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APPARENT HYDROXYL RADICAL GENERATION WITHOUT TRANSITION METAL CATALYSIS AND TYROSINE NITRATION DURING OXIDATION OF THE ANTI-TUBERCULAR DRUG, ISONICOTINIC ACID HYDRAZIDE

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Abstract—Aromatic hydroxylation and formation of thiobarbituric acid-reactive substances occurred in a mixture of isonicotinic acid hydrazide (isoniazid) and catalase. Since these reactions were stimulated by phytic acid (a potent metal chelator), rather than inhibited, transition metal-catalysed hydroxyl radical generation was not implicated. Hydroxylation also occurred with isoniazid and phytic acid in the absence of catalase, albeit to a lesser extent. The independent effects of catalase and phytic acid are related to their abilities to catalyse isoniazid oxidation. In the presence of tyrosine, both the isoniazid/phytic acid system and authentic peroxynitrite generated dityrosine. Authentic peroxynitrite, as well as a phytic acid-mediated isoniazid oxidation product, have absorbance maxima at 302 nm. The yield of this isoniazid-derived product increased with pH and in the presence of a superoxide-generating system. A good correlation existed between absorbance at 302 nm and aromatic hydroxylation. Acid-induced decomposition of the 302 nm absorbance in the presence of superoxide dismutase led to the formation of a product absorbing in the same region as peroxynitrite-modified superoxide dismutase (350 nm at acid pH). Catalase catalysed peroxynitrite-mediated, as well as isoniazid/phytic acid-mediated tyrosine nitration, which was accompanied by Compound II formation (ferryl-catalase) in both cases. We postulate that peroxynitrite or a similar species is formed during isoniazid oxidation.

Key words: isoniazid; peroxynitrite; phytic acid; catalase

The metabolism of the anti-tubercular drug, isonicotinic acid hydrazide or isoniazid† (see Fig. 9 for its structure), has been reported to produce hydrazine derivatives and free radicals which might contribute to the mycobactericidal activity of the drug or to its side-effects [1–3]. Spin-trapping evidence suggested that one of the most cytotoxic species, the hydroxyl radical (·OH), is generated during peroxidase-catalysed oxidation of isoniazid [3]. In another study, isoniazid added to a crude extract of *Mycobacterium tuberculosis*, catalysed the hydroxylation of phenol [4]—a reaction which is also characteristic for ·OH. The mechanism generally invoked for ·OH production in a biological system requires the simultaneous presence of superoxide, hydrogen peroxide and a transition metal ion. Interactions of compounds such as hydrazines with haem-containing proteins may result in haem splitting [5–7]. Iron thus released, as well as traces of transition metal ions in buffer solutions, could be involved in Fenton-type ·OH-generating reactions [7].

Recently, we have observed aromatic hydroxylation during the myeloperoxidase-oxidase oxidation of isoniazid in a transition metal-independent fashion [8]. Thus, a cytochrome P450-like mechanism has been suggested.

Although it is known from earlier work by Halfpenny and Robinson [9] that aromatic hydroxylation can be achieved with peroxynitrite in a chemical reaction at acid pH, this reaction has only recently been performed under more physiological conditions [10–12].

In this communication, we propose that an oxidative intermediate of isoniazid, reminiscent of peroxynitrite or a similar species, is involved in reactions frequently used to monitor generation of hydroxyl radical. To eliminate transition metal-catalysed ·OH generation, we have utilized the unique metal-chelating properties of phytic acid [13]. We also show that catalase catalysed both peroxynitrite- and isoniazid-mediated tyrosine nitration.

MATERIALS AND METHODS

Reagents. Catalase (donor H_2O_2: hydrogen peroxidase oxidoreductase, EC 1.11.1.6; sp. act. 65,000 U/mg) from bovine liver, SOD (donor superoxide: superoxide oxidoreductase, EC 1.15.1.1; sp. act, 5000 U/mg) from bovine erythrocytes and XO (donor xanthine: oxygen oxidoreductase, EC 1.1.3.22; sp. act. 1 U/mg) from cows' milk were

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† Abbreviations: isoniazid, isonicotinic acid hydrazide; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; XO, xanthine oxidase.

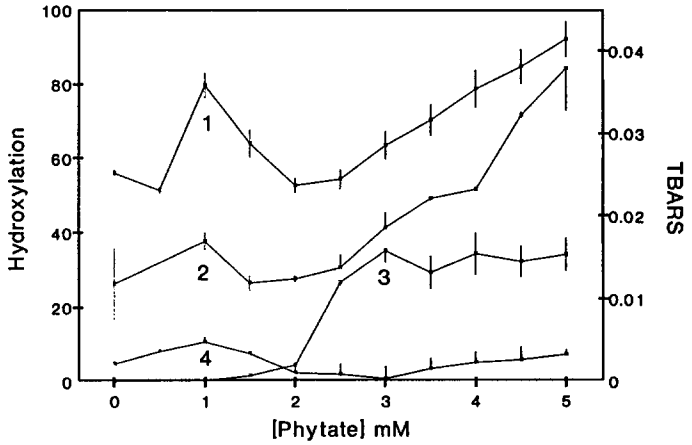

Fig. 1. Hydrazide-mediated benzoate hydroxylation and formation of TBARS as a function of phytic acid concentration. Isoniazid or nicotinic acid hydrazide (200 μM in reaction) was added to mixtures containing 1 mM benzoic acid or 0.8 mM deoxyribose in 0.01 M phosphate buffer (pH 7.4) in the absence and presence of 300 nM catalase. Fluorescence of hydroxylated benzoate was recorded after 6–10 hr (excitation and emission at 305 and 407 nm, respectively). The deoxyribose-containing mixtures were treated as described in Materials and Methods. Curves: 1 and 3, isoniazid-mediated hydroxylation expressed in relative fluorescence units in the presence and absence of catalase, respectively; 2, isoniazid-mediated TBARS formation in the presence of catalase (the chromogen was read at 532); 4, nicotinic acid hydrazide-mediated hydroxylation in the presence of catalase. Each data point represents the mean ± SD of four determinations.

obtained from Boehringer Mannheim (Mannheim, Germany). Isonicotinic acid hydrazide (isoniazid), nicotinic acid hydrazide (niazid) and phytic acid (dodecasodium salt hydrate) were products of the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.)

Spectroscopic analyses. UV absorption analyses were performed on a Beckman DU 640 spectrophotometric system and fluorescence measurements made with a Perkin–Elmer LS50B luminescence spectrometer.

Aromatic hydroxylation and the TBARS assay. Reactions were initiated by adding isoniazid (200 μM in reaction) to mixtures containing 300 nM catalase, 1 mM benzoic acid and increasing concentrations of phytic acid in 100 mM phosphate buffer (pH 7.4). Formation of fluorescent hydroxylated benzoic acid derivatives was measured after 2 hr (excitation and emission wavelengths at 305 and 407 nm, respectively) [14].

In the TBARS assay, reaction mixtures contained 0.8 mM deoxyribose instead of 1 mM benzoic acid. Incubations were for 15 min at 37° and TBA [1 mL of 1% (w/v)] was then added, plus 1 mL 2.8% (w/v) trichloroacetic acid. The solution was incubated at 100° for 10 min, cooled and the chromogen read at 532 nm. Appropriate corrections were made to account for the presence of phytic acid in the assay mixtures.

Preparation of peroxynitrite. Peroxynitrite was synthesized in a quenched-flow reactor from sodium nitrite and hydrogen peroxide [10, 15]; a 25-mL solution of 0.45 M $NaNO_2$ was rapidly mixed with 25 mL 0.3 M H_2SO_4 containing 1.17 mL 30% H_2O_2 ($\approx$0.45 M) and then quenched in 50 mL 1.25 M NaOH while stirring rapidly. To increase the peroxynitrite yield and decrease the violence of this potential hazardous synthesis, all solutions were cooled prior to reaction. The concentration of peroxynitrite was determined spectrophotometrically at 302 nm (ε = 1670 M/cm [10]). Peroxynitrite was also prepared by delayed quenching; i.e. NaOH was added when most of the ONOOH have decayed. This preparation was used to assess the effects of peroxynitrite contaminants (H_2O_2, nitrite and nitrate) in reaction systems.

RESULTS

Aromatic hydroxylation, TBARS and dityrosine formation

Figure 1 shows yields of hydroxylation (curve 1) and TBARS (curve 2) as a function of phytic acid concentration during isoniazid oxidation in the presence of catalase. Both curves have peaks at 1 mM phytic acid and upward trends above about 2 mM. In the absence of catalase, hydroxylation also occurred, but to a lesser extent and only at phytic acid concentrations above 1 mM (curve 3). It is also evident that hydroxylation can be mediated independently by catalase and by phytic acid. Nicotinic acid hydrazide, in the presence of catalase, was a relatively weak initiator of hydroxylation (curve 4).

The fluorescence spectra of a tyrosine solution, treated with an isoniazid/phytic acid mixture or authentic peroxynitrite (Fig. 2), are consistent with the presence of dityrosine [12].

Spectral alterations during isoniazid oxidation

Isoniazid has an absorbance peak at 262 nm (Fig. 3; spectrum 1) with a trailing edge at the descending limb. On addition of phytic acid, a small red shift

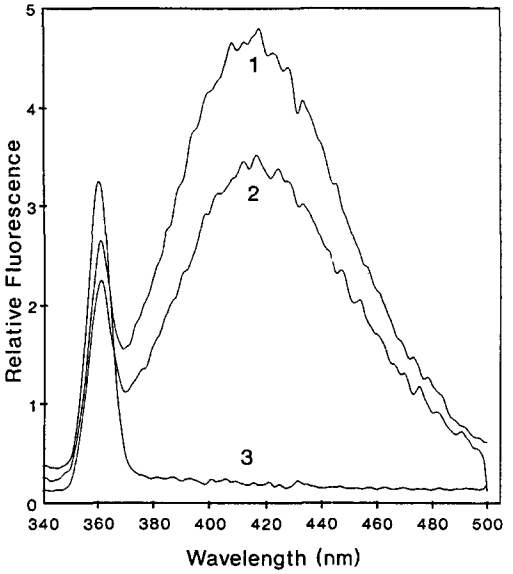

Fig. 2. Isoniazid- and peroxynitrite-mediated dityrosine formation. Fluorescence spectra of the following incubation mixtures were recorded (excitation 320 nm): 1, 200 μM tyrosine + 200 μM isoniazid + 5 mM phytic acid in 50 mM phosphate buffer (pH 7.4). 2, 200 μM tyrosine + 5 μM peroxynitrite in the same buffer. 3, 200 μM tyrosine.

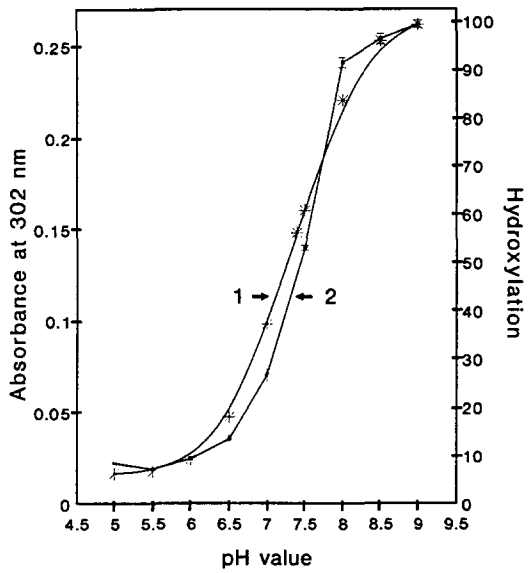

Fig. 4. Correlation between steady-state concentrations of the isoniazid-derived 302 nm—absorbing product formed at different pH values and end-point hydroxylation yields. Isoniazid (200 μM in reaction) was added to solutions at different pH values (100 mM phosphate buffer) containing 5 mM phytic acid and 1 mM benzoic acid. Absorbances were recorded after 1 min (curve 1). End-point fluorescence (hydroxylation) were recorded after 6–10 hr (curve 2).

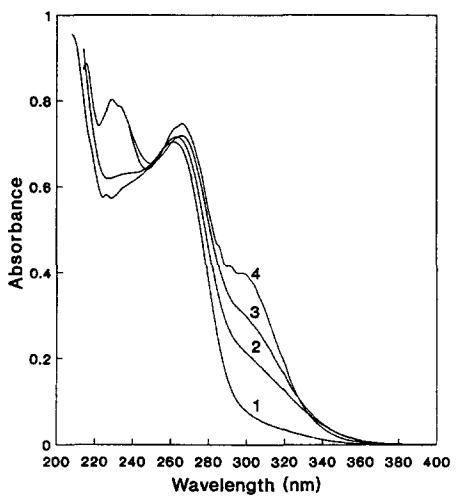

Fig. 3. Spectral alterations to the UV spectrum of isoniazid in 0.01 M phosphate buffer (pH 7.4) induced by phytic acid and gallic acid. Scans: 1, 200 μM isoniazid; 2, 1 min after adding phytic acid (5 mM in sample cuvette); 3 and 4, after adding gallic acid additionally (100 and 200 μM in sample cuvette, respectively).

occurred, while the trailing edge became a prominent shoulder at 302 nm (spectrum 2). Spectra 3 and 4 show a positive correlation between gallic acid concentration and absorbance at 302 nm. Inclusion of gallic acid in the isoniazid/phytic acid system led to a considerable increase in the rate of superoxide generation (results not shown).

Phytic acid-mediated oxidation of isoniazid was stimulated at alkaline pH. A close correlation existed between steady-state 302 nm absorbance and end-point aromatic hydroxylation yields (Fig. 4).

UV absorption profiles of peroxynitrite and isoniazid oxidation products

Figure 5A (curve 2) shows the UV spectrum of authentic peroxynitrite which has a peak at 302 nm [10]. Superimposed on this is curve 1, a difference spectrum showing the product formed during phytic acid-mediated isoniazid oxidation in 50 mM phosphate buffer (pH 9.0) containing SOD. Spectrum 3 was recorded after the pH of the reaction mixture had been adjusted to 6.5 with 1 M HCl. Bleaching of the 302 nm absorbance occurred with some recovery of the isoniazid spectrum at 260 nm. Of further interest is the increase in absorbance at the descending limb of curve 3, relative to curve 1. Subtracting scan 1 from scan 3, gives the difference scan of Fig. 5B. No absorbance increase at 350 nm occurred when SOD was omitted from the reaction mixture.

Peroxynitrite- and isoniazid-mediated tyrosine metabolism catalysed by catalase

Addition of peroxynitrite to a solution of catalase in 50 mM phosphate buffer (pH 7.4) resulted in an immediate decrease in Soret absorbance at 407 nm and a gradual blue shift (Fig. 6). Concomitantly a

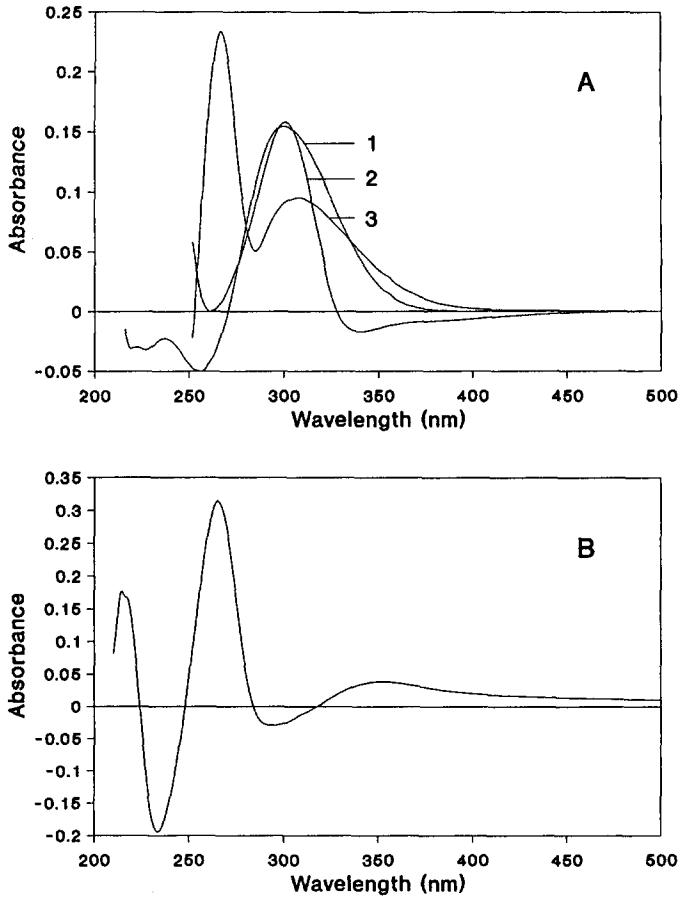

Fig. 5. UV spectral comparison of peroxynitrite and products formed during isoniazid oxidation. (A) Curve 1, spectrum of the product(s) formed during phytic acid-mediated isoniazid oxidation at alkaline pH. The reference solution contained 200 μM isoniazid and 3 μM SOD in 50 mM phosphate buffer (pH 9.0). The spectrum was recorded after adding phytic acid (5 mM in cuvette). Curve 3, spectrum recorded immediately after adjusting the pH to 6.5 with 1 M HCl. Curve 2, spectrum of authentic peroxynitrite. (B) The reference solution was a mixture at pH 9.0 similar to that of spectrum 1 in A (i.e. after phytic acid had been added and steady-state reached within 1 min). The spectrum was recorded immediately after acidifying the solution (i.e. a spectrum equivalent to curve 3 minus curve 1 in A).

peak at about 340 nm emerged which also gradually shifted towards lower wavelengths. In the presence of tyrosine (B), increases in absorbances around 350 and 450 nm occurred, which are characteristic of nitrotyrosine formation. A competing reaction under these conditions will be the simultaneous phenoxyl dimerization. The *o,o'*-dityrosine which forms predominantly (see also Fig. 2) will contribute to absorbance increases in the 300 to 350 nm region.

The kinetics of peroxynitrite-mediated benzoate hydroxylation (Fig. 7A) and nitrotyrosine formation (Fig. 7B) clearly show the stimulating effect of catalase. Absorbance at 440 nm (nitrotyrosine formation) reached a maximum after about 10 min of reaction and then gradually decreased (Fig. 7B). This two-phase effect is also evident during aromatic hydroxylation in the presence of catalase (A; curve 1). The initial rate of the reaction was constant up to about 10 min and then proceeded at a lower constant rate.

Isoniazid, in the presence of phytic acid, induced an immediate decrease of the Soret peak of catalase at 405 nm which was followed by a gradual red shift to 424 nm and an isosbestic point at 415 nm (Fig. 8A). Concomitantly, new bands at 534 and 567 nm appeared, while the peaks at 505 and 625 nm disappeared. These spectral changes are characteristic for formation of Compound II [16, 17].

In Fig. 6, considerable catalase haem modification occurred in the presence of 25 μM peroxynitrite. Peroxynitrite preparations can be contaminated with nitrate, nitrite and hydrogen peroxide. To determine the effects of these contaminants in the catalase system, a preparation was made by delayed quenching to allow decay of most of the generated peroxynitrite (see Materials and Methods). Addition of an aliquot of this preparation, which contained only 2.5 μM peroxynitrite in the same volume used in Fig. 6, was considerably less destructive to the catalase haem (Fig. 8C). Generation of Compound

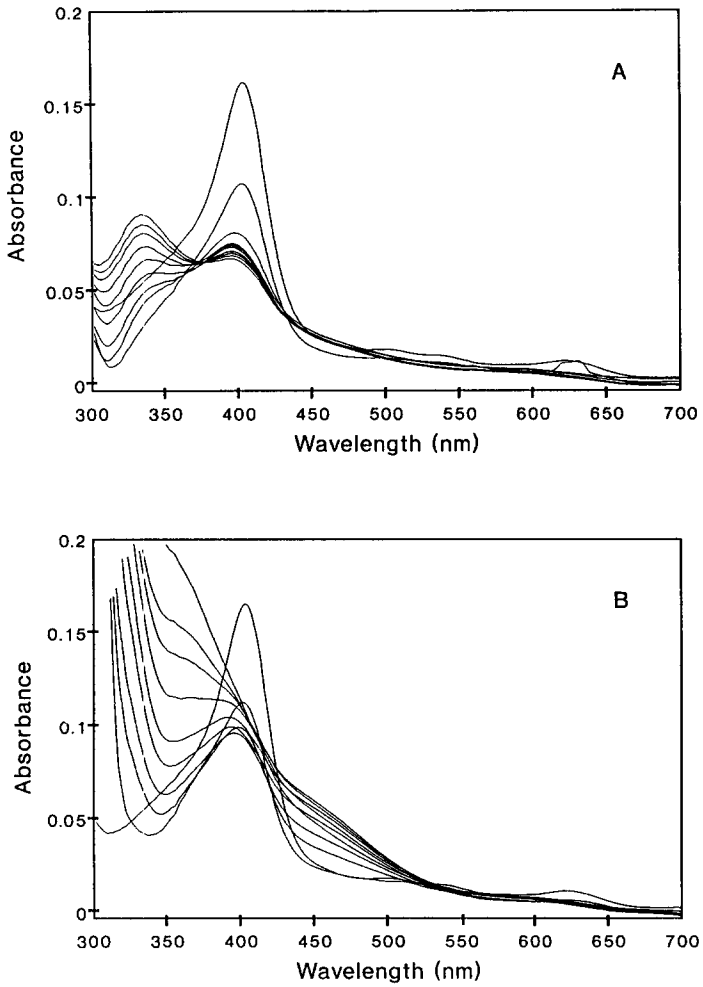

Fig. 6. Tyrosine nitration and spectral transformation of catalase induced by authentic peroxynitrite. (A) Peroxynitrite (40 μL), prepared as described in Materials and Methods, was added to a concentration of 25 μM to both the reference cuvette, and sample cuvette, which contained 250 nM catalase additionally. Scans were made immediately after peroxynitrite addition and then successively 1, 2, 4, 6, 10, 15, 20 and 30 min after peroxynitrite had been added. (B) Same as in A, but the sample solution additionally contained 500 μM tyrosine.

II is evident from the formation of peaks at 534 nm and 567 nm and the gradual red shift of the Soret band (scans 3–7). The peak at 585 nm formed immediately after addition of the peroxynitrite (scan 2) could be due to Compound III [18] formation which decayed within 1 min. Addition of hydrogen peroxide or nitrite (10 mM each), individually or in combination to a solution of 300 nM catalase, caused minimal haem loss and no Compound II could be detected (not shown).

In Fig. 8B, spectra of reaction mixtures of catalase, isoniazid and phytic acid in 50 mM phosphate buffer (pH 7.4), incubated in the absence and presence of tyrosine, are shown. Spectrum 1 is that of a control mixture of catalase, tyrosine, phytic acid and isoniazid at zero time. The reference cuvette contained the same concentrations of phytic acid and isoniazid in buffer solution. Reaction and reference cuvettes were incubated overnight and the spectrum recorded (scan 2). The reaction mixture of scan 3 was similar to that of scan 2, but without tyrosine. Scan 2 has shoulders at about 350 and 440 nm, which are absent in scan 3. Stimulation of dityrosine formation is also likely. The increase in absorbance near 300 nm (spectrum 2) will, at least in part, be due to formation of tyrosine polymers. It is difficult to verify this spectrofluorometrically, since the simultaneous presence of nitrotyrosine has a quenching effect on dityrosine fluorescence.

To investigate further tyrosine metabolism, another experiment was conducted. Reaction mixtures containing catalase and phytic acid at pH 7.4 with and without tyrosine were incubated overnight. UV scans were made after adjusting the pH of both the reference solution (without tyrosine) and the sample solution (with tyrosine) to 5 (Fig. 8D; scan 2). Scan 1 was recorded after adjusting the pH of the solutions to 11. The pH dependency of

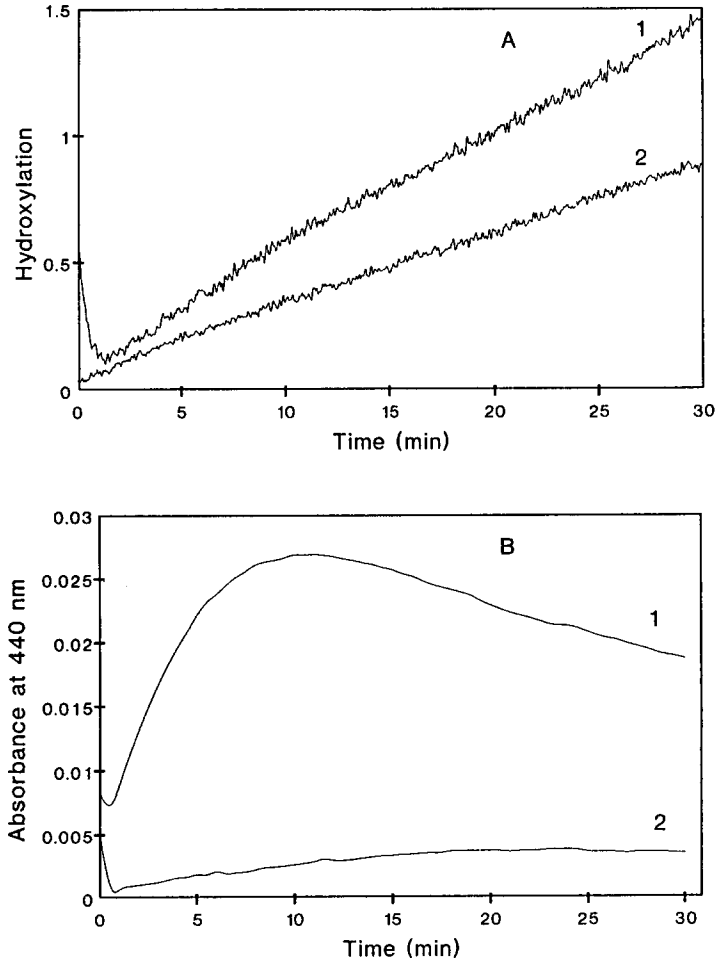

Fig. 7. Effect of catalase on peroxynitrite-mediated aromatic hydroxylation and nitrotyrosine formation. Peroxynitrite to a concentration of 25 μM was added to solutions of 1 mM benzoic acid or 500 μM tyrosine in 50 mM phosphate buffer (pH 7.4) at zero time. Fluorescence of hydroxylated benzoate was recorded at 407 nm (A) and absorbance of nitrotyrosine at 440 nm (B). Scans: 1, in the presence of 300 nM catalase; 2, in the absence of catalase.

the absorbance of the product formed is consistent with the presence of 3-nitrotyrosine [19]; i.e. absorbance maxima of 350 nm (acid pH) and 430 nm (shoulder at 430 nm; alkaline pH). In the absence of catalase, no corresponding pH-dependent spectral modifications occurred (not shown).

Superoxide requirement for benzoate hydroxylation

The effect of superoxide on aromatic hydroxylation is depicted in Table 1. SOD inhibited hydroxylation of the control mixture by more than 50% (addition 2). A XO-acetaldehyde superoxide-generating system, on the other hand, increased the hydroxylation yield nearly 40-fold (addition 3). SOD included in addition 3 suppressed the hydroxylation yield more than 10-fold (addition 4). The specific hydroxyl radical scavenger, mannitol, also suppressed hydroxylation. When 10 mM mannitol was included in addition 3 (addition 7), hydroxylation was inhibited by about 60%.

DISCUSSION

Phytic acid, or myo-inositol hexaphosphoric acid (Ins-P_6), is an abundant constituent of edible legumes, cereals, oil seeds, pollens and nuts [13]. It has several interesting features which make it a valuable tool in our investigations. By virtue of chelating free iron, phytic acid is a potent inhibitor of iron-driven hydroxyl radical formation [13]; it stimulates isoniazid-mediated superoxide generation [8]; it facilitates dissociation of oxygen from haemoglobin [13] and myeloperoxidase [8]. The latter property of phytic acid may well apply to other haem proteins, such as catalase. Phytic acid seems to be unique in its ability to affect the redox transformation of isoniazid [8] and to sustain haem spectral transformations in isoniazid-catalase systems (Fig. 8A), since other iron chelators, such as diethylenetriamine-pentaacetate (DTPA), were without effect (results not shown).

The increase in hydroxylation and TBARS

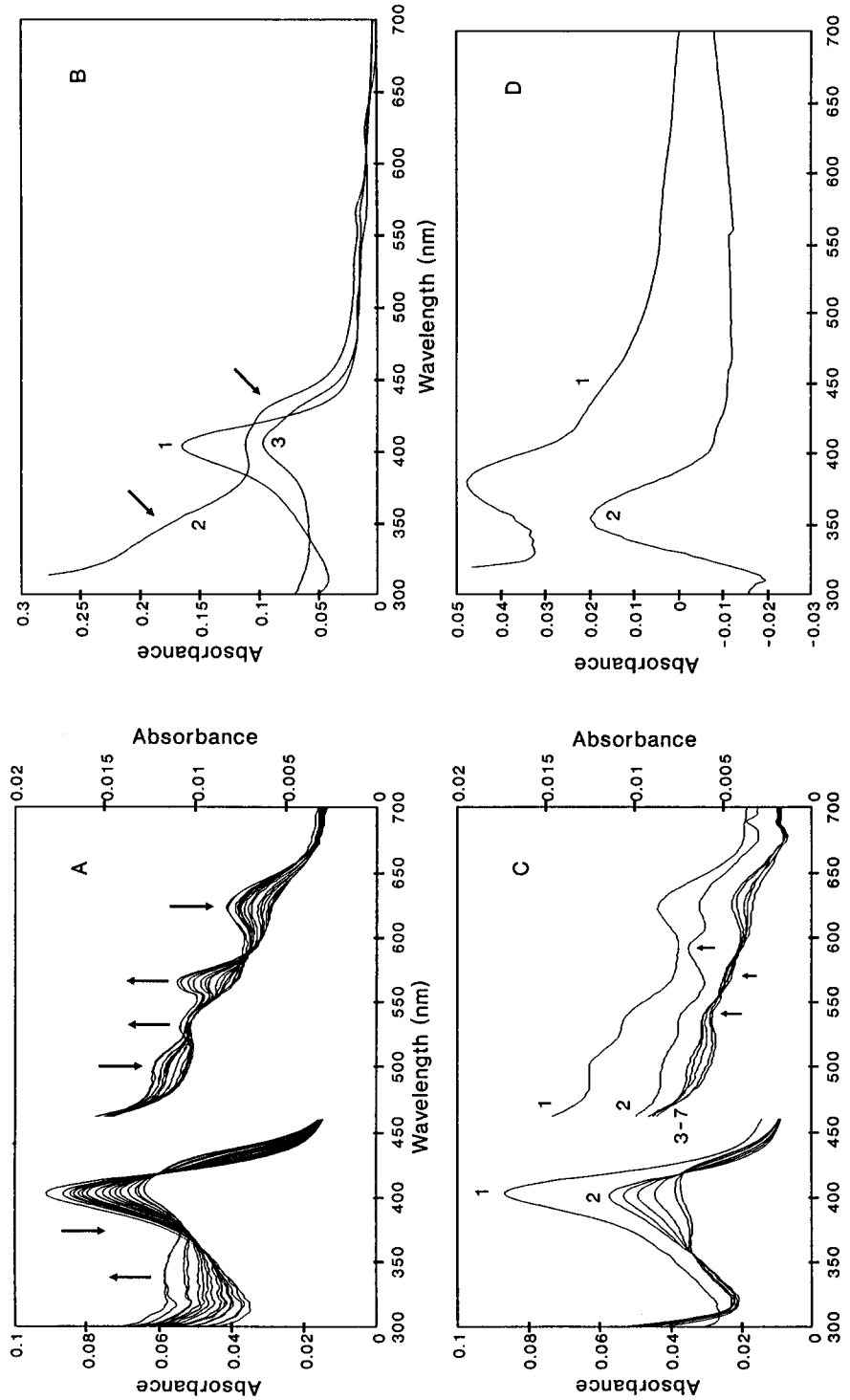

Fig. 8. Peroxynitrate- and isoniazid-mediated tyrosine nitration. (A) Isoniazid to a concentration of 200 μM was added to a solution of 300 nM catalase and 5 mM phytic acid in 0.01 M phosphate buffer at pH 7.4. A scan was recorded 30 sec later and then successively at 1 min intervals up to 5 min; at 5 min intervals up to 30 min and at 10 min intervals up to 70 min, after the isoniazid had been added. (C) Peroxynitrite (40 μL), synthesized by delayed quenching (see Materials and Methods), was added to a solution of 300 nM catalase in the same buffer as in A and successive overlay absorbance spectra recorded. The peroxynitrite concentration in the reaction was 2.5 μM. Spectra: 1, catalase control without peroxynitrite; 2, 30 sec after peroxynitrite addition; 3 to 7 at 2 min intervals. (B) Solutions containing 300 nM catalase, 5 mM phytic acid and 200 μM isoniazid with and without 500 μM tyrosine were incubated for 15 hr (overnight) at room temperature and UV spectra recorded. The reference cuvettes contained 200 μM isoniazid in buffer. Spectra: 1, catalase control; 2 and 3, in the presence and absence of tyrosine, respectively. (D) Reaction mixtures similar to that in C were incubated for 15 hr. Spectra were recorded using the reaction solution without tyrosine as reference. Spectra: 1, after adjusting the pH of both sample and reference solutions to 10; 2, after adjusting the pH of the solutions to 5.

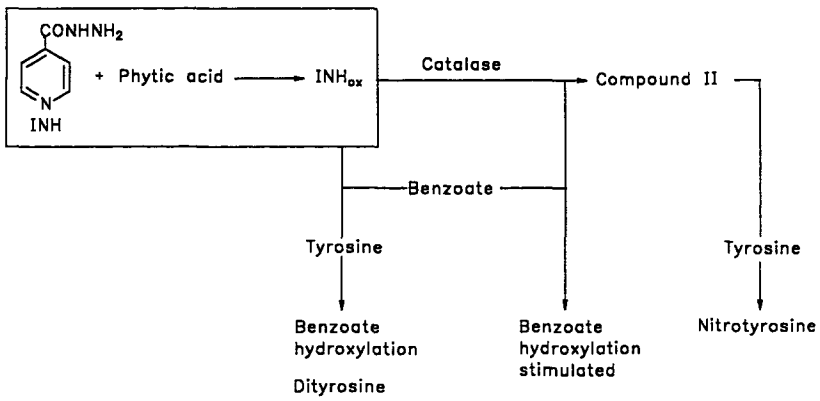

Fig. 9. Schematic representation of reactions used to monitor generation of hydroxylating and nitrating species during isoniazid oxidation. INH denotes isoniazid (isonicotinic acid hydrazide) and INH_{ox} its oxidation products (including superoxide). Our results suggest that the box in the scheme (upper left) can be replaced by peroxynitrous acid (ONOOH).

formation as a function of phytic acid concentration (Fig. 1), strongly argues against the involvement of transition metal-catalysed ·OH formation. Phytic acid has been shown to form a unique iron chelate which greatly accelerates Fe^{2+}-mediated oxygen reduction yet blocks iron-driven ·OH generation and suppresses lipid peroxidation [13]. Isoniazid-mediated hydroxylation (Fig. 1; curve 1) and TBARS formation (curve 2) in the presence of catalase had peak levels at 1 mM phytic acid and then increased again above about 2 mM. These results can be interpreted as follows: relatively low concentrations of phytic acid had a stimulating effect on catalase to promote isoniazid oxidation and thus hydroxylation and TBARS yields increased with phytic acid concentrations up to 1 mM. At higher phytic acid concentrations, catalase was increasingly inactivated due to the formation of compound II (Fig. 8A). Thus at high concentrations, phytic acid seems to be the only mediator of isoniazid oxidation. Compound II (ferryl catalase), however, was still capable of stimulating hydroxylation. This is suggested by the lower yields of hydroxylation in the absence of catalase (Fig. 1; curve 3) and the attainment of a plateau hydroxylation level at about 2.5 mM phytic acid. Of further interest is the fact that nicotinic acid hydrazide, the meta-isomer of isoniazid, was a relatively weak inducer of aromatic hydroxylation. We have recently shown that nicotinic acid hydrazide, in contrast to isoniazid, generated negligible amounts of $O_2^{\cdot}$ during its auto-oxidation [20]. Thus, a correlation between superoxide generation and aromatic hydroxylation might exist. Such a correlation is strengthened by the fact that a xanthine oxidase superoxide-generating system stimulated hydroxylation nearly 40-fold and that SOD suppressed hydroxylation (Table 1).

During phytic acid-mediated oxidation of isoniazid, the UV spectrum of the hydrazide showed an increase in absorbance at 302 nm (Fig. 3). With the additional presence of gallic acid, which increased the rate of superoxide generation, a further increase in 302 nm absorbance was noted. Furthermore, there was a good correlation between steady-state absorbance at 302 nm and end-point hydroxylation (Fig. 4). Our results thus suggest that superoxide is required to form the 302 nm-absorbing product, which decomposes to generate the hydroxylating species.

The question about the nature of the hydroxylating species still remains open, since phytic acid prevented ·OH generation in a possible iron-catalysed Haber–Weiss reaction. In a previous study we have suggested a cytochrome P450-like mechanism for hydroxylation during the myeloperoxidase-oxidase oxidation of hydrazines [8]. We cannot rule out a P450 hydroxylation mechanism for the catalase-mediated oxidation of isoniazid (Fig. 1), although the catalase haem was much more resistant to redox transformation than myeloperoxidase. During myeloperoxidase-catalysed oxidation of isoniazid, 1.8 mM phytic acid was sufficient to transform all enzyme to its Compound III form within 1 min [8]. With catalase, transformation to Compound II in the presence of 5 mM phytic acid and 200 μM isoniazid, still continued after 1 hr (Fig. 8A). In any case, a P450 mechanism cannot explain aromatic hydroxylation in the non-enzymatic system (Fig. 1; curve 3). The fact that mannitol inhibited hydroxylation to some extent (Table I) suggests that ·OH could be involved.

Another mechanism for generation of a hydroxyl radical-like species, has been proposed [10]. Nitric oxide reacts with superoxide at close to a diffusion-controlled rate of $(6.7 \pm 0.9) \times 10^9$ 1 mol/sec [21] to form peroxynitrite ($ONOO^-$) which, on protonation, yields peroxynitrous acid:

$$NO^{\cdot} + O_2^{\cdot} \rightarrow ONOO^- \quad (1)$$

$$ONOO^- + H^+ \rightarrow ONOOH. \quad (2)$$

Peroxynitrous acid has been suggested to decompose into highly reactive hydroxyl radicals [10, 11]:

$$ONOOH \rightarrow NO^{\cdot} + {}^{\cdot}OH. \quad (3)$$

However, more recent analyses based on thermodynamic considerations, preclude formation of free

·OH from ONOOH [22]. It has been suggested that a transitory intermediate formed in the isomerization reaction of peroxynitrous acid to form nitrate, may be the active species responsible for ·OH-like reactions:

$$ONOOH <---> O{=}N{-}O\cdots OH---> H^+ + NO_3^-. \quad (4)$$

Peroxynitrite can also decompose via a nitronium cation (NO_2^+) pathway which will be thermodynamically favoured in the presence of SOD or transition metals [19, 23]. The nitronium ion is a strong oxidant and nitrating agent [22]. Our results show that peroxynitrite-mediated tyrosine nitration was also catalysed by catalase (Figs 6B and 7B) and it is possible that the nitronium ion was also involved. The fact, however, that catalase also promoted the hydroxylation reaction (Fig. 7A) and that Compound II (ferryl catalase) was formed (Fig. 8C), suggests an alternative mechanism. Since peroxynitrite is a strong oxidant, it might oxidize catalase to its ferryl state, releasing nitrogen dioxide ($NO_2^{\cdot}$; reaction 3). Compound II thus formed can readily oxidize phenolics to phenoxyl radicals which then react with nitrogen dioxide to give nitrophenols [24]. Floris *et al.* [25] found that peroxynitrite reacted with peroxidases to form Compound II. In the case of catalase, however, the rate of decomposition of peroxynitrite, monitored at 302 nm, was unaffected. The reason for this negative result, in contrast to ours, is not known, but it may be mentioned that addition of 25 μM peroxynitrite to catalase (Fig. 6A), led to considerable haem modification and absorbance increases which can interfere with peroxynitrite monitoring at 302 nm. Peroxynitrite preparations can be contaminated with hydrogen peroxide, nitrite and nitrate. To determine the effects of these contaminants on haem destruction, a delayed-quenched preparation was made (i.e. in the synthesis, most of the peroxynitrite was allowed to decompose before quenching the reaction with NaOH). Figure 8C indicates that this preparation, which contained only 2.5 μM peroxynitrite in the same volume used in the more concentrated preparation described above, was considerably less destructive to the catalase haem. Furthermore, in contrast to the finding with the more concentrated peroxynitrite preparation, formation of Compound II could be observed with the delayed-quenched preparation. The results thus suggest that haem modifications observed in Figs 6A and 8C are due to peroxynitrite. These haem modifications could also not be reproduced by adding up to 10 mM of hydrogen peroxide or nitrite, individually or in combination, to native catalase.

Several typical peroxynitrite-mediated reactions could be reproduced in the isoniazid/phytic acid system. These are summarized in the reaction scheme of Fig. 9. A variety of nitroso compounds that form effectively under neutral physiological conditions can be conveniently viewed as nitric oxide carriers [26]. These include the nitrosamines (or nitrosamides) which can be formed as oxidation products of hydrazines (or hydrazides) [27, 28]. Nitrosamides, which could be an intermediate in the oxidation of isoniazid, are unstable at physiological pH and can decompose to form nitric oxide [29].

The product formed during phytic acid-mediated oxidation of isoniazid has a spectrum similar to that of authentic peroxynitrite (Fig. 5A), which has a peak maximum at 302 nm [10]. Superoxide necessary to form peroxynitrite is produced during isoniazid oxidation and absorbance at 302 nm increases with an increase in the rate of superoxide generation (Fig. 3). Peroxynitrite is stable in alkaline solutions, but decomposes readily at lower pH values [10]. We have also observed that bleaching of the 302 nm absorbance formed during isoniazid oxidation was favoured at acid pH (Fig. 5A).

Peroxynitrite has been reported to mediate tyrosine nitration catalysed by SOD. This resulted in a stable yellow adduct of SOD with absorbance maximum of 356 nm at pH 6 [19]. Evidence for the formation of an absorbance peak at about 350 nm was obtained when isoniazid was oxidized in the presence of SOD at pH 9 (Fig. 5). Acidification of the solution led to bleaching of 302 nm absorbance and formation of 350 nm absorbance which coincided with that of peroxynitrite-modified SOD [19]. The pH dependency of the isoniazid/phytic acid/catalase-mediated tyrosine modification (Fig. 8B and D) suggests the presence of nitrotyrosine. In addition, stimulation of the isoniazid/phytic acid-mediated aromatic hydroxylation and TBARS formation by catalase and the concurrent generation of ferryl catalase, suggest a hydroxylation and nitration mechanism similar to that mediated by peroxynitrite.

Nitric oxide is thought to be the bioregulatory factor in many different biological systems, such as vascular smooth muscle relaxation, platelet deaggregation and neural communication [30, 31]. A class of vasodilator drugs include numerous compounds of diverse structure, but one common chemical property among these compounds is their capability of generating nitric oxide under various conditions [32]. Of interest is that hydralazine (also a hydrazine) is a widely used peripheral vasodilator.

It has been suggested that rodent macrophages produce nitric oxide as part of their cytotoxic armamentarium [33]. Since *Mycobacterium tuberculosis* is phagocytosed by the macrophage, the mycobactericidal drug, isoniazid, may aid in augmentation of macrophage activity by generating peroxynitrite. The potent bactericidal activity of peroxynitrite has recently been reported [34]. The macrophage contains oxidative enzymes (including catalase), as well as a superoxide-generating capacity which could generate peroxynitrite or a similar species from isoniazid. In addition, isoniazid-sensitive mycobacteria contain a haem enzyme with peroxidatic and catalytic activity [35, 36] which could also be involved in isoniazid-mediated anti-mycobacterial activity.

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