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Is the isonicotinoyl radical generated during activation of isoniazid by Mn^{III}-pyrophosphate?

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This article is dedicated to the memory of John Osborn.

Abstract – The antituberculosis drug isoniazid (INH) is quickly oxidised by stoichiometric amounts of manganese(III)-pyrophosphate and the following products were identified: isonicotinic acid **1**, isonicotinamide **2** and isonicotinaldehyde **3**, the acid being the major product. The oxidation of INH with Mn^{III}-pyrophosphate was carried out in either $H_2{}^{16}O$, or $H_2{}^{18}O$ or D_2O and under varied atmosphere composition (argon, air, O_2 or $^{18}O_2$). LC–MS analyses of isotope incorporation suggest the simultaneous presence of two competitive pathways leading to the formation of acid **1**, with the isonicotinoyl radical as a common intermediate. One route is oxygen-dependent and the other is water-dependent. Analyses of isotope incorporation in amide **2** and aldehyde **3** also support this mechanism. ***To cite this article: M. Nguyen et al., C. R. Chimie 5 (2002) 325–330***

tuberculosis / isoniazid / oxidative activation / Mn^{III}-pyrophosphate / isonicotinoyl radical

Résumé – Le radical isonicotinoyle, intermédiaire réactif formé lors de l'activation de l'isoniazide par le pyrophosphate de manganèse(III) ? Utilisé en quantité stœchiométrique, le pyrophosphate de manganèse(III) oxyde très efficacement l'isoniazide (INH), un antibiotique antituberculeux, avec formation d'acide isonicotinique **1**, d'isonicotinamide **2** et d'isonicotinaldéhyde **3**, l'acide étant le produit majoritaire. L'oxydation de l'INH par le pyrophosphate de manganèse(III) a été effectuée à la fois dans $H_2{}^{16}O$, $H_2{}^{18}O$ ou D_2O, sous différentes compositions d'atmosphère (argon, air, O_2 ou $^{18}O_2$). Les expériences isotopiques réalisées en présence de $H_2{}^{18}O$ et/ou $^{18}O_2$ ont montré l'existence de deux voies compétitives, oxygéno-dépendante et aqua-dépendante, conduisant à l'acide **1**, ces deux voies comportant un intermédiaire commun, le radical isonicotinoyle. L'une de ces voies est oxygéno-dépendante, tandis que l'autre est aqua-dépendante. Les résultats d'incorporation isotopique observés pour l'amide **2** et l'aldéhyde **3** sont en accord avec ce schéma réactionnel. ***Pour citer cet article : M. Nguyen et al., C. R. Chimie 5 (2002) 325–330***

tuberculose / isoniazide / oxydation / Mn^{III}-pyrophosphate / radical isonicotinoyl

1. Results and discussion

1.1. Generalities

Isoniazid (INH), an antibiotic used in the treatment of tuberculosis [1, 2], is considered as a prodrug requiring activation by the *Mycobacterium tuberculosis* KatG enzyme [3, 4]. This catalase–peroxidase hemoprotein [4–7] catalyses the oxidation of INH but, despite recent efforts, the exact nature of the activated intermediate and the activation mechanism of this drug are still a matter of debate. Since none of the stable derivatives observed in KatG-dependent conversion of INH i.e. isonicotinic acid **1**, isonicotinamide **2** and isonicotinaldehyde **3** (Scheme 1) have demonstrated bactericidal effect [8], the mechanism proposed for INH activity supposes the reaction of a reactive intermediate species with the β-nicotinamide adenine dinucleotide (NAD^+/NADH), which is the cofactor of

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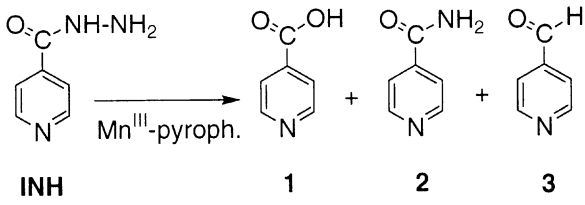


Scheme 1.

the long-chain 2-trans-enoyl-acyl carrier protein reductase InhA [9, 10], a key enzyme involved in the biosynthesis of mycolic acids, specific components of the mycobacterial cell wall [11, 12]. The formation of covalent adduct(s) INH-NAD(H) as competitive inhibitor(s) might explain the loss of InhA activity [13].

Recently, we have shown that a stoichiometric amount of Mn^{III}-pyrophosphate is able to replace the catalysis by the KatG protein in the initial activation of INH and, in the presence of NAD^+, to allow the formation of INH-NAD(H) adducts, which are effective inhibitors of the InhA protein [14, 15]. The present work, based on isotopic studies involving D_2O, $H_2{}^{18}O$ or $^{18}O_2$ and analyses of INH oxidation products, indicates the simultaneous presence of two reaction pathways, leading to the formation of the isonicotinic acid, with the isonicotinoyl radical as a common intermediate. The isotope content analyses of the amide **2** and the aldehyde **3** are also supporting this mechanism.

Since isonicotinic acid **1** and isonicotinamide **2** do not exchange their carbonyl oxygen with water (Table 1, runs 1 and 2), we could determine the origin of the oxygen atoms in these products and obtain information on their mode of formation. Isonicotinaldehyde **3** exchanged rapidly its carbonyl oxygen atom with water and so is not a convenient reporter for ^{18}O mechanistic study. However, in this case, some data were obtained from reaction performed in D_2O. In addition, we checked that neither amide **2** nor aldehyde **3** was transformed in acid **1** by Mn^{III}-pyrophosphate in the experimental conditions described.

1.2. Formation of isonicotinic acid 1

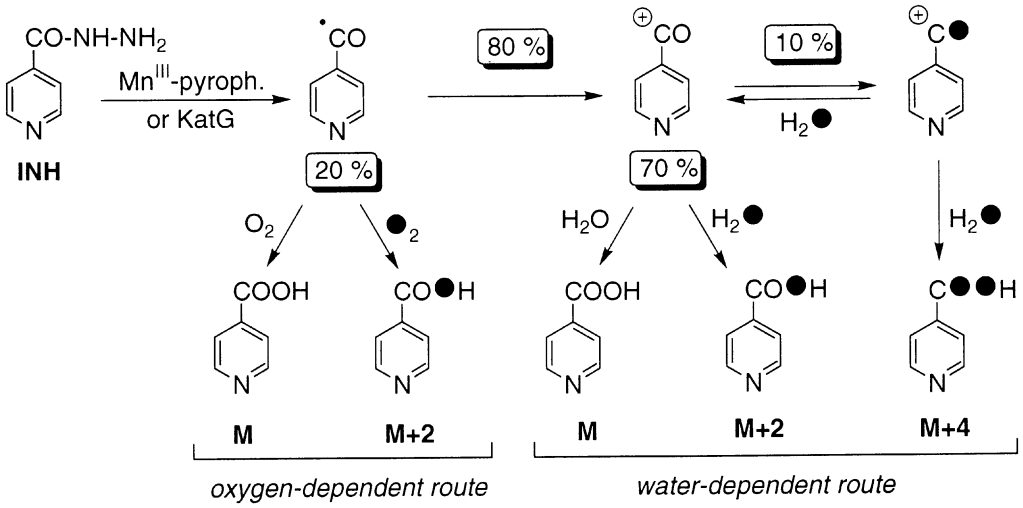
As shown in Table 1, the incorporation rate of ^{18}O in isonicotinic acid **1** to give M+2 and M+4 species during oxidation of INH by Mn^{III}-pyrophosphate (this work, runs 5 and 8) or by KatG (from ref. 2, runs 3 and 7) is rather similar, suggesting a similar formation pathway with probable common reactive intermediate(s). The level of incorporation of ^{18}O label in **1** differs, in a complementary mode, depending on the use of labelled water (run 5, 81% of labelling) or labelled dioxygen (run 8, 20% of labelling). These results support the existence of two independent routes, the major one (80%) corresponding to the incorporation of oxygen from water and the second one (20%) resulting from the incorporation of oxygen from molecular dioxygen (Scheme 2). In addition:

– (*i*) in the oxygen-dependent route, the partial incorporation of a maximum of one oxygen atom from dioxygen in compound **1** suggests the formation of an intermediate species, like the isonicotinoyl radical, able to trap dioxygen and giving the acid without exchanging the carbonyl oxygen initially present in INH; this route might involve the formation of a peroxydic radical and its further conversion to **1** catalysed by manganese salts (Scheme 3);

– (*ii*) in experiments with $H_2{}^{18}O$, when the partial pressure of dioxygen increases (from about zero, to 0.2 atm and 1 atm, runs 4, 5 and 6, respectively), a concomitant decrease of ^{18}O-incorporation from water (91, 81 and 65%, respectively) was observed – these data suggest that a common intermediate is at the origin of the oxygen- and water-dependent pathways; the difficulty in performing the reaction under an

Table 1. Incorporation of ^{18}O (●) in isonicotinic acid **1** and isonicotinamide **2** during oxidation of INH by Mn^{III}-pyrophosphate (this work) or KatG [2] (runs 3 and 7) in the presence of H_2● or ●$_2$. INH does not exchange its oxygen with water. *The pressure was adjusted to 1 atm with nitrogen. The isotopic M+1 contribution (less than 5%) has been included in the M peak.

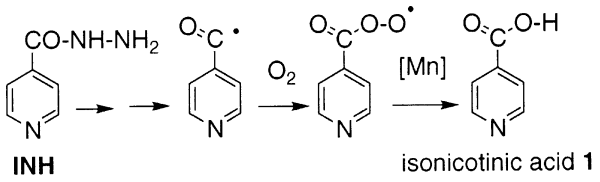
Run	Conditions	Oxidation system	Isonicotinic acid **1**			Isonicotinamide **2**	
			M CO	M + 2 C●	M + 4 ●–C=●	M CO	M + 2 C●
1	H_2O/air	—	100%	—	—	100%	—
2	H_2●/air	—	100%	—	—	100%	—
3	H_2●/air	KatG	33%	50%	17%		—
4	H_2●/Ar (1 atm)	Mn^{III}pyro.	9%	69%	22%	100%	—
5	H_2●/air	Mn^{III}pyro.	19%	72%	9%	97%	3%
6	H_2●/O_2 (1 atm)	Mn^{III}pyro.	35%	55%	10%	97%	3%
7	H_2O/●$_2$ (1 atm)	KatG	65%	35%	—		—
8	H_2O/●$_2$ (0,2 atm)*	Mn^{III}pyro.	80%	20%	—	100%	—



Scheme 2.

atmosphere completely deprived of dioxygen is illustrated by the 9% value of unlabelled acid **1** observed under argon atmosphere –;

– (*iii*) to explain the observed partial double labelling of **1** in experiments performed in $H_2{}^{18}O$ (runs 3–6, Table 1), we must suppose the existence of a reaction intermediate allowing the exchange of the carbonyl oxygen atom initially present in INH; this intermediate could be the isonicotinoyl cation generated by oxidation of the isonicotinoyl radical (Scheme 2); the nucleophilic attack of water on this carbocation (Scheme 4) should give the corresponding hydrate which can either loose one proton to give the isonicotinic acid with a single label (pathway 1) or, alternatively, release one water molecule to come back to the carbocation with partial incorporation of a labelled oxygen (pathway 2); finally, the incorporation of a labelled oxygen on this partially labelled cation through addition of another labelled water molecule leads to compound **1** with a partial double ^{18}O labelling; in the usual experimental conditions (run 5), the level of exchange with water of the initial oxygen atom present in INH could be estimated to 10%, based on the amount of detected double-labelled acid **1**.



Scheme 3.

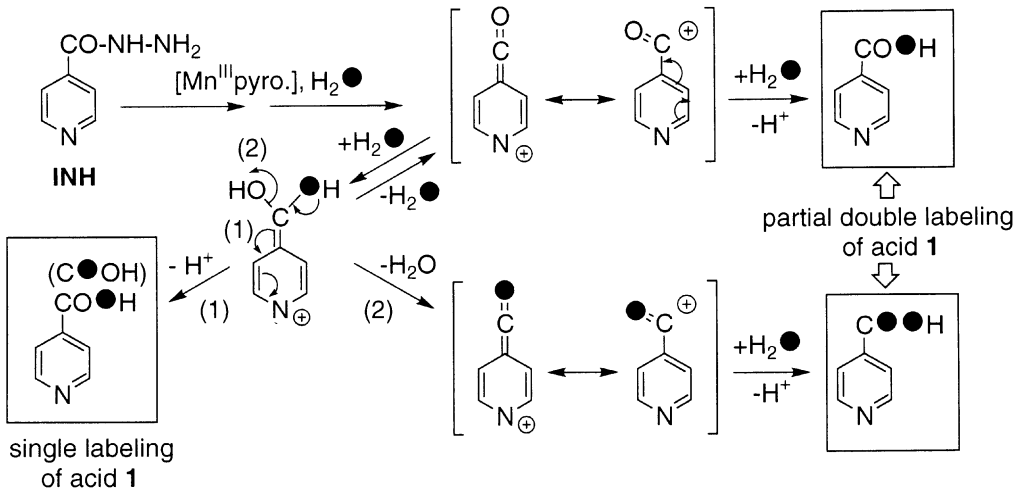
An overall representation of these different ways for generating acid **1** is shown in Scheme 2. Literature data [3] (runs 3 and 7 in Table 1) suggest that the oxygen-dependent way is slightly more important when the reaction is catalysed by KatG. In addition, both the isonicotinoyl radical and the peroxidic radical could be detected in EPR spin-trapping experiments performed during KatG-mediated oxidation of INH [16].

1.3. Formation of isonicotinamide 2

Isonicotinamide **2** did not incorporate ^{18}O atom in the presence of $H_2{}^{18}O$ or $^{18}O_2$ (Table 1, runs 2, 4–6, 8). The detection of small levels of 1,2-diisonicotinoylhydrazine in the reaction medium supports a formation pathway with a cleavage of the nitrogen–nitrogen bond, as displayed in Scheme 5 (route **a**; splitting of the N–N bond to generate amide **2** was also suggested by Schultz [3]) but does not exclude the route proposed by Bodiguel et al., with an initial carbon–nitrogen bond cleavage (route **b**) [17].

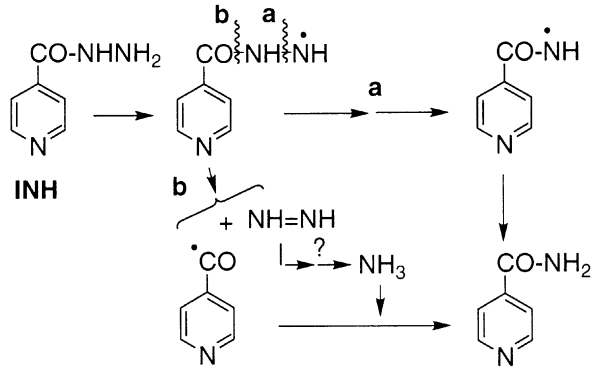
1.4. Experiments in D_2O

When the reaction was performed in D_2O, neither acid **1** nor amide **2** incorporated deuterium. On the opposite, the aldehyde **3** (detected as the *O*-carboxymethyloxime derivative, since the free aldehyde could not be directly analysed) showed 100% incorporation of deuterium (Table 2, runs 1 and 2). This observation supports the formation of **3** through a radical intermediate, the isonicotinoyl radical, able to abstract $D^{\bullet}$ from D_2O or more likely from deuterated INH (fast exchange of the mobile protons of the hydrazide moiety of INH with D_2O) (Scheme 6).



Scheme 4.

In conclusion, this isotope labelling study provides evidence that the INH oxidation by Mn^{III}-pyrophosphate produces isonicotinic acid **1** through two competitive ways: an oxygen-dependent route and a water-dependent one, both pathways involving the isonicotinoyl radical as a common intermediate. This conclusion is also in agreement with the formation of isonicotinaldehyde **3** through abstraction of an $H^{\bullet}$ from INH by this radical. Since the chemical INH activating system Mn^{III}-pyrophosphate was shown to be a good mimick of the KatG catalase-peroxidase, which activates INH in *M. tuberculosis*, the isonicotinoyl radical appears likely to be the active species responsible for the activity of INH, and so it should be involved in the formation of the INH-NAD(H) adducts acting as strong inhibitors of key enzymes in the biosynthesis of mycolic acids of bacteria.



Scheme 5.

2. Experimental section

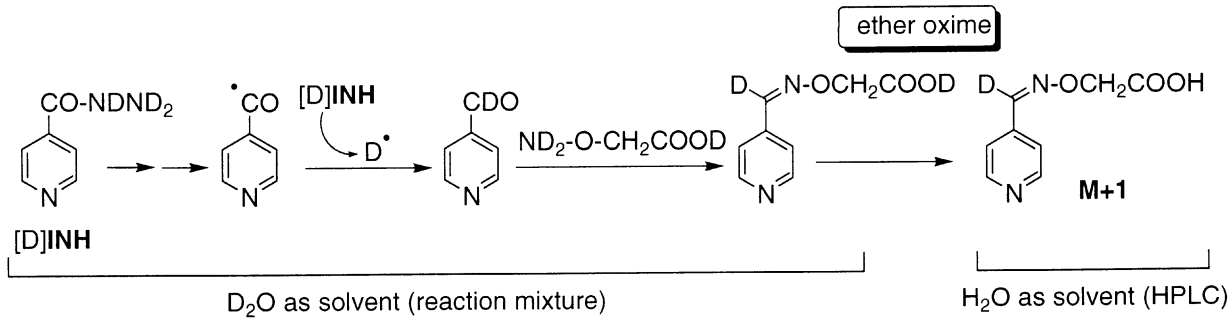
2.1. Analytical methods

2.1.1. HPLC analyses

Analyses were performed on a reverse-phase C18 column (nucleosil, 10 µm, 250 × 4.6 mm) using a 5:95 (v/v) methanol/NH_4OAc 5 mM, pH 4.5 solution as eluent (flow rate: 1 ml min^{-1}). The column was coupled to a diode array detector (Kontron) for the detection of products at 260 nm and the monitoring of UV-Vis spectra. Yields were calculated for INH and compounds **1**–**3** by comparison with authentic sample calibration curves (in order to detect **3** as its *O*-carboxymethyl oxime derivative, some reaction samples were analysed after 5 min quenching with 40 mM NH_2OCH_2COOH).

Table 2. Incorporation of deuterium in compounds **1**, **2** and **3** during oxidation of INH by Mn^{III}-pyrophosphate with D_2O as solvent. *Detected as ether oxime derivative during LC–MS (*Scheme 6*). All labile deuterium atoms incorporated during the reaction were exchanged for protons during the LC analysis.

Run	Conditions	Isonicotinic acid **1**		Isonicotinamide **2**		Isonicotinaldéhyde **3***		
		M	M+1	M	M+1	M	M+1	M+2
1	D_2O/air	95%	5%	93%	7%	—	96%	4%
2	D_2O/N_2	95%	5%	95%	5%	—	94%	6%



Scheme 6.

2.1.2. LC–TIS–MS analyses

The analyses were performed in the conditions indicated above, but with a quaternary pump. Only 50% (split 1/2) of the flow eluted from the column was introduced into the electrospray turbo ionisation source (TIS). Nitrogen at 480 °C (ultrahigh purity) was used as spray and drying gas. The ESI–MS spectrometer was a Perkin-Elmer SCIEX API 365 and the analyses were performed in the positive mode.

2.2. Materials

Manganese(III)-pyrophosphate, $Mn^{III}(H_2P_2O_7)_3]Na_3$, was prepared according to previously described method [14, 18]. Isoniazid, isonicotinic acid, isonicotinamide, and isonicotinaldehyde were obtained from Sigma-Aldrich. $H_2{}^{18}O$ (97.1 atom%) was supplied by Eurisotop, D_2O (> 99.9 atom%) by SDS (Peypin, France) and $^{18}O_2$ (96.8 atom%) by Leman (Saint-Quentin-en-Yvelines, France).

2.3. Oxidation conditions

The reaction medium (final volume of 1 ml of water for classical HPLC analysis or 100 µl for LC–TIS–MS analysis), containing 50 mM phosphate buffer pH 7.5, 0.5 mM INH and 1 mM Mn^{III}-pyrophosphate (introduced in five consecutive additions of 200 µM each, every 2 min) was stirred at room temperature for 10 min and then analysed by HPLC or LC–TIS–MS after 5 min quenching with 40 mM NH_2OCH_2COOH (200 µM 2-nitrobenzoic acid was used as internal standard only for HPLC analysis). In a typical reaction, the yields of compounds 1, 2 and 3 were 45, 12 and 4% , respectively.

2.4. Experiments with $H_2{}^{18}O$

Solutions of required concentrations and volume of isoniazid (0.5 mM) plus phosphate buffer (50 mM, pH 7.5), Mn^{III}-pyrophosphate (1 mM) and NH_2OCH_2COOH (40 mM) were prepared separately and lyophilised using a Speed-Vac. Appropriate volumes of $H_2{}^{18}O$ were added (50 µl for isoniazid in buffer sample, 40 µl for Mn^{III}-pyrophosphate sample and 10 µl for NH_2OCH_2COOH sample; the total volume was 100 µl) and the reaction was carried out as described above. In case of reaction under dioxygen or argon, the reaction medium was placed in a Schlenk tube connected to a pressure detector and degassed by three consecutive freeze-thaw cycles under vacuum. It was then repressurised with 1 atm of dioxygen or argon. The solution of Mn^{III}-pyrophosphate (previously degassed) was added in five consecutive additions via a gastight syringe. After 10 min, the reaction medium was quenched with a previously degassed solution of NH_2OCH_2COOH. After 15 min, the sample resulting from isoniazid oxidation was directly analysed by LC–TIS–MS. The percentage of ^{18}O incorporated in each product was determined by the relative abundances of the peaks (peak surfaces) at *m/z* 124, 126 or 128 ($M+H^+$ of isonicotinic acid **1** with no, one or two ^{18}O atom incorporated) and *m/z* 123 or 125 ($M+H^+$ of isonicotinamide **2** with no or one ^{18}O atom incorporated). The values obtained were corrected considering the ^{18}O content of labelled water. We also checked (except for isonicotinaldehyde, which was derivatised with NH_2OCH_2COOH in ether oxime) that there was no oxygen exchange with $H_2{}^{18}O$ of isoniazid or its oxidation products **1** and **2**.

2.5. Experiments with D_2O

The conditions were the same as for experiments with $H_2{}^{18}O$, but in this case appropriate volumes of D_2O were added. The incorporation of deuterium in each product was followed by the relative abundances of the peaks (peak surfaces) at *m/z* 124 and 125 ($M+H^+$ of isonicotinic acid **1** with no and one deute-

rium incorporated) and *m*/*z* 123 and 124 ($M+H^+$ of isonicotinamide **2** with no and one deuterium incorporated).

2.6. Experiments with $^{18}O_2$

The conditions were the same as for experiments with $H_2{}^{18}O$ under dioxygen or argon but, in this case, after three consecutive freeze-thaw cycles, the reaction medium was repressurised with the desired pressure of $^{18}O_2$ (0.2 atm) and nitrogen was then added to have a final pressure of 1 atm. After 15 min, the sample was analysed by LC–TIS–MS. The values obtained for ^{18}O incorporations were corrected considering the ^{18}O content of the labelled dioxygen.

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