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Mutagenic products are promoted in the nitrosation of tyramine

Mario González-Jiménez [1], Jorge Arenas-Valgañón, María del Pilar García-Santos, Emilio Calle, Julio Casado *

Departamento de Química Física, Universidad de Salamanca, Plaza de los Caídos, 1-5, E-37008 Salamanca, Spain

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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.

* Corresponding author.
E-mail address: jucali@usal.es (J. Casado).
[1] Present address: School of Chemistry, University of Glasgow, G12 8QQ Glasgow, UK.

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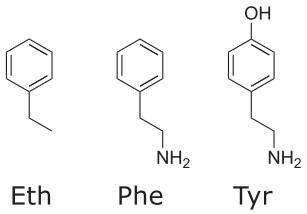

Fig. 1. Compounds studied in this work.

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 $[Nit] = [HNO_2]$