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Nitration of Catecholamines with Nitrogen Oxides in Mild Conditions: *a Hypothesis for the Reactivity of NO in Physiological Systems*

Marie-Laure de la Bretèche, Claudine Servy, Maryse Lenfant and Claire Ducrocq*

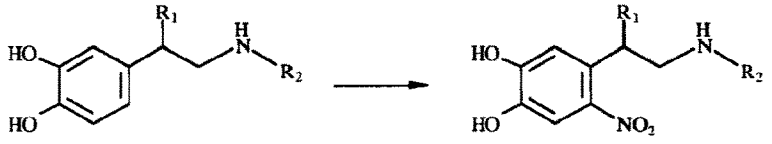
Institut de Chimie des Substances Naturelles, avenue de la Terrasse, C.N.R.S. F-91198 Gif-sur-Yvette (France)

Abstract: Dopamine, norepinephrine and epinephrine react at room temperature, in acetate buffer (3<pH<6) with sodium nitrite or in non-deaerated phosphate buffer (pH 7.4) with NO. The corresponding 6-nitro derivatives are formed.

Numerous studies have shown that nitration of phenols occurs at room temperature by treatment with nitrate in strongly acidic conditions [1]. Catalytic amounts of nitrous acid which generates highly reactive NO_2^+ / NO^+ entities have been shown to accelerate this process [2,3]. Various mechanisms have been proposed: i) electrophilic attack by NO^+ [4]; ii) radical reaction implicating phenoxy radicals, NO and/or NO_2 [5]. However the functionalisation of phenols in mild conditions has been reported i. e formation of ortho-nitrotyrosine from tyrosine and nitrous acid at pH 2-4 [6].

We have evaluated the possibility that this type of reaction might be applied to the nitration of physiological polyfunctional compounds presenting phenolic functions. We report here that, in the presence of nitrite and in mild conditions (pH 4-5), norepinephrine **1a**, epinephrine **1b** and dopamine **1c**, are quantitatively converted into their 6-nitro derivatives (**2a, 2b, 2c**). Identical products are obtained when these hormones are treated with NO in non deaerated buffer at pH 7.4.

Reactivity of Catecholamines with Sodium Nitrite: When **1a, 1b** or **1c** were treated at pH 7.4 with sodium nitrite [7], no reaction was observed by HPLC analysis [8] even after a 7-hour incubation period. Conversely, the nitration of catecholamines occured at a pH lower than pH 6. The kinetics of the transformation were pH-dependent (results not shown) and for instance a quantitative nitration of **1b** was obtained in 2 min at pH 4. HPLC purified derivatives obtained from **1a, 1b** and **1c** were caracterized by NMR [9] and mass spectroscopy techniques [10]. Data obtained were in agreement with the structure of the corresponding 6-nitro-catecholamines **2a, 2b, 2c**:



1a R_1 = OH, R_2 = H norepinephrine
1b R_1 = OH, R_2 = CH_3 epinephrine
1c R_1 = H, R_2 = H dopamine

2a R_1 = OH, R_2 = H
2b R_1 = OH, R_2 = CH_3
2c R_1 = H, R_2 = H

Reactivity of Catecholamines with Nitric Oxide in a non-deaerated Buffer at pH 7.4: NO [11] was bubbled for 10 to 15 min into a non-deaerated solution of catecholamines at pH 7.4 at room temperature. In all cases, HPLC analysis indicated a quantitative conversion into the 6-nitro-derivatives **2a,**

2b, 2c. These structures were confirmed by Electrospray MS analysis [10] presenting respectively molecular ion peaks MH^+ at 215, 229 and 199. In all cases, no reaction was detected in absence of oxygen.

NO has been shown to be an important mediator of physiological processes [12], but few data are actually available concerning its reactivity. The reversible formation of phenoxy radicals have been detected by EPR when NO reacted with hindered phenols [13]. However in presence of O_2 in aqueous solution, NO led exclusively to the formation of nitrite [14,15]. This reaction was dependent on NO concentration according to a second-order rate kinetics and the generation of an electrophilic N_2O_4 entity, which split rapidly into NO_2^- ion was postulated. In our conditions, this intermediate should react preferentially upon the C-6 nucleophilic center of the catecholamines to give the respective C-6 nitro derivatives.

The possibility that such a reaction might occur *in vivo* is appealing. Indeed, among the potent lung vasoconstrictor circulating hormones, catecholamines exhibit in physiological fluids, a life-span similar to that reported for NO. Furthermore, NO and catecholamines exert rapid and totally reversible effects. In fact, it has been observed during the treatment of respiratory distress syndrome that NO introduced directly into the patients' lungs, reduces pulmonary vasoconstriction [16]. In this particular therapeutic use, both norepinephrine, epinephrine and NO are present in oxygenated blood at pH 7.4. In this case, it might be postulated that the pharmacological effects of NO could be mediated by an inactivation of the endogenous vasoconstrictors through a direct reaction. This hypothesis is supported by the observation of decrease of norepinephrine concentration in the pig pulmonary blood after NO inhalation [17]. The biological significance of such a reactivity of NO with hormones presenting phenolic function is currently under study.

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7. **Reactivity of Catecholamines with Sodium Nitrite:** Catecholamine (20 mM) and sodium nitrite (100 mM) in 0.2M acetate (pH 3 to 6) or 0.1M phosphate (pH 7.4) buffers were stirred at room temperature, for periods of time varying from few seconds to 7 hours. Aliquots were analysed by HPLC.
8. **HPLC System:** aliquots of incubation medium were analysed in a Waters gradient H P L C system (WISP injector 712, UV detector 484, integrator Epson EL plus) using a 250 x 4.6 mm Hypersil *Sup Rs Classic* column. Elution was performed using a gradient consisting of 0,05% TFA in water (solvent A) and 0,05% TFA in 20% acetonitrile (solvent B) at a flow rate of 1 ml/min. The elution profile included an isocratic elution with 100% solvent A for 15 min and a linear gradient from 100% solvent A to 100% solvent B over 10 min. Products were detected by UV absorbance at 280 nm.
9. **^{1}H-NMR** (DMSO-d_6, 300MHz) spectra obtained on a Brucker apparatus exhibited respectively peaks at 2.86 (1H, dd, J_1=9Hz, J_2=2Hz), 3.35 (1H, dd, J_1 = 3Hz, J_2=12Hz), 4.47 (1H, dd, J_1=3Hz, J_2=9Hz), 7.30 (1H, s), 7.58 (1H, s) for **2a**; 2.72 (3H, s), 3.02 (1H, t, J=6Hz), 3.32 (1H, t, J=6Hz), 5.55 (1H, d, J=6Hz), 7.34 (1H, s), 7.65 (1H, s) for **2b** and 2.71 (2H, t, J=6Hz), 3.76 (2H, t, J=6Hz), 6.75 (1H, s), 7.64 (1H, s) for **2c**.
10. **Molecular Mass Determination** was performed on a VG Platform mass spectrometer using an electrospray ion source and a quadrupole mass analyzer with an upper mass limit of m/z = 3000 (VG Biotech, Manchester, UK). Ten-microliter samples were introduced into the source at a flow rate of 5ml/min (carrier solvent : 50% acetonitrile, 49% water, 1% formic acid); 4000 V were applied on the capillary and 450 V on the counter electrode. The sampling cone voltage was adjusted to obtain the best sensitivity and the smallest number of fragmentations. The quadrupole was calibrated using a mixture of polyethylene glycol 300 and 600. Experiments were carried out by J.-P. Le Caer (Institut Alfred Fessard, Gif-sur-Yvette-France).
11. The NO gas was provided either by Setic Labo (Magny-les-Hameaux, France), or formed *in situ* by reacting sulfuric acid with sodium nitrite in a vessel previously and vigorously flushed with argon; the gas was passed through soda pellets and bubbled in the phosphate buffer (1M, pH 7.4)
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