Sorbate−Nitrite Interactions: Acetonitrile Oxide as an Alkylating Agent

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Because chemical species with DNA-damaging and mutagenic activity are formed in sorbate−nitrite mixtures and because sorbic acid sometimes coexists with nitrite occurring naturally or incorporated as a food additive, the study of sorbate−nitrite interactions is important. Here, the alkylating potential of the products resulting from such interactions was investigated. Drawn were the following conclusions: (i) Acetonitrile oxide (ACNO) is the compound responsible for the alkylating capacity of sorbate−nitrite mixtures; (ii) ACNO alkylates 4-(*p*-nitrobenzyl)pyridine (NBP), a trap for alkylating agents with nucleophilic characteristics similar to those of DNA bases, forming an adduct (AD; $\varepsilon = 1.4 \times 10^4$ M^{-1} cm^{-1}; $\lambda = 519$ nm); (iii) the NBP alkylation reaction complies with the rate equation, $r = d[AD]/dt = k_{alk}^{ACNO}[ACNO][NBP] - k_{hyd}^{AD}[AD]$, k_{alk}^{ACNO} being the NBP alkylation rate constant for ACNO and k_{hyd}^{AD} the rate constant for the adduct hydrolysis reaction; (iv) the small fraction of ACNO forming the adduct with NBP, as well as the small magnitude of the quotient ($k_{alk}^{ACNO}/k_{hyd}^{ACNO}$) as compared with those reported for other alkylating agents, such as some lactones and *N*-alkyl-*N*-nitrosoureas, reveals the ACNO effective alkylating capacity to be less significant; (v) the low value of the NBP−ACNO adduct life (defined as the total amount of adduct present along the progression of the NBP alkylation per unit of alkylating agent concentration) points to the high instability of this adduct; and (vi) the obtained results are in accordance with the low carcinogenicity of ACNO.

Introduction

Sorbic acid and its salts (henceforth sorbate) are used worldwide as preservatives in foods, feeds, pharmaceuticals, and cosmetics (*1, 2*) because of their inhibitory effects on yeasts, fungi, and bacteria (*3*). Sorbic acid and potassium sorbate are classified as "Generally Recognized as Safe" (GRAS) by the FDA (*4*). While the majority of investigations have not revealed mutagenic properties for sorbic acid and sorbate, some reports have indicated the possible genotoxic potential of these compounds (*5*−*8*).

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* (*9*). Because sorbic acid also inhibits *C. botulinum* growth (*10*) as well as the formation of nitrosamines, it has been proposed as a partial replacement for nitrite in meat curing (*11*).

Chemical species with DNA-damaging and mutagenic activity for bacterial test systems have been detected in sorbate−nitrite mixtures (*12*−*18*). Sorbate−nitrite interactions seem to be important, since sorbic acid sometimes coexists with nitrite, occurring naturally or incorporated as a food additive. One of the products with direct-acting genotoxic activity has been identified as ethylnitrolic acid (ENA) (*12*).

ENA exhibits relatively low activity in the Ames reverse-mutation *Salmonella* assay (*15*) but is highly active in the *rec* assay (*12*). This compound is at least 40-fold more active than sorbic acid alone and at least 30-fold more active than sodium nitrite alone in the bacterial *rec* assay (*12*), and it is also a direct-acting mutagen in a *Salmonella* forward-mutation assay (*19*).

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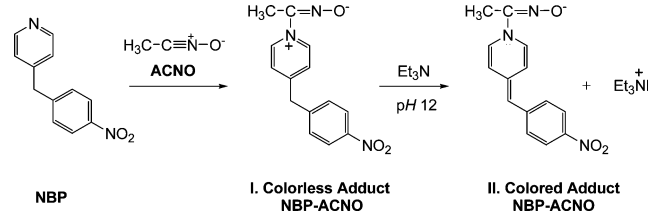
Scheme 1. Method for Monitoring the NBP Alkylation of ACNO



Because, (i) in previous work, some correlation was found between the alkylating potential of strong and weak carcinogenic compounds such as lactones (*20*−*25*) and sorbates (*26, 27*) and their carcinogenicity and, (ii) to our knowledge, the alkylating potential of ENA has not been studied in quantitative chemical terms, here, we were prompted to investigate its alkylating capacity. The nucleophile 4-(*p*-nitrobenzyl)pyridine (NBP), a trap for alkylating agents (*28*) with nucleophilic characteristics similar to those of DNA bases (*29*), was used to measure the alkylating capacity of ENA.

Experimental Procedures

Alkylation reactions were performed in the 4−10 pH range. Acetate and borate buffers were used to maintain pH constant. To render NBP soluble, the alkylation mixtures (ENA + NBP) were prepared in 7:3 (v/v) water:dioxane medium.

To monitor the alkylation reactions, 2.4 mL aliquots of the alkylation mixture were removed at different times and added to a cuvette containing 0.6 mL of 99% triethylamine reagent (Et_3N) to stop the alkylation process (Scheme 1), after which the absorbance was measured at the wavelength of maximum absorption ($\lambda = 519$ nm). Detailed reaction conditions are given in the figure and table legends.

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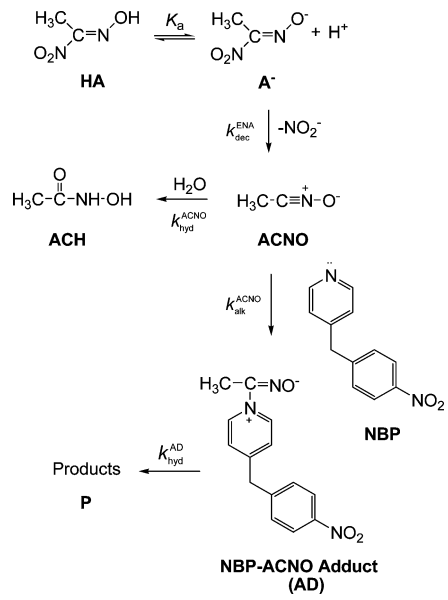
Scheme 2. Alkylation of NBP by ACNO



A Shimadzu UV-2401-PC spectrophotometer with a thermoelectric six-cell holder temperature control system (±0.1 °C) was used. A Metrohm 827 pH meter was used for pH measurements (±0.001). The reaction temperature was kept constant (±0.05 °C) with a Lauda Ecoline RE120 thermostat.

Identification of the adduct NBP−ACNO and its hydrolysis product was performed in 7:3 water:dioxane medium with the same concentration of both reagents: [ENA] = [NBP] = 10^{-3} M. Mode electrospray ionization mass spectra were recorded on a Waters ZQ4000 spectrometer.

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

Water was deionized with a Milli-Q-Gradient (Millipore). NBP was a Sigma product; 99% Et_3N was obtained from Aldrich, and dioxane was purchased from Panreac (Barcelona, Spain).

ENA was obtained from the nitrosation reaction of nitroethane in acidic aqueous solution, as described before (*30*). 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 sulphuric acid (5 M) was added slowly to maintain the pH of the reaction mixture acidic. The aqueous solution was extracted with three 50 mL portions of ether and evaporated to dryness in vacuo to give the product, which was recrystallized in dichloromethane−hexane. Because of its low thermal stability, it was stored at 0 °C. UV λ_{max} in nm (ε, M^{-1} cm^{-1}): 240 (4341 ± 29) in acid media and 328 (8237 ± 17) in alkaline medium. ^{1}H NMR ($CDCl_3$): δ 2.46 (s, 3H, CH_3), 9.3 (broad, 1H, OH).

Sodium nitrite was a Panreac product (Barcelona, Spain). Nitroethane was obtained from Aldrich. NMR proton spectra were obtained with a Varian spectrometer model Mercury VS2000 (200 MHz).

Results

Reaction Mechanism. The mechanism shown in Scheme 2 for the alkylation of the NBP can be proposed on the basis of (i) the fact that the stability of ENA decreases with increasing pH (*30, 31*); (ii) the dissociated ENA molecule (A^-; Scheme 2) undergoes NO_2^- loss, this being the limiting step in the mechanism (*32*); and (iii) the fact that nitrile oxides (in this case, acetonitrile oxide, ACNO) react with neutral nucleophiles such as amines (*33, 34*) and that they also undergo hydrolysis, forming the corresponding hydroxamic acids (*35*) (in the present case, acetohydroxamic acid, ACH).

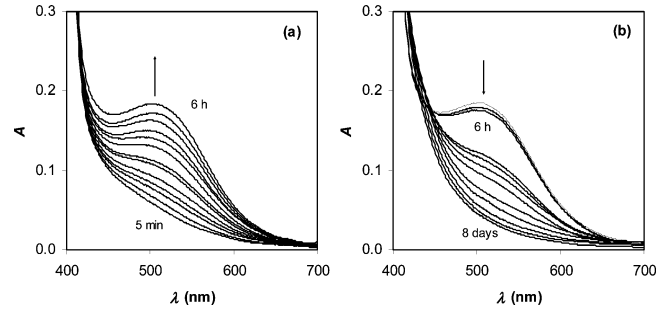


Figure 1. (a) Formation of the NBP−ACNO adduct over time (λ = 519 nm). (b) Hydrolysis of the adduct with time. pH 9.2, $[ENA]_o$ = 8.4×10^{-4} M, $[NBP]_o = 2.6 \times 10^{-2}$ M, and T = 30.0 °C.

The measured value of the molar absorption coefficient of the NBP−ACNO adduct in 7:3 (v/v) water:dioxane medium was $\varepsilon_{AD} = 1.4 \times 10^4$ M^{-1} cm^{-1} (λ = 519 nm). The fact that the wavelength of maximum absorption is short as compared with that of other NBP adducts (*36, 37*) suggests that the adduct molecule has a double bond conjugated with the aromatic ring. The electrospray ionization mass spectrum of the NBP−ACNO adduct shows an m/z = 272, in agreement with the suggested structure. Figure 1 shows the increase in absorption caused by the formation of the adduct over time and the decrease in absorbance due to its hydrolysis to form the corresponding hydrate, which shows an m/z = 290.

From the mechanism in Scheme 2, eq 1 can be deduced.

$$\text{rate} = \frac{\mathrm{d[AD]}}{\mathrm{d}t} = k_{alk}^{ACNO}[ACNO][NBP] - k_{hyd}^{AD}[AD] \quad (1)$$

Because the formation of the ACNO intermediate is slow and its disappearance is fast (due to the alkylation and hydrolysis reactions), steady-state treatment (*38*) can be applied to its concentration, such that,

$$[ACNO] = \frac{k_{dec}^{ENA}[A^-]}{k_{hyd}^{ACNO}[H_2O] + k_{alk}^{ACNO}[NBP]} \quad (2)$$

Because the total concentration of ENA is the sum of the concentrations of its nondissociated HA plus the dissociated form A^-, the concentration of the latter can be expressed as a function of the ENA equilibrium dissociation constant K_a:

$$[A^-] = \frac{K_a}{K_a + [H^+]}[ENA] \quad (3)$$

Because the pH was kept constant, eq 1 can be written in the form:

$$\text{rate} = \frac{\mathrm{d[AD]}}{\mathrm{d}t} = \frac{k_{alk}^{ACNO}[NBP]k_{obs}^{ENA}[ENA]}{k_{hyd}^{ACNO}[H_2O] + k_{alk}^{ACNO}[NBP]} - k_{hyd}^{AD}[AD] \quad (4)$$

where k_{obs}^{ENA} is the ENA decomposition pseudofirst-order rate constant defined as follows:

$$k_{obs}^{ENA} = k_{dec}^{ENA}\frac{K_a}{K_a + [H^+]} \quad (5)$$

The concentration of ENA can be expressed as a function of k_{obs}^{ENA}:

$$[\mathrm{ENA}] = [\mathrm{ENA}]_o e^{-k_{obs}^{ENA} t} \quad (6)$$

such that eq 4 can be converted to eq 7:

$$\text{rate} = \frac{d[\mathrm{AD}]}{dt} = \frac{k_{alk}^{ACNO}[\mathrm{NBP}]k_{obs}^{ENA}}{k_{hyd}^{ACNO}[\mathrm{H_2O}] + k_{alk}^{ACNO}[\mathrm{NBP}]}[\mathrm{ENA}]_o e^{-k_{obs}^{ENA} t} - k_{hyd}^{AD}[\mathrm{AD}] \quad (7)$$

Equation 8 shows the result of the integration of eq 7, A_t, ε_{AD}, and l, respectively, being the absorbance of the adduct at time t, the molar absorption coefficient, and the cuvette light path.

$$A_t = \frac{\varepsilon_{AD} l k_{alk}^{ACNO}[\mathrm{NBP}]k_{obs}^{ENA}[\mathrm{ENA}]_o}{(k_{hyd}^{ACNO}[\mathrm{H_2O}] + k_{alk}^{ACNO}[\mathrm{NBP}])(k_{hyd}^{AD} - k_{obs}^{ENA})} (e^{-k_{obs}^{ENA} t} - e^{-k_{hyd}^{AD} t}) \quad (8)$$

To handle eq 8 in a more simple form, it can be written as in eq 9.

$$y = a(e^{-cx} - e^{-bx}) \quad (9)$$

a, b, and c being parameters obtained by a nonlinear fitting of results, and defined as:

$$a = \frac{\varepsilon_{AD} l k_{alk}^{ACNO}[\mathrm{NBP}]k_{obs}^{ENA}[\mathrm{ENA}]_o}{(k_{hyd}^{ACNO}[\mathrm{H_2O}] + k_{alk}^{ACNO}[\mathrm{NBP}])(k_{hyd}^{AD} - k_{obs}^{ENA})} \quad (10)$$

where b and c are the hydrolysis rate constants of the adduct, $b = k_{hyd}^{AD}$, and the decomposition rate constant of ENA, $c = k_{obs}^{ENA}$, respectively. The value of the latter has been determined by us previously (*30, 31*). Figure 2 shows the good fit of the results to eq 9.

Because of the small value of k_{obs}^{ENA} in the 4−7 pH range and because $k_{obs}^{ENA} \ll k_{hyd}^{AD}$ ($k_{obs}^{ENA} = 3.77 \times 10^{-5}$ min^{-1} (*30*) and $k_{hyd}^{AD} = 1.89 \times 10^{-3}$ min^{-1}; pH 6.25), eq 8 can be converted into eq 11:

$$A_t = \frac{\varepsilon_{AD} l k_{alk}^{ACNO}[\mathrm{NBP}]k_{obs}^{ENA}[\mathrm{ENA}]_o}{(k_{hyd}^{ACNO}[\mathrm{H_2O}] + k_{alk}^{ACNO}[\mathrm{NBP}])k_{hyd}^{AD}} (1 - e^{-k_{hyd}^{AD} t}) \quad (11)$$

Equation 11 can be written in the form:

$$y = m(1 - e^{-nx}) \quad (12)$$

where m and n are defined as follows:

$$m = \frac{\varepsilon_{AD} l k_{alk}^{ACNO}[\mathrm{NBP}]k_{obs}^{ENA}[\mathrm{ENA}]_o}{(k_{hyd}^{ACNO}[\mathrm{H_2O}] + k_{alk}^{ACNO}[\mathrm{NBP}])k_{hyd}^{AD}} \quad (13)$$

and n is the rate constant for the hydrolysis reaction of the adduct, $n = k_{hyd}^{AD}$. Figure 3 shows the good fit of the results to eq 12.

Alkylation of NBP and Hydrolysis of the NBP−ACNO Adduct. The quotient between the NBP alkylation rate constant of ACNO and the ACNO hydrolysis rate constant ($k_{alk}^{ACNO}/k_{hyd}^{ACNO}$) has been used as a reference for the ACNO alkylating potential. To determine its value, we proceeded as follows.

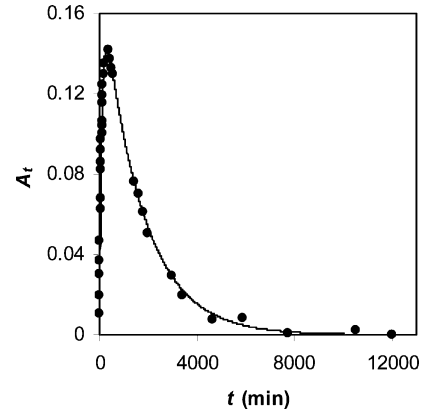


Figure 2. Formation and hydrolysis of the NBP−ACNO adduct in 7:3 water:dioxane; $\lambda = 519$ nm. pH 9.2, $[\mathrm{ENA}]_o = 8.4 \times 10^{-4}$ M, $[\mathrm{NBP}]_o = 2.6 \times 10^{-2}$ M, and $T = 30.0$ °C.

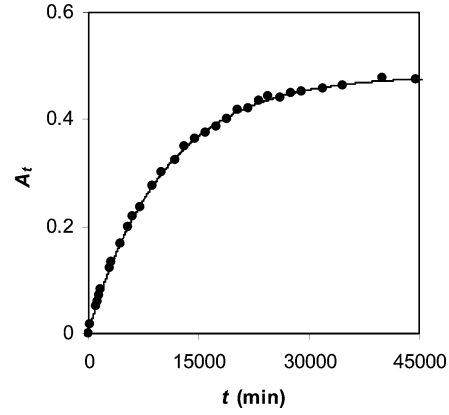


Figure 3. Formation of the NBP−ACNO adduct in 7:3 water:dioxane. pH 4.8, $[\mathrm{ENA}]_o = 8.0 \times 10^{-4}$ M, $[\mathrm{NBP}]_o = 2.0 \times 10^{-2}$ M, and $T = 30.0$ °C.

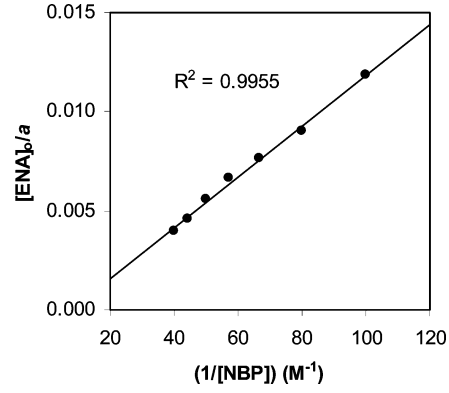


Figure 4. Fitting of the results to eq 14 in 7:3 water:dioxane. pH 9.2, $[\mathrm{ENA}]_o = 8.0 \times 10^{-4}$ M, and $T = 37.5$ °C.

The reciprocal of the preexponential factor of eq 8 can be written as in eq 14:

$$\frac{1}{a}[\mathrm{ENA}]_o = \frac{[\mathrm{H_2O}](k_{hyd}^{AD} - k_{obs}^{ENA})}{\varepsilon_{AD} l k_{obs}^{ENA}} \left(\frac{k_{hyd}^{ACNO}}{k_{alk}^{ACNO}}\right) \frac{1}{[\mathrm{NBP}]} + \frac{(k_{hyd}^{AD} - k_{obs}^{ENA})}{\varepsilon_{AD} l k_{obs}^{ENA}} \quad (14)$$

Figure 4 shows the good fit of the experimental results to eq 14.

Moreover, the reciprocal of the preexponential factor of eq 11 is given by eq 15:

$$\frac{1}{m}[\mathrm{ENA}]_o = \frac{[H_2O]k_{hyd}^{AD}}{\varepsilon_{AD} l k_{obs}^{ENA}}\left(\frac{k_{hyd}^{ACNO}}{k_{alk}^{ACNO}}\right)\frac{1}{[NBP]} + \frac{k_{hyd}^{AD}}{\varepsilon_{AD} l k_{obs}^{ENA}} \quad (15)$$

Figure 5 shows the good fit of the results to eq 15.

The values of ($k_{alk}^{ACNO}/k_{hyd}^{ACNO}$) were calculated with the slope and intercept of eqs 15 and 16, at alkaline and acidic pH, respectively.

The fraction f of ACNO forming the adduct is

$$f = \frac{k_{alk}^{ACNO}[NBP]}{k_{alk}^{ACNO}[NBP] + k_{hyd}^{ACNO}[H_2O]} \quad (16)$$

Equation 16 can be written in the form:

$$\frac{1}{f} = 1 + \frac{1}{(k_{alk}^{ACNO}/k_{hyd}^{ACNO})}\frac{[H_2O]}{[NBP]} \quad (17)$$

Equation 17 shows that the value of f can be known once the quotient ($k_{alk}^{ACNO}/k_{hyd}^{ACNO}$) has been calculated.

The hydrolysis of the NBP−ACNO adduct was also investigated. To do so, we defined adduct life (AL) as the total amount of adduct present along the progression of the reaction per unit of alkylating agent concentration, such that:

$$\mathrm{AL} = \frac{\int_0^\infty [\mathrm{AD}]\,dt}{[\mathrm{ENA}]_o} = \frac{k_{alk}^{ACNO}[NBP]}{(k_{alk}^{ACNO}[NBP] + k_{hyd}^{ACNO}[H_2O])k_{hyd}^{AD}} = \frac{f}{k_{hyd}^{AD}} \quad (18)$$

Tables 1 and 2 give the values of ($k_{alk}^{ACNO}/k_{hyd}^{ACNO}$), f, and AL at different pH values and temperatures.

Discussion

As can be observed in Table 1, ($k_{alk}^{ACNO}/k_{hyd}^{ACNO}$) increases with decreasing pH. This variation with pH must be caused by the change in k_{hyd}^{ACNO} with the acidity of the medium.

The value of the fraction f of ACNO that forms the adduct decreases with increasing pH. In the 4−10 pH range, the value of f is less than 50%. The hydrolyzed fraction of ACNO is maximum.

To evaluate the stability of the NBP−ACNO adduct, its hydrolysis was investigated at different pH values (Table 1) and was found to increase with the increase in pH. This variation, together with the value of f, is reflected in the adduct life value, which decreases from 2600 min at pH 4.83 to 3 min at pH 9.68.

Comparison of the (k_{alk}/k_{hyd}), f, and AL values for NBP alkylation by ACNO with those determined with other alkylating agents is of interest. Table 3 depicts the values obtained here together with those measured previously by us for NBP alkylation by lactones such as β-propiolactone (BPL), β-butyrolactone (BBL), and diketene (DIK) and several *N*-alkyl-*N*-nitrosoureas in which the alkylating agent is the corresponding alkyldiazonium ion (*39*).

The small fraction of ACNO forming the adduct with NBP, as well as the small magnitude of the quotient $k_{alk}^{ACNO}/k_{hyd}^{ACNO}$ as compared with those for other alkylating agents such as some lactones and *N*-alkyl-*N*-nitrosoureas (Table 3), shows the ACNO effective alkylating capacity to be less significant.

With regard to the stability of the NBP−ACNO adduct, the low value of its AL (as well as for the NBP−DIK adduct; see Table 1) shows the high instability of both adducts. These results are in accordance with the low carcinogenicity of both compounds (*12, 40*).

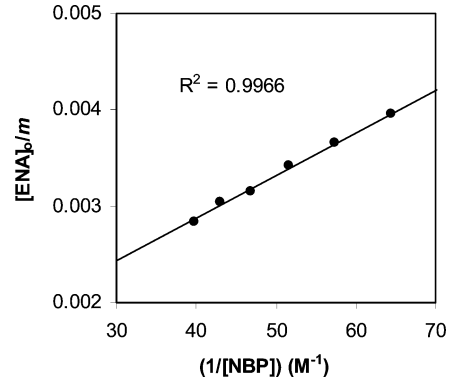


Figure 5. Fitting of the results to eq 15 in 7:3 water:dioxane. pH 5.8, $[\mathrm{ENA}]_o = 8.0 \times 10^{-4}$ M, and $T = 37.5$ °C.

Table 1. Alkylation of NBP by ACNO in 7:3 Water:Dioxane at Different pH Values and $T = 37.5$ °C[a]

pH	$k_{alk}^{ACNO}/k_{hyd}^{ACNO}$[d]	f	$10^3 k_{hyd}^{AD}$ (min^{-1})[d]	AL (min)
4.8[b]	648 ± 7	0.25	0.096 ± 0.001	2608.20
5.3[b]	688 ± 9	0.26	0.262 ± 0.007	996.20
5.8[b]	538 ± 5	0.22	0.66 ± 0.02	326.85
6.3[b]	270 ± 15	0.12	1.89 ± 0.05	64.45
8.7[c]	372 ± 11	0.16	9.3 ± 0.5	17.28
9.2[c]	301 ± 8	0.13	19 ± 1	6.96
9.7[c]	179 ± 24	0.08	28 ± 2	2.97

[a] $[\mathrm{ENA}]_o = 8.0 \times 10^{-4}$ M and $[\mathrm{NBP}]_o = 2.0 \times 10^{-2}$ M. [b] Acetate buffer. [c] Borate buffer. [d] Values are given with their standard deviations.

Table 2. Alkylation of NBP by ACNO in 7:3 Water:Dioxane as a Function of Temperature[a]

T (°C)	$k_{alk}^{ACNO}/k_{hyd}^{ACNO}$[b]	f	$10^2 k_{hyd}^{AD}$ (min^{-1})	AL (min^{-1})
37.5	301 ± 8	0.13	1.9 ± 0.1	6.96
35.0	391 ± 8	0.17	1.59 ± 0.08	10.51
32.5	283 ± 13	0.13	1.14 ± 0.05	11.10
30.0	264 ± 18	0.12	1.02 ± 0.03	11.69
27.5	259 ± 7	0.12	0.76 ± 0.08	15.58
25.0	238 ± 57	0.11	0.56 ± 0.02	19.43

[a] $[\mathrm{ENA}]_o = 8 \times 10^{-4}$ M and $[\mathrm{NBP}]_o = 2 \times 10^{-2}$ M. Borate buffer:dioxane mixtures at pH 9.2. [b] Values are given with their standard deviations.

Table 3. Alkylating Capacity of ACNO as Compared with That of Other Alkylating Agents[a]

alkylating agent	$10^{-3}(k_{alk}/k_{hyd})$	f	AL (min)
BPL[b]	5	0.76	stable adduct
BBL[b]	3	0.62	stable adduct
alkyldiazonium ions[c]	3−13	0.60−0.90	stable adducts
DIK[d]	500	1	1.2
ACNO[e]	0.4	0.17	10.5

[a] $T = 35.0$ °C; 7:3 water:dioxane medium. $[\mathrm{NBP}]_o = 2.0 \times 10^{-2}$ M. [b] Values are taken from ref *23*. [c] Ref *39*. [d] Ref *25*. [e] This work; pH 9.2.

Conclusions

The following conclusions can be drawn. (i) ACNO is the compound responsible for the alkylating capacity of sorbate−nitrite mixtures. (ii) ACNO alkylates NBP, a trap for alkylating agents with nucleophilic characteristics similar to DNA bases,

forming an adduct (AD; $\varepsilon = 1.4 \times 10^4$ M^{-1} cm^{-1}). (iii) The NBP alkylation reaction complies with the rate equation, $r = d[AD]/dt = k_{alk}^{ACNO}[ACNO][NBP] - k_{hyd}^{AD}[AD]$, k_{alk}^{ACNO} being the NBP alkylation rate constant of ACNO and k_{hyd}^{AD} being the rate constant of the adduct hydrolysis reaction. (iv) The small fraction of ACNO forming the adduct with NBP, as well as the small magnitude of the quotient $k_{alk}^{ACNO}/k_{hyd}^{ACNO}$ as compared with those for other alkylating agents such as some lactones and *N*-alkyl-*N*-nitrosoureas, shows the ACNO effective alkylating capacity to be less significant. (v) The low value for the NBP−ACNO adduct life (defined as the total amount of adduct present along the progression of the NBP alkylation per unit of alkylating agent concentration) points to the high instability of this adduct. (vi) The results obtained are in accordance with the low carcinogenicity of ACNO.

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