to NO_2 and that ascorbate effectively prevents N-nitrosation during CH_2Cl_2 extraction. Large amounts of *N*-nitroso-2,6-dimethylmorpholine (NDMM) were found when 2,6-dimethylmorpholine (DMM) was added prior to NO_2 exposure. Much smaller yields were observed when the DMM was added after NO_2 exposure, but prior to ascorbate addition. The addition of ascorbate prior to DMM addition further reduced the yield to background levels, suggesting that ascorbate neutralizes residual NO_2 or other nitrosating agents formed during NO_2 exposure.

Keefer and Roller (1973) showed that aldehydes can catalyze N-nitrosation by nitrite. To determine whether the observed enhancement of *N*-nitrosation by vanillin was due to the aldehyde group, the experiment was repeated with guaiacol. The results were virtually identical with those for vanillin, indicating that the aldehyde group of vanillin is not essential to the N-nitrosation reaction. Added phenol (Figure 3) also enhanced N-nitrosation but required higher concentrations, and the maximum increase in NMOR formation was 7-fold. The ability of the *o*-methoxy group to enhance the effect of phenol could be significant in light of its common occurrence in many flavors. Interestingly, Virk and Issenberg (1985) reported that 2,6-dimethoxyphenol inhibits the acid-catalyzed N-nitrosation of morpholine by nitrite, while Kuenzig et al. (1984) found that ferulic acid (4-hydroxy-3-methoxycinnamic acid) inhibited in vivo formation of *N*-nitrosodimethylamine from aminopyrine and nitrite.

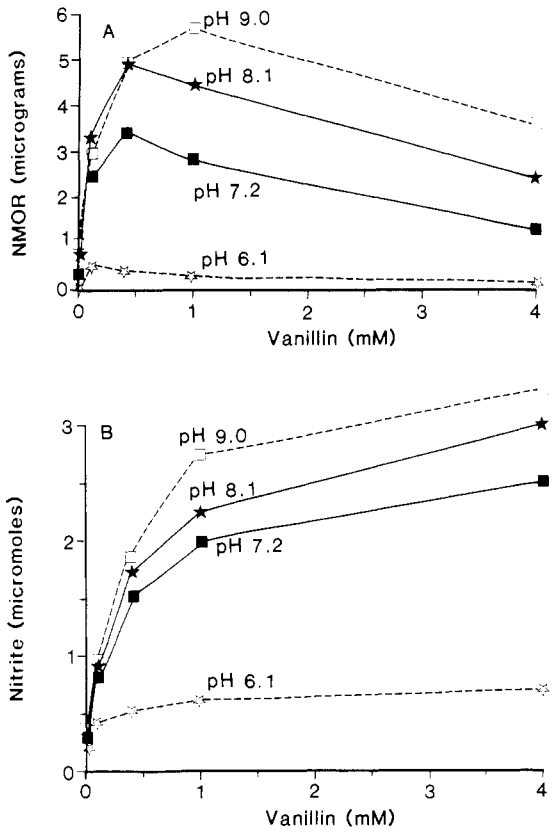


Figure 5. Influence of pH on the enhancement of *N*-nitrosamine and nitrite formation by vanillin. Samples containing 10 mM morpholine and vanillin at varying pH were exposed to 99 ppm NO_2 as described above for 12 min. The formation of NMOR (A) and nitrite (B) are plotted as a function of vanillin concentration.

The effect of hydroquinone on the N-nitrosation reaction is shown in Figure 4. Another dihydroxybenzene, catechol, gave results almost identical with those for hydroquinone. These results are analogous to those seen in the acid-catalyzed reaction with nitrite as reviewed by Challis and Challis (1982). The strong inhibition observed with hydroquinone and catechol is probably related to their greater ease of oxidation to yield quinones. In contrast, resorcinol (which cannot form a resonance-stabilized quinone) gave results quite similar to those for vanillin and guaiacol, but it was 20% less efficient, and the peak enhancement of N-nitrosation occurred around 0.1 mM. The *o*- and *p*-hydroxyphenols appear to be more efficient than monohydroxyphenols and resorcinol in removing NO_2 from air as nitrite, with yields approaching 100% at the highest phenol concentrations.

Increasing pH significantly increases the ability of vanillin to trap NO_2 as nitrite (Figure 5). The absolute amount of NMOR formed at a given vanillin concentration increases as a function of pH, which is probably related to the increased concentration of amine free base. However, the *ratio* of NMOR formed in the presence of vanillin relative to its control decreases. At very high pH (0.1 M NaOH) vanillin did not affect NMOR formation, and resorcinol inhibited the reaction (data not shown). This may be the result of destruction of the phenolic N-nitrosating intermediate by OH^- at high pH. Alternatively, at high pH, high phenoxide concentrations may reduce NO_2 to nitrite before the phenolic N-nitrosating intermediate can be formed (Scheme II).

It appears that phenols such as vanillin may react directly with the NO_2 radical in an oxidation–reduction reaction to yield nitrite and phenoxyl radical (Scheme II)

Scheme II

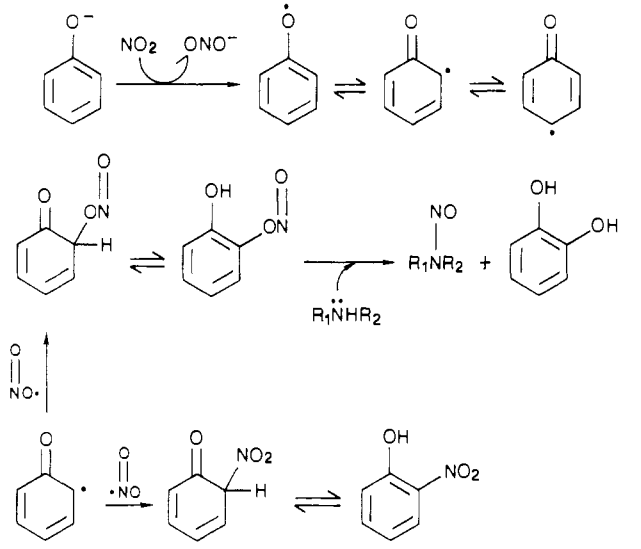


Table II. Effect of 2,4,6-Trimethylphenol on the N-Nitrosation of Morpholine by NO_2[a]

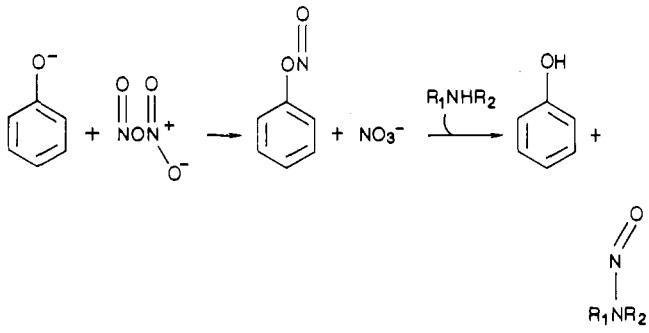
2,4,6-trimethylphenol concn, mM	yield NMOR, ng
0	462 ± 5
0.0010	424 ± 48
0.0025	421 ± 11
0.010	445 ± 36
0.050	896 ± 26
0.20	1393 ± 81
1.0	4309 ± 401

[a] NO_2 (99 ppm in air) was bubbled into 5.0-mL samples of 10 mM MOR (pH 7.4) containing 2,4,6-trimethylphenol as indicated for 15 min at a flow rate of 69 mL/min as described in Materials and Methods. Values represent the mean and SD of duplicate samples.

as described by Prutz et al. (1985). Thus, enhancement of nitrite formation by increasing pH could be due to either increased concentrations of the phenol anion, enhanced hydrogen abstraction due to removal of a proton from formed HONO, or both. The latter mechanism seems unlikely since un-ionized phenol concentrations would be reduced at high pH. If the reaction involved nucleophilic attack by the phenol anion on N_2O_4, equal amounts of nitrate and nitrite would be formed (Davies et al., 1980). Although nitrate levels were not measured, the amounts of nitrite formed in the presence of phenols approached the theoretical maximum, based on the measured NO_2 levels and gas flow rate (Figure 5). Thus, Scheme II appears to provide the best explanation for the enhancement of nitrite formation by phenols.

The mechanism proposed by Davies et al. (1980) for the phenolic catalysis of N-nitrosation by nitrite at acid pH (Scheme I) may not be the same as that for NO_2 at neutral or basic pH. Evidence against this mechanism for the reaction with NO_2 is shown in Table II. The ability of 2,4,6-trimethylphenol to enhance N-nitrosation by NO_2 would appear to preclude the formation of the postulated quinone monoxime tautomer. It is more likely that an intermediate alkyl nitrite is formed either by reaction of NO_2 with the phenoxyl radical as proposed by Hartshorn et al. (1985) to yield either the *o*- or *p*-phenol nitrite (Scheme II) or through nucleophilic attack by the phenol anion on N_2O_4 to yield benzene nitrite plus nitrate anion (Scheme III). As postulated by Hartshorn et al. (1985) the phenolic nitrite may undergo rapid hydrolysis. However, in the presence of a nucleophilic amine this unstable nitrite intermediate might act as an N-nitrosating agent.

Scheme III



In the case of 2,4,6-trimethylphenol, an alkyl nitrite may form that is probably slightly less reactive yet should be an excellent nitrosating agent analogous to cholesterol nitrite described by Mirvish et al. (1986). It is possible that phenols may affect nitrite formation and N-nitrosation through different mechanisms (both Schemes II and III may be operative). Alternatively, only Scheme II may be involved.

Evidence in support of a radical mechanism for phenol-enhanced N-nitrosation is provided by Prutz et al. (1985) who demonstrated that phenoxyl radicals are formed from NO_2 and tyrosine in aqueous solution and that *C*-nitrotyrosine is the predominant product. Although they did not report the formation of tyrosine nitrite, one would predict its formation through an analogous mechanism (Scheme II). It appears that any nitrophenols formed are not responsible for the observed enhancement of N-nitrosation, since *p*-nitrophenol did not affect NMOR formation under our experimental conditions (data not shown). In addition, *p*-nitrophenol did not increase absorption of gaseous NO_2 as observed with other phenols; the amount of nitrite formed was the same regardless of *p*-nitrophenol concentration. The absence of any effect on N-nitrosation and nitrite formation is probably due to the electron-withdrawing effect of the nitro group on the aromatic ring, which would lessen its nucleophilic and electron-donating characteristics.

Phenolic compounds are present in many foods, spices, and drugs; consequently, their ability to enhance N-nitrosation by NO_2 may be cause for some concern. The reported ability of *o*-methoxyphenols such as vanillin, syringol, and ferulic acid to inhibit acid-catalyzed N-nitrosation by nitrite (Gray and Dugan, 1975; Virk and Issenberg, 1985; Kuenzig et al., 1984) and the inability of guaiacol to affect acid-catalyzed N-nitrosation by nitrite (Pignatelli et al., 1982) may indicate that different mechanisms are involved. The results reported here indicate a need to further examine the aqueous N-nitrosation reactions of NO_2 in order to assess the potential for carcinogen formation in vivo as a result of exposure to NO_2. The ability of phenols to enhance N-nitrosation suggests that reducing phenol intake or content may be advisable where exposure of humans or foods to NO_2 is likely to occur. It addition, the type of N-nitrosating species must be considered when evaluating the likely effect of phenols in *N*-nitrosamine formation, e.g. in smoked or direct-heat-dried foods where phenols may enhance N-nitrosation during the processing but inhibit endogenous N-nitrosation in the stomach.

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Registry No. NO_2, 10102-44-0; 2,4,6-$Me_3C_6H_2OH$, 527-60-6; *p*-HOC_6H_4OH, 123-31-9; *o*-HOC_6H_4OH, 120-80-9; *m*-HOC_6H_4OH, 108-46-3; *o*-$MeOC_6H_4OH$, 90-05-1; PhOH, 108-95-2; *p*-$O_2NC_6H_4OH$, 100-02-7; morpholine, 110-91-8; *N*-nitrosomorpholine, 59-89-2; *N*-nitromorpholine, 4164-32-3; vanillin, 121-33-5.

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