Reactivity of Some Products Formed by the Reaction of Sorbic Acid with Sodium Nitrite: Decomposition of 1,4-Dinitro-2-methylpyrrole and Ethylnitrolic Acid

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Sorbic acid reacts with nitrite to yield mutagenic products such as 1,4-dinitro-2-methylpyrrole (NMP) and ethylnitrolic acid (ENA). In order to know the stability of these compounds, a kinetic study of their decomposition reactions was performed in the 6.0—9.5 pH range. The conclusions drawn are as follows: (i) The decomposition of NMP occurs through a nucleophilic attack by OH⁻ ions, with the rate equation as follows: \( r = k_{dec}^{NMP}[\text{OH}^-][\text{NMP}] \) with \( k_{dec}^{NMP} \) (37.5 °C) = 42 ± 1 M⁻¹ s⁻¹. (ii) The rate law for the decomposition of ENA is as follows: \( r = k_{dec}^{ENA}[\text{ENA}][\text{K}_a/(\text{K}_a + [\text{H}^+])] \), with \( K_a \) being the ENA dissociation constant and \( k_{dec}^{ENA} \) (37.5 °C) = (7.11 ± 0.04) × 10⁻⁴ s⁻¹. (iii) The activation energies for NMP and ENA decomposition reactions are, respectively, \( E_a = 94 ± 3 \) and \( 94 ± 1 \) kJ mol⁻¹. (iv) The observed values for the decomposition rate constants of NMP and ENA in the pH range of the stomach lining cells, into which these species can diffuse, are so slow that they could be the slow determining step of the alkylation mechanisms by some of the products resulting from NMP and ENA decomposition. Thus, the current kinetic results are consistent with the low mutagenicity of these species.

KEYWORDS: Nitrite; sorbic acid; 1,4-dinitro-2-methylpyrrole; ethylnitrolic acid.

INTRODUCTION

Sorbic acid (trans,trans-2,4-hexadienoic acid) and its potassium, sodium, and calcium salts are used as preservatives in a wide range of foodstuffs, such as cheese, pickles, sauces, and wine. They inhibit the growth of fungi and yeasts and have antibacterial activity (1). Accordingly, many analytical methods have been proposed to determine the amount of sorbic acid and sorbates in different types of samples (2, 3). Sorbates have been reported to be more efficient and less toxic than benzoate (4) and are classified as "Generally Recognized as Safe" (GRAS) additives by the U.S. Food and Drug Administration (FDA) (5). Nevertheless, a weak genotoxic potential of stored sorbate solutions has been reported (6–11). In light of this, the alkylation potential of the sorbic acid has been investigated previously (12, 13).

Nitrite is widely used in the curing of meat, where, in conjunction with sodium chloride, it inhibits the growth and toxin production of Clostridium botulinum (14). Since the discovery in 1956 that nitrosamines are powerful carcinogens, there has been a noteworthy increase in studies addressing nitrosamine chemistry, in particular the formation and reactions of these substances. This area continues to be one of major concern, since nitrosamines can readily be formed under acidic conditions (e.g., in the human stomach) from many naturally occurring amines and ingested nitrate and nitrite (15–17).

Because sorbic acid also inhibits Clostridium botulinum growth as well as the formation of nitrosamines, it has been proposed as a partial replacement for nitrite in meat curing. However, this practice may lead to other toxicological problems since sorbic acid reacts with nitrite to yield certain mutagenic products (18) (Scheme 1). The main mutagens seem to be 1,4-dinitro-2-methylpyrrole (NMP) and ethylnitrolic acid (ENA) (19).

Since (i) a comparison of the alkylation capacity of NMP and ENA with their mutagenic potential could be of interest and (ii) knowledge of the kinetic parameters of the decomposition reactions of those species (and, hence, of their stability) is

\[ \text{Sorbic acid} + \text{NaNO}_2 \rightarrow \text{NMP} \]

\[ \text{Sorbic acid} \]

\[ \text{NMP} \]

\[ \text{ENA} \]

Scheme 1. Reaction of Sorbic Acid with Sodium Nitrite

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necessary as a previous step in the investigation of their alkylation potential, here we were prompted to address this issue.

MATERIALS AND METHODS

To monitor the NMP and ENA decomposition reactions (Schemes 2 and 3), their absorbances at the wavelengths of maximum absorption (\(\lambda = 269\) nm and \(\lambda = 328\) nm, respectively) were followed. The hydrolysis reaction of acetonitrile oxide (ACNO) was monitored spectrophotometrically at \(\lambda = 210\) nm, where the product acetohydroxamic acid (ACH) absorbs. A Shimadzu UV-2401-PC spectrophotometer with a thermoelectric six-cell holder temperature control system (\(\pm 0.1^\circ\)C) was used. Detailed reaction conditions are given in the figure and table legends.

Reactions were carried out in the 6.0–9.5 pH range. Phosphate and borate buffers were used to maintain constant pH. A Crison Micro pH 2000 pH-meter was used for pH measurements (\(\pm 0.01\)). The reaction temperature was kept constant (\(\pm 0.05^\circ\)C) with a Lauda Ecoline RE120 thermostat.

Titrations were performed using a pH-Stat Metrohm 718 STAT Titrino, which releases NaOH, previously normalized with potassium hydrogen phthalate. An QSTAR XL TOF-MS system (Applied Biosystems) was used for the determination of accurate masses. NMR proton spectra were obtained with a Varian spectrometer Mod. Mercury VS2000 (200 MHz).

Water was deionized with a MilliQ-Gradient (Millipore). Sorbic acid and sodium nitrite were from Panreac. Nitroethane and D2O (98%) were obtained from Aldrich.

All kinetic runs were performed in triplicate. Numerical treatment of the data was performed using the 7.1.44 Data Fit software.

Procedures: Synthesis of 1,4-Dinitro-2-methylpyrrole and Ethynitrolic Acid. 1,4-Dinitro-2-methylpyrrole was obtained from the reaction between sorbic acid and sodium nitrite in aqueous solution at pH \(= 3.5\) (18, 20). A solution of sodium nitrite (11.0 g) was added to a partially suspended solution of sorbic acid (2.2 g) in distilled water (200 mL). Keeping the pH of the mixture constant at 3.5 with dilute H2SO4, the mixture was stirred at 60°C for 2 h. The mixture was extracted with four 50 mL portions of CH2Cl2. The combined extracts were washed with water, dried over Na2SO4, and evaporated to dryness in vacuo to give the residue. The residue was recrystallized in chloroform ether.

UV \(\lambda_{\text{max}}\) in nm (\(\varepsilon, \text{M}^{-1} \cdot \text{cm}^{-1}\)):

- 209 (11621 ± 123), 229 (17428 ± 135), 270 (13551 ± 107), 329 (8764 ± 66), 385 (1806 ± 19).
- 1H NMR (CDCl3): \(\delta\) 2.64 (s, 3H, CH3), 7.9 (d, 1H, CH–C–NO2), 8.8 (d, 1H, CH–N–NO2).

Ethylnitrolic acid was obtained from the nitrosation reaction of nitroethane in acidic aqueous solution. Sodium nitrite (8.9 g) was added to a solution of nitroethane (8 mL) in aqueous sodium hydroxide (4.3 g in 100 mL) at 0°C. Simultaneously to sodium nitrite addition, a solution of sulfuric acid (5 M) was slowly added to maintain an acidic pH in the reaction mixture. The aqueous solution was extracted with three 50 mL portions of ether and evaporated to dryness in vacuo to give the product. The product was recrystallized in dichloromethane hexane. Because of its low thermal stability, it was stored at 0°C.

UV \(\lambda_{\text{max}}\) in nm (\(\varepsilon, \text{M}^{-1} \cdot \text{cm}^{-1}\)):

- 240 (4341 ± 29) in acid media and 328 (8237 ± 17) in alkaline medium.
- 1H NMR (CDCl3): \(\delta\) 2.46 (s, 3H, CH3), 9.3 (broad, 1H, OH).

RESULTS AND DISCUSSION

Decomposition of 1,4-Dinitro-2-methylpyrrole. 1,4-Dinitro-2-methylpyrrole undergoes decomposition, and an inverse
kinetic isotope effect, $k_{\text{dec H}_2\text{O}}/k_{\text{dec D}_2\text{O}} = 0.7$, was observed. This result was interpreted in terms of a nucleophilic attack of the OH$^-$ on the NMP molecule. Since OD$^-$ is a stronger nucleophile than OH$^-$, the direct nucleophilic attack on the electrophilic carbon (Scheme 2) would be expected to proceed faster in D$_2$O ($^{21}$), as was observed.

The presence of nitro groups on the pyrrole ring may play a significant role in lowering the high π-electron density of the ring and in favoring the attack of the nucleophilic reagent ($^{20}$, $^{22}$). This explains the formation of the Meisenheimer-type adduct due to the addition of OH$^-$ on the NMP molecule.

The following rate equation was observed for NMP decomposition,

$$r = -\frac{d[\text{NMP}]}{dt} = k_{\text{dec}}[\text{OH}^-][\text{NMP}] = k_{\text{obs}}[\text{NMP}]$$

(1)

where $k_{\text{obs}}$ is the pseudofirst-order rate constant:

$$k_{\text{obs}} = k_{\text{dec}}[\text{OH}^-]$$

(2)

By designating the absorbance values of NMP as $A_0$, $A_t$, and $A_\infty$ at times, respectively, zero, $t$, and infinity (end of the reaction), integration of eq 1 yields eq 3:

$$A_t = A_\infty + (A_0 - A_\infty)e^{-k_{\text{obs}}t}$$

(3)

Figure 1 depicts the variation in the absorption of NMP with time. Figure 2 shows a typical kinetic run for the decomposition reaction of NMP in aqueous solution.

Experiments were performed at different pH’s (Figure 3), with the slope $\alpha = 1$ revealing first order with respect to the concentration of OH$^-$ (eq 2).

By fitting the absorbance values against those of time (eq 3), the rate constant $k_{\text{obs}}$ was found to be $k_{\text{obs}} (T = 32.5 \, ^\circ\text{C}) = (7.22 \pm 0.05) \times 10^{-4}$ s$^{-1}$ at pH 9.47. Using eq 2, at different pH values, the NMP decomposition rate constant was found to be $k_{\text{dec}} (T = 32.5 \, ^\circ\text{C}) = 22 \pm 1$ M$^{-1}$ s$^{-1}$. The values of $k_{\text{dec}}$ at different temperatures are reported in Table 1.

**Decomposition of Ethylnitrolic Acid.** Since (i) the stability of ethylnitrolic acid decreases with increasing pH and (ii) the dissociated acid molecule undergoes NO$_2^-$ loss, this being the limiting step ($^{23}$), the mechanism shown in Scheme 3 for the decomposition of ENA was investigated.

Because (i) all the kinetic experiences were carried out in the pH = 6–9.5 range and (ii) the ACH pK$_a$ = 9.31 ($^{24}$, $^{25}$)
that the undissociated (HA) and dissociated (A) forms of ENA show maximum absorption at 240 and 328 nm, respectively. Since the ethylnitrolic acid concentration [ENA] can be expressed as the sum of the concentration of nondissociated ethylnitrolic acid (HA) plus that present as anion (A⁻),

\[
[\text{ENA}] = [\text{HA}] + [\text{A}^-]
\]

and because \( K_a = ([\text{A}^-][\text{H}^+])/(\text{HA}) \), eq 7 is readily deduced from Scheme 3,

\[
\frac{\text{d[ENA]}}{\text{dt}} = k_{\text{obs}}^\text{ENA} \frac{K_a}{K_a + [\text{H}^+]} [\text{ENA}] = k_{\text{obs}}^\text{ENA} [\text{ENA}]
\]

where \( k_{\text{obs}}^\text{ENA} \) is the pseudofirst-order rate constant:

\[
k_{\text{obs}}^\text{ENA} = k_{\text{dec}}^\text{ENA} \frac{K_a}{K_a + [\text{H}^+]} \]

By designating the absorbance values of ENA as \( A_\infty \), \( A_0 \), and \( A_t \) at times, respectively, zero, \( t \), and infinity (end of the reaction), the integration of eq 7 yields eq 9:

\[
A_t = A_\infty + (A_0 - A_\infty)e^{-k_{\text{obs}}^\text{ENA} t}
\]

Figure 7 shows the variation in the absorption of ENA with time.

Figure 6a represents a typical kinetic run for the decomposition reaction of ENA in aqueous solution.

By fitting the absorbance values against those of time (eq 9), the rate constant was found to be \( k_{\text{obs}}^\text{ENA} (T = 32.5 \degree C) = (3.96 \pm 0.03) \times 10^{-5} \text{ s}^{-1} \) at \( pH = 9.52 \). Using eq 8, ENA decomposition rate constant was found to be \( k_{\text{dec}}^\text{ENA} (T = 32.5 \degree C) = (3.99 \pm 0.03) \times 10^{-5} \text{ s}^{-1} \).
According to the above mechanism, the rate equation for the light path.

\[ k \text{ is the absorbance of acetohydroxamic acid at time } t \text{ and } k_{\text{obs}} \text{ is the pseudofirst-order hydrolysis rate constant, defined as follows:} \]

\[ k_{\text{obs}} = k_{\text{hyd}} [\text{H}_2\text{O}] \]

According to the general kinetic scheme for consecutive first-order reactions (27) and the boundary condition \([\text{ACNO}]_0 = 0\), the concentration of acetonitrile oxide at time \(t\) is given by eq 12:

\[ [\text{ACNO}] = \frac{k_{\text{obs}} [\text{ENA}]_0}{k_{\text{obs}} - k_{\text{obs}}} (e^{-k_{\text{obs}}t} - e^{-k_{\text{obs}}t}) \]

Integration of eq 10 and expression of the result in terms of absorbance leads to the following:

\[ A_t = \varepsilon_{\text{ACH}} [\text{ENA}]_0 \left( 1 + \frac{e^{-k_{\text{obs}}t} - e^{-k_{\text{obs}}t}}{k_{\text{obs}} - k_{\text{obs}}} \right) \]

where \(A_t\) is the absorbance of acetylhydroxamic acid at time \(t\), \(\varepsilon_{\text{ACH}}\) is the absorption coefficient of this species, and \(l\) is the light path.

Figure 7b shows the good fit of the experimental results to eq 13, which allows, with the above calculated \(k_{\text{obs}}\) values, to know the value of the decomposition rate constant \(k_{\text{obs}}\) immediately affords that of \(k_{\text{obs}}\) (eq 11).

\[ k_{\text{obs}} = k_{\text{hyd}} [\text{H}_2\text{O}] \]

Thus, the current kinetic results are consistent with the low mutagenicity of NMP and ENA. In any case, further experimental work is necessary to check the possible existence of a correlation between the chemical reactivity of NMP/ENA and their mutagenicity, as has been demonstrated with other alkylating agents such as lactones (29–31).

With the \(k_{\text{NMP}}^\text{obs}\) and \(k_{\text{ENA}}^\text{obs}\) values reported in Table 1, the following energies of activation were obtained for the respective reactions: \(E_a\) (decomposition of NMP) = 94 ± 3 kJ mol\(^{-1}\); \(E_a\) (decomposition of ENA) = 94 ± 1 kJ mol\(^{-1}\); and \(E_a\) (hydrolysis of ACNO) = 73 ± 5 kJ mol\(^{-1}\).

From the present study, the following conclusions may be drawn:

1. The decomposition of NMP occurs through a nucleophilic attack by OH\(^-\) ions, with the rate equation being the following:

\[ r = \frac{d[A]}{dt} = k_{\text{NMP}} [\text{ACNO}[\text{H}_2\text{O}] = k_{\text{obs}} [\text{ACNO}] \]

2. The rate law for the decomposition of ENA is as follows:

\[ r = k_{\text{ENA}} [\text{ENA}]_0 [\text{H}_2\text{O}] \text{ with } k_{\text{ENA}} = k_{\text{obs}} \]

3. The activation energies for NMP and ENA decomposition reactions are, respectively, \(E_a\) = 94 ± 3 and 94 ± 1 kJ mol\(^{-1}\).

4. The observed values for the decomposition rate constants of NMP and ENA in the pH range of stomach-lining cells, into which these species can diffuse, are so slow that they could be the slow determining step of the alkylation mechanisms by some of the mutagenic products resulting from NMP and ENA decomposition (18, 28).

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