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Mutagenic products are promoted in the nitrosation of tyramine

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This article is dedicated to the memory of Dr. Jorge Francisco Gaspar.

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ABSTRACT

Tyramine is a biogenic compound derived from the decarboxylation of the amino acid tyrosine, and is therefore present at important concentrations in a broad range of raw and fermented foods. Owing to its chemical properties, tyramine can react with nitrite, a common food additive, in the acidic medium of stomach to form *N*- and *C*-nitroso compounds. Since toxicology studies have shown that the product of C-nitrosation of tyramine is mutagenic, in the present article tyramine nitrosation mechanisms have been characterized in order to discern which of them are favoured under conditions similar to those in the human stomach lumen. To determine the kinetic course of nitrosation reactions, a systematic study of the nitrosation of ethylbenzene, phenethylamine, and tyramine was carried out, using UV–visible absorption spectroscopy. The results show that, under conditions mimicking those of the stomach lumen, the most favoured reaction in tyramine is C-nitrosation, which generates mutagenic products.

1. Introduction

Tyramine (4-(2-aminoethyl)phenol, Fig. 1) is a biogenic aromatic monoamine compound derived from the decarboxylation of the amino acid tyrosine (Andersen, 1977; Marcobal, De las Rivas, Landete, Tabera, & Muñoz, 2012). Tyramine can accumulate in high concentrations in a broad range of raw and fermented foods, such as fish, meat, fruits, cheese, soybean products, and wine (Bayram, 2008; Linares, Martín, Ladero, Álvarez, & Fernández, 2011; Prester, 2011; Stratton, Hutkins, & Taylor, 1991). When these products are consumed, tyramine can react with nitrite – a common food additive used to inhibit the growth of *C. botulinum* – in the acidic medium of stomach, to form nitroso compounds (Lijinsky, 2011; Mysliwy, Wick, Archer, Shank, & Newberne, 1974; Wishnok, 1977). The chemistry of nitroso compounds has attracted considerable research owing to their proven toxic, carcinogenic, mutagenic, and teratogenic effects (Casado, 1994; García-Santos, González-Mancebo, Hernández-Benito, Calle, & Casado, 2002; Mirvish, 1995). Nitroso compounds are unique among carcinogenic agents in that they are active in all living species and have an unparalleled spectrum of target cells and organs in which they can induce cancer (Lijinsky, 2011).

Since (i) Biological studies of tyramine after nitrite treatment have confirmed the mutagenicity of the reaction products (Laires et al., 1993; Ochiai, Wakabayashi, Nagao, & Sugimura, 1984), and in fact an association between the nitroso compounds generated from foodstuffs rich in tyramine and the risk of nasopharyngeal cancer has been found (Wakabayashi et al., 1985; Ward et al., 2000), (ii) nitrosation reactions involve electrophilic intermediates, tyramine can be nitrosated at two sites: the amine group (N-nitrosation) and the carbons of the aromatic ring (C-nitrosation) (Williams, 2004), (iii) the absence of mutagenicity in the nitrosation products of phenethylamine (2-phenethylamine, Fig. 1) (Laires et al., 1993) implies that only the products of tyramine C-nitrosation are mutagenic, as phenethylamine and tyramine are analogous molecules and the only products of nitrosation that they do not have in common are the products of C-nitrosation (substantial aromatic activation of the nitrosatable substrate by the hydroxyl group is necessary (Williams, 2004)) and (iv) to our knowledge no kinetic investigation has been performed to determine the different mechanisms of nitrosation that the tyramine molecule can undergo, including the reaction responsible for the mutagenicity of tyramine nitrosation products, or to discern which products are favoured in conditions similar to the human stomach lumen, here we were prompted to address these issues. With this objective, the nitrosation reactions of ethylbenzene, phenethylamine and tyramine (Fig. 1) were investigated.

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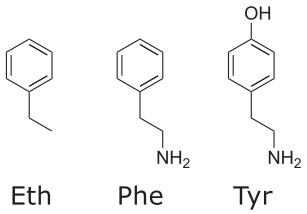


Fig. 1. Compounds studied in this work.

2. Materials and methods

2.1. Chemicals and materials

Ethylbenzene (>99.0%) and phenethylamine (>99.0) were obtained from Fluka (Steinheim, Germany). Tyramine (>99%) was purchased from SAFC (Steinheim, Germany), and deuterium oxide (99.8%) from Acros (Geel, Belgium). Sodium nitrite (ultrapure), copper sulphate (AS), diethyl ether (AS), and perchloric acid (AS) were obtained from Panreac (Barcelona, Spain). Sodium perchlorate (AS) was from Merck (Darmstadt, Germany).

Reactions were monitored by UV-spectroscopy in a Shimadzu UV2401 PC with a thermoelectric six-cell holder temperature control system (±0.1 °C). Electrospray ionization mass spectra were recorded on a Waters ZQ4000 spectrometer by direct injection. A Crison Micro pH 2000 pH meter was used to perform pH measurements (±0.01). Water was deionized with a Millipore MilliQ-Gradient device.

2.2. Nitrosation of ethylbenzene

0.016 ml of ethylbenzene was dissolved in 100 ml of water by sonication and 20 ml of this solution was mixed with 3 ml of a solution of 0.5 M sodium nitrite and 2 ml of 0.14 M perchloric acid to obtain a solution with a concentration of ethylbenzene of 1.04×10^{-3} M and pH = 3.07. Temperature was kept constant at 25 °C (±0.05 C) with a Lauda Ecoline RE120 thermostat and the changes occurring in solution were monitored by UV spectroscopy. After 48 h, a liquid-liquid extraction of 20 ml of aqueous reaction solution with 10 ml of diethyl ether was performed and the organic phase was analysed by gas chromatography – mass spectroscopy in a Shimadzu QP5000 apparatus.

2.3. Nitrosation of phenethylamine

The reaction was followed by using the initial rate method to avoid the decomposition of nitrous acid (Arenas-Valgañón et al., 2014), measuring the absorbance of the nitrous acid/nitrite system at λ = 371 nm (the absorbance of phenethylamine was very weak). To determine reaction orders and rate constants, an excess of phenethylamine was used. The $pK_a = 9.78$ of this compound (Tuckerman, Mayer, & Nachod, 1959) required the use of a buffer solution of potassium hydrogen phthalate (KHP) and perchloric acid (KHP does not interfere with the nitrosation reaction) (Fernández-Liencres, Calle, González-Mancebo, Casado, & Quintero, 1997). Ionic strength was controlled with sodium perchlorate. It should be pointed out that perchloric acid and sodium perchlorate were used because other acids and anions form nitrosyl compounds that catalyse nitrosation reactions, thus they would affect our kinetic studies (Morrison & Turney, 1960).

The kinetic reaction mixtures were prepared by combining a sodium nitrite solution (0.69 M), a phenethylamine solution (0.21 M, very close to saturation), a $NaClO_4/HClO_4$ solution (1.00 M and 0.74 M, respectively) and the KHP solution (0.25 M) in a 50 ml volumetric flask. All kinetic runs were performed in triplicate.

2.4. Nitrosation of tyramine

Nitrosation reactions were monitored by measuring the absorbance of the reaction product (λ = 405 nm). The initial rate method and an excess of nitrite were used to determine the reaction rate constants and partial orders. Since no buffer solution was necessary to control the pH of the solutions, pH was adjusted with perchloric acid. Ionic strength was controlled with sodium perchlorate. Deuterated tyramine was obtained by deuteration of tyramine with deuterium oxide. The kinetic reaction mixtures (KRM) were prepared by combining a tyramine solution (3.0×10^{-2} M), a sodium nitrite solution (0.30 M) and a $NaClO_4/HClO_4$ solution (1.00 M and 0.20 M, respectively) in a 50 ml volumetric flask. To prove the product of reaction, when the reaction was finished, 1.00 M copper (II) sulphate solution was added such that copper was in excess, and the solution was allowed to react for 2 days at room temperature (Masoud, Haggag, Ramadan, & Mahmoud, 1998). All kinetic runs were performed in triplicate.

3. Results and discussion

3.1. General

To characterize the nitrosation mechanisms of tyramine it was first necessary to study the reaction of nitrous acid with two analogous compounds, namely ethylbenzene and phenethylamine (Fig. 1); this would enable us to investigate the different potential processes of nitrosation in the tyramine molecule. Ethylbenzene is the simplest compound and allows the determination of the C-nitrosation rate of its relatively poorly activated aromatic ring. Once this reaction had been characterized, it was possible to study the N-nitrosation of the amine moiety of phenethylamine and hence to investigate the C-nitrosation of the aromatic ring of tyramine, activated by the mesomeric effect of the phenol group.

3.2. Nitrosation of ethylbenzene

Because of the poor activation of the aromatic ring of ethylbenzene for aromatic substitution, its reaction with nitrite was investigated under the most advantageous conditions for aromatic C-nitrosation: a high excess of sodium nitrite and mild acidic conditions. After 48 h no sign of a reaction was observed in the UV spectrum. To confirm the absence of reactions, a gas chromatogram and mass spectrogram of the sample resulting from a diethyl ether extraction of the KRM were obtained and compared with a sample resulting from a diethyl ether extraction of a solution of ethylbenzene at the same concentration (see Materials and methods). In both cases only a peak at 2 min and $m/z = 106$ appeared, such that it may be concluded that the activation of the aromatic ring of ethylbenzene by the ethyl group is insufficient to permit the reaction of this compound with a weak electrophilic compound such as sodium nitrite.

3.3. Nitrosation of phenethylamine

The absence of nitrosation in the aromatic ring of ethylbenzene and the formation of bubbles in the reaction medium (resulting from the decomposition of the primary nitrosamine formed) suggest that, under the experimental conditions used, nitrite only reacts with the amine group of phenethylamine. Study of the dependence of the reaction rate on the concentration of reagents led to the experimental rate Eq. (1), where $[Nit] = [HNO_2]$

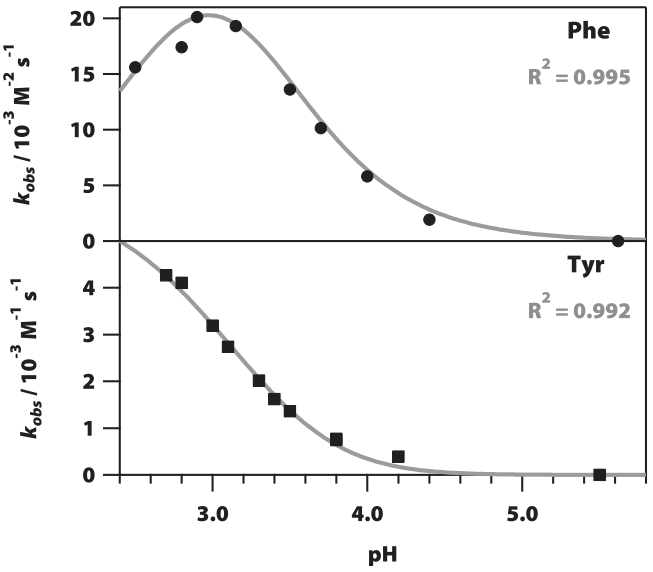


Fig. 2. Top: Influence of pH on the rate constant of the phenethylamine nitrosation reaction. $[PHE]_o = 6.311 \times 10^{-2}$ M, $[NIT]_o = 2.7 \times 10^{-2} - 5.5 \times 10^{-2}$ M, [FTA] $= 5.00 \times 10^{-2}$ M, I = 0.34 M, $T = 20.0$ °C. Bottom: Influence of pH on the rate constant of the tyramine nitrosation reaction. $[TYR]_o = 3 \times 10^{-4} - 3.0 \times 10^{-3}$ M, $[NIT]_o = 3 \times 10^{-3} - 3.0 \times 10^{-2}$ M, I = 0.2 M, $T = 20.0$ °C.

+ $[NO_2^-]$. A strong dependence of k_{obs} on pH was observed (Fig. 2) and no effect of ionic strength was detected within the I_c = 0.37 – 0.67 M range.

$$r_N = k_{obs}[\mathrm{Nit}]^2[\mathrm{Phe}] \quad (1)$$

These results are akin to those observed in the nitrosation of other amines (Arenas-Valgañón et al., 2014; García-Santos et al., 2002), in which the reaction rate was diffusion-controlled and the effective nitrosating agent was dinitrogen trioxide, N_2O_3 (Arenas-Valgañón et al., 2012; Casado, Castro, Leis, López-Quintela, & Mosquera, 1983). Accordingly, an analogous mechanism is proposed here (Scheme 1a), from which the following theoretical rate equation can be deduced:

$$r_N = k_a K_1 K_2 K_3 K_I \frac{[\mathrm{H}^+]^2[\mathrm{Nit}]^2[\mathrm{Phe}]}{([\mathrm{H}^+] + K_I)([\mathrm{H}^+] + K_1)^2} \quad (2)$$

Upon comparing the experimental and theoretical rate equations, respectively (1) and (2), and taking into account that the value of K_I is much smaller than the concentration of protons ($K_I = 1.65 \times 10^{-10}$ M) (Tuckerman et al., 1959), Eq. (3) can easily be obtained, with $\alpha = k_a K_1 K_2 K_3 K_I$ and $\beta = K_1$.

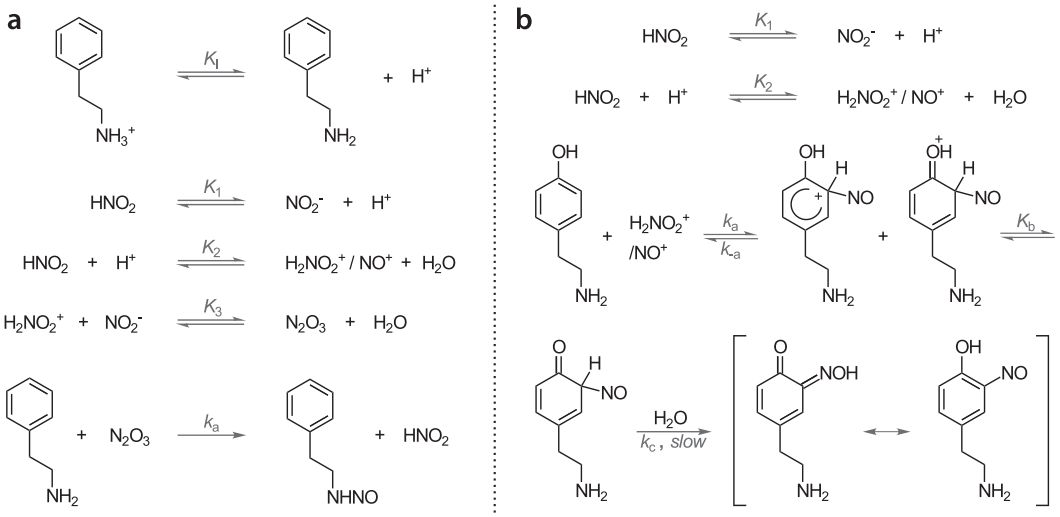
$$k_{obs} = \alpha \frac{[\mathrm{H}^+]}{([\mathrm{H}^+] + \beta)^2} \quad (3)$$

From the least-squares fit, the values $\alpha = (8.6 \pm 0.4) \times 10^{-5}$ $M^{-1}\ s^{-1}$ and $\beta = (1.09 \pm 0.05) \times 10^{-3}$ M were determined. The excellent fit of the experimental data to Eq. (3) and the good agreement of the value of the nitrous acid pK_a deduced from β ($pK_1 = 2.98 \pm 0.05$) with that reported in the literature ($pK_1 = 3.138$) (Tummavouri & Lumme, 1968) support the proposed mechanism. Since $K_I K_2 K_3$ is the Markovits constant ($K_M = (3.03 \pm 0.23) \times 10^{-3}\ M^{-1}$) (Markovits, Schwartz, & Newman, 1981), the value of the rate constant for the nitrosation reaction $k_a = (1.72 \pm 0.08) \times 10^8\ M^{-1}\ s^{-1}$ was determined. The order of magnitude of k_a suggests that the attack of the N_2O_3 on the amine group (Scheme 1a) should be diffusion-controlled (Ridd, 1978).

The activation parameters $\Delta^{\ddagger}H^{\circ} = 56 \pm 4$ kJ mol^{-1} ($E_a = 58 \pm 5$ kJ mol^{-1}) and $\Delta^{\ddagger}S^{\circ} = -104 \pm 16$ J K^{-1} mol^{-1} for the phenethylamine nitrosation reaction were obtained by fitting the values of k_{obs} measured at different temperatures (Fig. 3) to the Eyring equation: (Espenson, 1995)

$$\ln k_{obs} = \ln \frac{k_B T}{h} - \frac{\Delta^{\ddagger}G^{\circ}}{RT} = \ln \frac{k_B T}{h} - \frac{\Delta^{\ddagger}H^{\circ}}{RT} + \frac{\Delta^{\ddagger}S^{\circ}}{R} \quad (4)$$

The observed enthalpy, $\Delta^{\ddagger}H^{\circ}_{obs}$, is the combination of the enthalpy of activation of N_2O_3 attacking the free amine group, $\Delta^{\ddagger}H^{\circ}_a$ (see Scheme 1a), the enthalpy of deprotonation of the phenethylamine amine group, ΔH°_{dp}, and the enthalpy associated with the Markovits constant ΔH_M. Since the value of $\Delta H_M = 5.9 \pm 0.5$ kJ mol^{-1} (Casado et al., 1983), and considering that $\Delta H^{\circ}_{dp} = 41.5 \pm 0.1$ kJ mol^{-1} (ΔH°_{dp} was not determined for phenethylamine, so the value corresponding to the deprotonation of the amine group in the analogous molecule L-phenyl alanine (Hamborg, Niederer, & Versteeg, 2007) was used), it may be deduced that $\Delta^{\ddagger}H^{\circ}_a \approx 8.6$ kJ mol^{-1}. This enthalpy lies within the generally permitted range for diffusion-controlled processes (Challis & Ridd, 1962; Ridd, 1978) and also supports the proposed mechanism for the phenethylamine nitrosation reaction.



Scheme 1. Mechanisms of (a) N-nitrosation of phenethylamine and (b) C-nitrosation of tyramine.

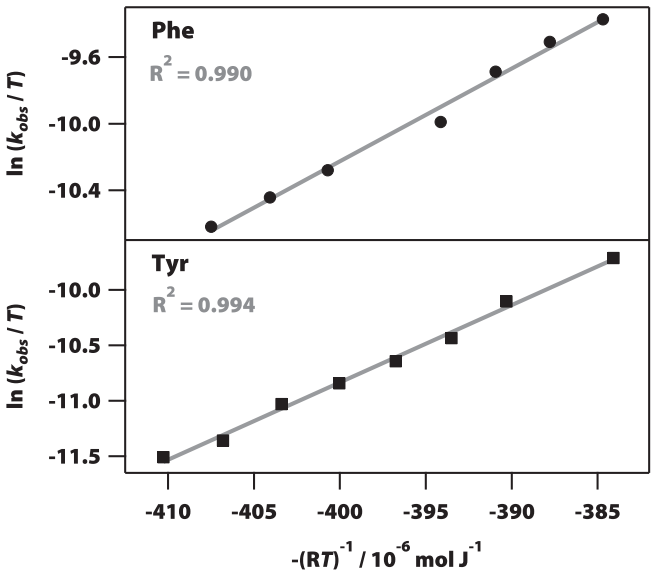


Fig. 3. Eyring plot for the determination of the activation parameters for the nitrosation reactions of phenethylamine (Top, $[PHE]_o = 6.31 \times 10^{-2}$ M, $[NIT]_o = 2.7 \times 10^{-2} - 5.5 \times 10^{-2}$ M, [FTA] = 5.00×10^{-2} M, pH = 3.80, I = 0,34 M) and tyramine (Bottom, $[TYR]_o = 3 \times 10^{-4} - 3.0 \times 10^{-3}$ M, $[NIT]_o = 3 \times 10^{-3} - 3.0 \times 10^{-2}$ M, pH = 3.1).

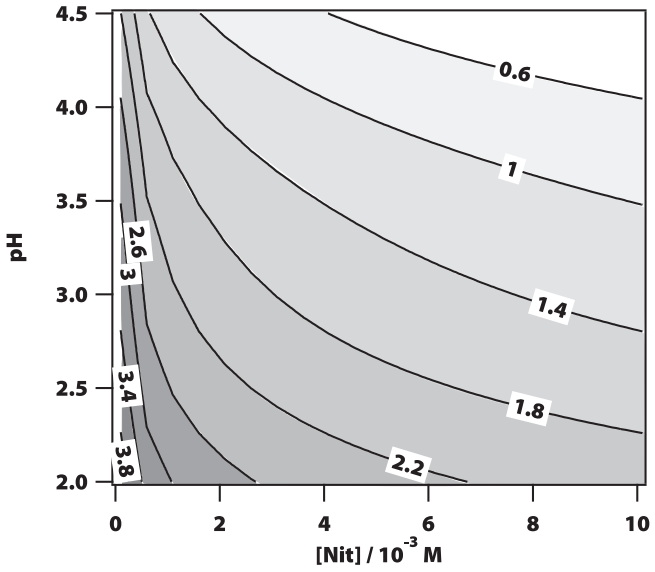


Fig. 4. Influence of the pH of the medium of nitrosation reactions and nitrite concentration on the r_C/r_N ratio (Eq. (9)). Isolines correspond to different values of the z parameter.

3.4. Nitrosation of tyramine

In its structure the tyramine molecule has a phenol group that drives the electrophilic reaction to the ortho and para positions. Since the para position is occupied by the aminoethyl group, nitrosation of the aromatic ring only can occur in one of the two equivalent ortho positions. The tyramine C-nitrosation reaction was monitored by following the yellow colour that appears over time. This reaction is much faster than that of the N-nitrosation of the amine, assuming that its rate is at least as fast as the N-nitrosation of phenethylamine. The absence of bubbles in the KRM during the experiments supports this assumption. Using the initial rate method, the following experimental rate equation for the nitrosation of tyramine was obtained:

$$r_C = k_{obs}[\mathrm{Nit}][\mathrm{Tyr}] \tag{5}$$

The first-order in nitrite suggest that the effective nitrosating agents are nitrosonium (NO^+) or nitrosacidium ($H_2NO_2^+$) ions, which are kinetically indistinguishable (Challis & Lawson, 1971). There was no effect of the ionic strength on the reaction rate in the $I_c = 0.02 - 0.26$ M range, and the influence of pH was appreciable (Fig. 2).

In light of these results, a mechanism of aromatic electrophilic substitution by $H_2NO_2^+/NO^+$ in the ortho position of tyramine, whose rate-determining step is the deprotonation of the Wheland intermediate, can be proposed (Scheme 1b). From this mechanism, the following rate equation is readily achieved:

$$r_C = \frac{K_2 k_a [\mathrm{Nit}][\mathrm{Tyr}][\mathrm{H}^+]^2}{([\mathrm{H}^+] + K_1)\left(1 + \frac{k_{-a}}{K_b k_c}[\mathrm{H}^+]\right)} \tag{6}$$

The experimental data shown in Fig. 2 were fitted to Eq. (7), obtained from a comparison of experimental Eq. (5) and theoretical Eq. (6) rate equations.

$$k_{obs} = \frac{\alpha[\mathrm{H}^+]^2}{([\mathrm{H}^+] + K_1)(1 + \beta[\mathrm{H}^+])} \tag{7}$$

where $\alpha = K_2 k_a$ and $\beta = k_{-a}/K_b k_c$. Using the value of K_1 measured at 25 °C by Tummavuori and Lumme ($K_1 = 6.652 \times 10^{-4}$ M) (Tummavouri & Lumme, 1968), the parameters $\alpha = 47 \pm 9\ \mathrm{M}^{-2}\ \mathrm{s}^{-1}$ and $\beta = 7800 \pm 700\ \mathrm{M}^{-1}$ were obtained. Since the value of K_2 was known ($K_2 = 3 \times 10^{-7}\ \mathrm{M}^{-1}$) (Turney & Wright, 1958), a value for $k_a = (1.6 \pm 0.3) \times 10^8\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$ was obtained. This value is consistent with those obtained for other *C*-nitrosation reactions (González-Jiménez, Arenas-Valgañón, Calle, & Casado, 2011).

Because the rate-determining step in the proposed mechanism is a C—H proton transfer in the Wheland intermediate (k_c in Scheme 1b), the replacement of that hydrogen by a deuterium atom should show a primary kinetic isotope effect (KIE) $k_c^{H_2O}/k_c^{D_2O} > 1$ (Connors, 1990), as has been observed previously for the nitrosation of several aromatic and heteroaromatic substrates (Challis & Higgins, 1973; Dix & Moodie, 1986; González-Jiménez et al., 2011; González-Mancebo, García-Santos, Hernández-Benito, Calle, & Casado, 1999).

To check the existence of a primary KIE, k_{obs} has been measured in water and deuterated water at pH = 2.1, obtaining $k_{obs}^{H_2O}/k_{obs}^{D_2O} = 1.07$. At that pH, Eq. (7) leads to the expression:

$$\frac{k_{obs}^{H_2O}}{k_{obs}^{D_2O}} = \frac{K_2^{H_2O}}{K_2^{D_2O}} \frac{k_c^{H_2O}}{k_c^{D_2O}} \tag{8}$$

Because $K_2^{D_2O}/K_2^{H_2O} = 2.7$(Casado et al., 1983), the primary KIE for the nitrosation of tyramine is $k_c^{H_2O}/k_c^{D_2O} \simeq 3$. This value, which is analogous to those found for the nitrosation of different phenols (González-Jiménez et al., 2011), confirms that a C—H proton transfer is involved in the slow kinetic step.

Since the existence of an isokinetic relationship can be used to support the argument that the reactions of a series of reagents share a common mechanism (Exner, 1988; Leffler & Grunwald, 1989; Senent, 1986), this possibility was tested in order to gain further evidence in support of the proposed mechanism. The activation parameters of the reaction were determined, using the Eyring equation, measuring the k_{obs} values at different temperatures (Fig. 3). With the $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values obtained here for the nitrosation of tyramine ($\Delta H^{\ddagger} = 70 \pm 2\ \mathrm{kJ\ mol^{-1}}$ and $\Delta S^{\ddagger} = -54 \pm 8\ \mathrm{J\ K^{-1}\ mol^{-1}}$) and those previously determined for a series of C-nitrosation reactions occurring through electrophilic attack on the nitrosatable substrates by $H_2NO_2^+/NO^+$, the plot of $\Delta H^{\ddagger}/\Delta S^{\ddagger}$ was drawn (Fig. 5). The results are consistent with the existence of an isokinetic relationship.

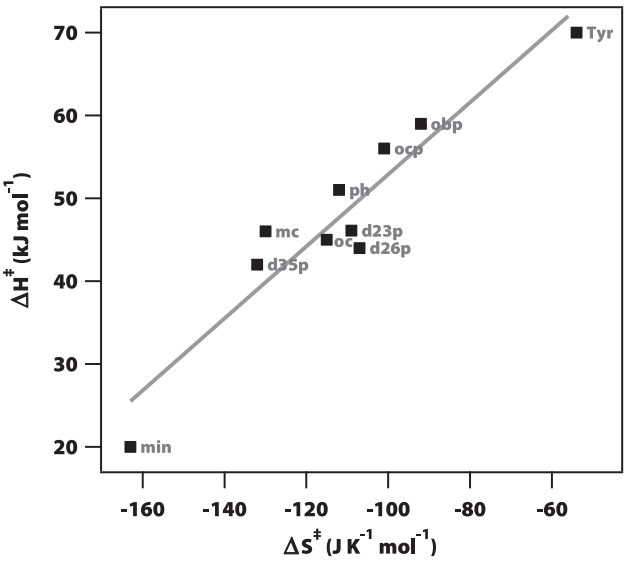


Fig. 5. $\Delta H^{\ddagger}/\Delta S^{\ddagger}$ isokinetic relationship for the C-nitrosation reactions of tyramine (Tyr) and other nitrosatable substrates: phenol (ph), m-cresol (mc), o-cresol (oc), 2,3-dimethylphenol (d23p), 2,6-dimethylphenol (d26p), 3,5-dimethylphenol (d35p), o-chlorophenol (ocp), o-bromophenol (obp) and minoxidil (min) (González-Jiménez et al., 2011; González-Mancebo et al., 1999).

The nitrosation of tyramine was also analysed by mass spectroscopy to confirm the proposed reaction product. After the reaction had finished, the mass spectrum displayed a peak at a mass/charge ratio of $m/z = 165.9$, corresponding to the nitrosated tyramine. To check nitrosation in the ortho position with respect to the phenol group, a complexation reaction with copper was used (Masoud, Haggag, Ramadan, & Mahmoud, 1998), leading to the appearance of a brown colour that corresponded to the copper complex.

3.5. Comparison of C- and N-nitrosation rates

Once the reaction rates r_N (Phe) and r_C (Tyr) for N- and C-nitrosation have been determined (Eqs. (2) and (6), respectively), their values can be compared in order to know the conditions (pH, nitrite concentration) in which either C- or N-nitrosation is prevalent. To accomplish this, and because the position of the amine group in both nitrosatable substrates allows one to assume that r_N (Phe) $\approx r_N$ (Tyr), the r_C/r_N ratio can be estimated. Fig. 4 shows a contour plot representing the values of this ratio as a function of pH and nitrite concentrations. For the purposes of clarity the common logarithm of this ratio (Eq. (9), resulting from Eqs. (2) and (6)) is plotted.

$$z = \log_{10}\frac{r_C}{r_N} \tag{9}$$

Fig. 4 shows the ranges in which either C- or N-nitrosation was prevalent. As can be seen, at pH > 4 and with significant concentrations of nitrite the N-nitrosation of tyramine is more important. These conditions are quite unlike those found in the lumen of the stomach where low concentrations of nitrite and high acidity favour the mutagenic products of C-nitrosation at the expense of innocuous N-nitrosation products.

4. Conclusions

1. No aromatic reaction is observed between sodium nitrite and ethylbenzene, after 48 h in the most advantageous conditions for aromatic C-nitrosation of this compound: a strong excess of sodium nitrite and mild acidic conditions.
2. The N-Nitrosation of phenethylamine occurs through a diffusion-controlled mechanism in which dinitrogen trioxide is the effective nitrosating agent.
3. The aromatic C-Nitrosation of tyramine occurs through a mechanism in which $H_2NO_2^+/NO^+$ are the effective nitrosating agents and its rate-determining step is the deprotonation of the Wheland intermediate.
4. In the chemical conditions of the lumen of the stomach, the most favoured nitrosation reaction of tyramine is C-nitrosation, which generates mutagenic products.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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