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ISONIAZID-MEDIATED IRREVERSIBLE INHIBITION OF THE MYELOPEROXIDASE ANTIMICROBIAL SYSTEM OF THE HUMAN NEUTROPHIL AND THE EFFECT OF THYRONINES

JOHANN M. VAN ZYL, KAREN BASSON, REINHARD A. UEBEL and BEN J. VAN DER WALT*
Department of Pharmacology, Medical School, University of Stellenbosch, Tygerberg 7505, South Africa

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Abstract—During aerobic myeloperoxidase-catalysed oxidation of isoniazid at pH 7.8, compound III was generated. Oxidation of isoniazid or hydrazine sulphate at pH values of 6.5 or 7.8 in a myeloperoxidase–H_2O_2 system caused considerable haem loss, which was associated with compound III formation. Haem loss and also compound III formation could be inhibited when 8 μM thyroxine was included in the reaction mixtures. During the reaction with isoniazid, an intense pink-coloured pigment with maximum absorbance at 500 nm was formed which could be bleached with ascorbate or hypochlorous acid. The pigment was more stable at pH 7.8 than at pH 6.5. A similar pink colour was generated when a mixture of isoniazid and thyroxine in alkaline solution was irradiated with light of wavelength > 300 nm. A possible product of thyroxine oxidation, 3,5-diiodotyrosine, could not protect the enzyme against isoniazid-mediated haem loss and no colour formation was observed. Haem loss was most extensive when isoniazid was oxidised in a myeloperoxidase system at pH 7.8 in the presence of 0.1 M NaCl. Thyroxine (8 μM), however, could still inhibit haem loss under these conditions. A good correlation was found between haem loss and irreversible loss of peroxidase activity.

The treatment of tuberculosis with isoniazid (INH)† is associated with a high incidence of hepatotoxicity [1]. A relationship between INH hepatotoxicity and metabolism, which leads to covalent binding to liver proteins, has been found [2]. Many hydrazines and hydrazides (such as INH) are irreversible inhibitors of monoamine oxidase and nitrogen oxidation is thought to be a key step in forming the inhibiting chemical species [3]. A number of hydrazine and hydrazide derivatives are also capable of interacting with, and inhibiting the function of, the haemoprotein, cytochrome P-450 [4–6] and spectral interactions have been described for INH [7]. Particular attention was directed towards those hydrazines which are also potent monoamine oxidase inhibitors [4]. Loss of CO-reactive cytochrome P-450 induced by INH metabolism was also found to be associated with haem loss [6].

Hydrazines (or hydrazides) are readily oxidised to diazenes in air and in the presence of mild oxidising agents. Diazenes are also unstable and are readily oxidised to nitrogen and the alkane (or the aldehyde) [8]. Haemoglobin, for example, will react with phenyldiazene, formed from phenylhydrazine oxidation, to generate nitrogen and an arylated haem [9].

Oxidation of INH by peroxidase of *Mycobacterium tuberculosis* seems to contribute to the action of the drug [10, 11]. The horseradish peroxidase system has been used in a number of studies as a model for the metabolism of INH [12–14].

In the present study, the possible role of INH as a suicide substrate in the myeloperoxidase MPO–Cl^-–H_2O_2 antimicrobial system of the polymorphonuclear leukocyte was investigated.

After specific membrane perturbation by particulate or soluble stimuli, neutrophils exhibit a burst in oxygen consumption and start to generate reactive oxygen metabolites. Particles are sequestered in both sealed and unsealed phagocytic vacuoles [15]. Within 5 min after initiation of phagocytosis, the intravacuolar pH was found to be alkaline, exhibiting a pH of about 7.8 [15, 16]. The acidity subsequently increased to reach pH values of 6 or even lower [15, 16].

In the neutrophil, hypochlorous acid (HOCl) is produced by the oxidation of Cl^- by H_2O_2. This reaction is catalysed by MPO [17], a haem enzyme which is confined to the azurophylic granules of the unstimulated cell [18, 19], but can be demonstrated within the phagocytic vacuole following degranulation [20].

In this communication, MPO-mediated metabolism of INH was studied at pH values of 7.8 and 6.5. A pH of 7.8 is close to the value which may be expected shortly after the respiratory burst, while a pH of 6.5 may be expected within 30 min after the burst [15]. Leukocytes are capable of taking up the thyroid hormones, triiodothyronine and thyroxine [21], and their turnover is enhanced during illness states such as leukaemia and bronchopulmonary infection [22]. Thyroxine was found to stimulate the oxidation of a number of substances by hydrogen peroxide in the presence of peroxidase [23–25]. Thy-

* To whom correspondence should be addressed.

† Abbreviations used: INH, isoniazid; MPO, myeloperoxidase; T_4, L-thyroxine; T_3, 3,3′,5-triiodo-L-thyronine; 3,5-T_2, 3,5-diiodo-L-thyronine; T_0, D,L-thyronine; DIT, 3,5-diiodotyrosine.

roid hormones bind to haemoproteins in a poorly dissociable manner [26] and an affinity chromatography procedure for the preparation of MPO was based on this observation [27]. Peroxidases also form spectroscopically distinct complexes with hydrazides [28]. Furthermore, T_4 stimulates the chlorinating activity of MPO.* Hydrazines (or hydrazides) are known scavengers of chlorinating agents. Martindale Extra Pharmacopoeia [29] even specifies hypochlorites and INH as incompatible.

These considerations prompted us to investigate the metabolism of INH in the MPO–Cl^-–H_2O_2 system in the presence of thyronines.

MATERIALS AND METHODS

Materials. Isonicotinic acid hydrazide (isoniazid); nicotinic acid hydrazide; phenylhydrazine; hydrazine sulphate; nicotinamide adenine dinucleotide (reduced form); L-thyroxine (T_4); 3,3',5-triiodo-L-thyronine (T_3); 3,5-diiodo-L-thyronine (3,5-T_2); D,L-thyronine (T_0) and 3,5 diiodo tyrosine (DIT) were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium hypochlorite (approx 0.1 N in NaOH) was from BDH. Hypochlorous acid was prepared by adjusting NaOCl to pH 6.2 with dilute H_2SO_4 and its concentration determined iodometrically [30].

MPO was isolated from human neutrophils as described previously [27] and had a RZ of 0.75.

Methods. Modifications of the haem spectrum during oxidation of various compounds by MPO were monitored on a Cary 219 recording spectrophotometer.

To remove reactants and products from MPO, aliquots (50 μl) of reaction mixtures were passed through Sephadex G-25 columns (2 × 1.3 cm) and peroxidase activity was measured by the guaiacol assay.

In the photolysis studies, mixtures of INH and a thyronine (in 0.01 N NaOH) were irradiated for 1 min at 0° with a xenon lamp (500 W) at a distance of 9 cm from the light source. Irradiation below about 300 nm was filtered out with a borosilicate glass plate (2 mm thickness).

RESULTS

Compounds I and II have been well established as catalytic intermediates of a peroxidatic cycle [31]. While photometric recordings of compound I require special techniques (e.g. stopped-flow), compound II and the oxyperoxidase form, compound III, can be identified by their characteristic UV absorbance maxima [32]. When either NADH (200 μM) or INH (100 μM), respectively, was added to MPO (1.6 μM) at pH 7.8, difference scans were recorded which had inflection points at the same wavelengths, i.e. at 625 and 455 nm with troughs at 422 nm (Fig. 1). These peak maxima are characteristic of MPO compound III [32]. Since auto-oxidation of hydrazides is base-catalysed [33] and that of NADH acid-catalysed [34], compound III formation induced by INH will proceed more readily at pH 7.8 than compound III formation induced by NADH at the same pH. This fact is also demonstrated in Fig. 2(A and B). Thirty sec after adding 100 μM INH to MPO at pH 6.5, the haem moiety was hardly affected (Fig. 2A; scan 2), while at pH 7.8 under similar conditions, a considerable red shift as well as a decrease in maximum absorbance were evident (Fig. 2B; scan 2). At both pH values some compound III was formed on addition of H_2O_2 to the MPO–INH mixture (Fig. 2A and B, tracing 3). Ascorbate was used to decompose compound III to resting ferric MPO since it is a scavenger of superoxide [35]. Quantities of ascorbate sufficient to decompose compound III did not cause any haem loss. Tracing 4 of Fig. 2A and B, respectively, shows the UV scans when ascorbate was added 7 min after mixtures of MPO and INH were supplemented with 100 μM H_2O_2. It is evident that the haem loss at pH 7.8 was significantly greater than at pH 6.5 (30% vs 20%, respectively). When INH was oxidised in the MPO–H_2O_2 system in the presence of T_4, a pink-coloured pigment with maximum absorbance at 500 nm was formed at both pH values (Fig. 2C and D; scan 1). This demonstrates that 30 sec after initiating the reactions with H_2O_2, the maximum absorbance of the pigment formed at pH 7.8 was about twice the absorbance of pigment formed at pH 6.5. The pigment gradually decomposed. Scan 2 of Fig. 2C and D was recorded 2 min after recording scan 1, respectively. From these results it is evident that not only considerable more pigment is formed at pH 7.8 than at pH 6.5, but that it is also more stable at the higher pH. Furthermore, when ascorbate was added to the reaction mixtures, the pigment was bleached (Fig. 2C and D; tracing 4). This demonstrates that T_4 largely protected MPO against haem loss, when compared to the scan of unreacted MPO (Fig. 2C and D; tracings 3).

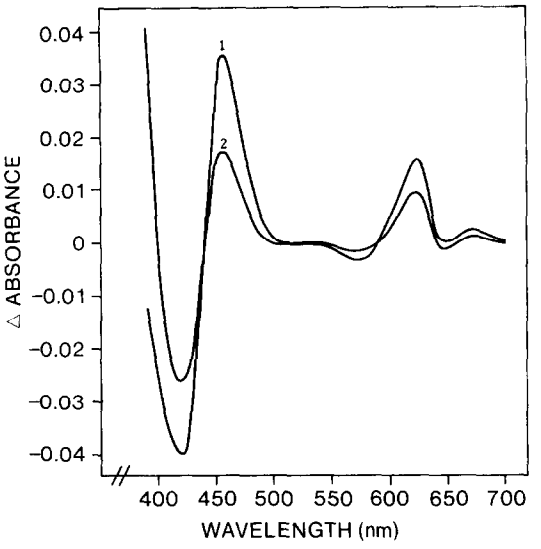

Fig. 1. Difference scans of MPO compound III in 50 mM phosphate (pH 7.8). Compound III was formed by addition of 100 μM INH (scan 1) or 200 μM NADH (scan 2) to sample cuvettes containing 1.6 μM MPO.

* van Zyl, Basson and van der Walt, submitted for publication.

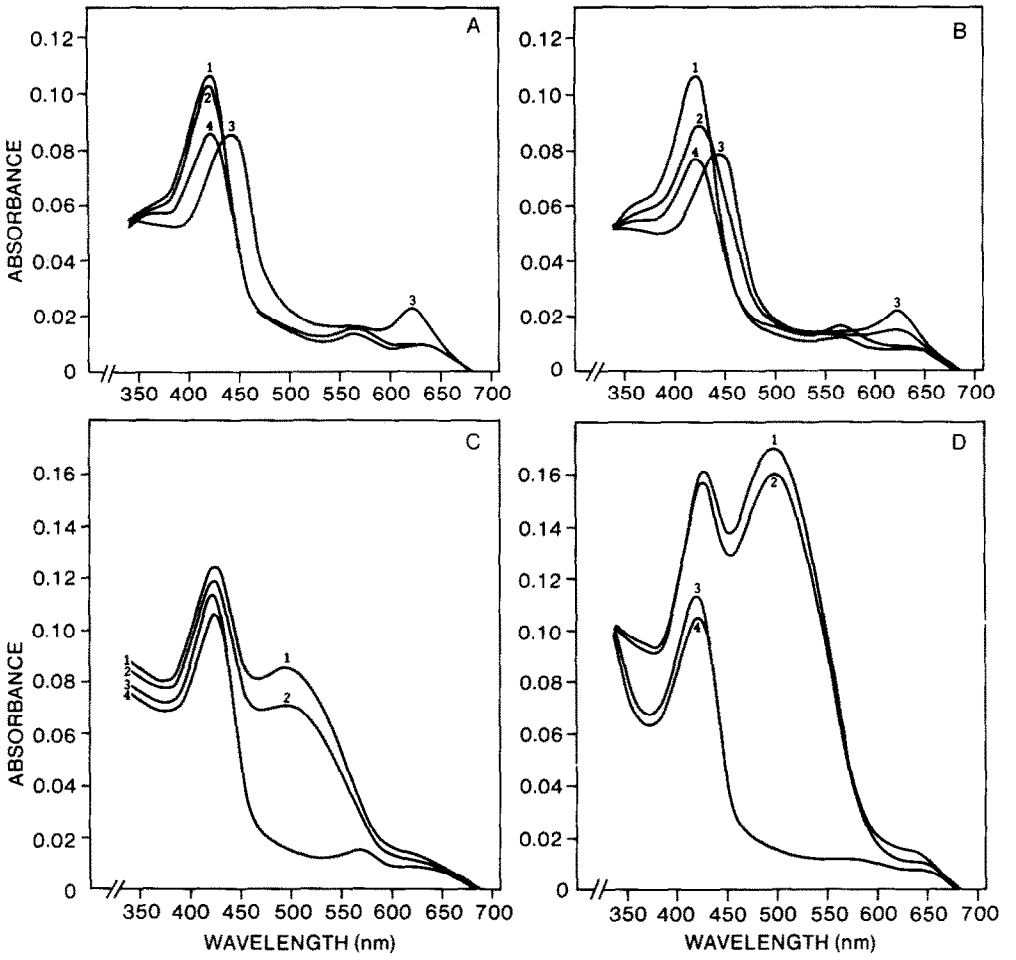

Fig. 2. Effects of INH and T_4 on the absorbance spectrum of MPO. (A) MPO (1.6 μM) was dissolved in 50 mM phosphate (pH 6.5). Scan 1: Unreacted MPO; Scan 2: MPO + 100 μM INH; Scan 3: 30 sec after adding 100 μM H_2O_2 to MPO + 100 μM INH; Scan 4: Ascorbate added 6.5 min after scan 3 was recorded. (B) The same as (A), but MPO (1.6 μM) was dissolved in 50 mM phosphate (pH 7.8). (C) MPO (1.6 μM) was dissolved in 50 mM phosphate (pH 6.5). Scan 1: 30 sec after adding 100 μM H_2O_2 to MPO + 8 μM T_4 + 100 μM INH; Scan 2: 2 min after recording scan 1; Scan 3: Unreacted MPO; Scan 4: Ascorbate added 6.5 min after recording scan 1. (D) Same as (C), but MPO was dissolved in 50 mM phosphate (pH 7.8).

In the oxidative metabolism of T_4, the non-phenolic ring iodines are believed to remain largely intact [36–39]. The effect of the number of iodines in the phenolic ring on the photometric characteristics of pigment formed in the presence of INH was subsequently investigated. For this purpose, T_4, T_3 and 3,5-T_2 which contain 2, 1 and 0 iodines in their respective phenolic rings, were used as model compounds. A pigment with a pinkish tint was also formed in the case of the T_3/INH MPO-mediated interaction. Its absorbance at 500 nm (Fig. 3A; scan 2), however, was only about half of that formed from T_4/INH interaction (Fig. 3A; scan 1). In contrast, with the 3,5-T_2/INH oxidation, the reaction mixture acquired a light tan colour exhibiting continuous absorbance without indication of a peak near 500 nm (Fig. 3A; scan 3). When T_0 was used, essentially the same scan as with 3,5-T_2 was produced which suggests the involvement of iodines predominantly in the phenolic ring. Scan 4 (Fig. 3A) shows the UV absorbance of unreacted MPO.

To support the contention that complex formation is dependent on the iodine substituents in the 3′ and 5′ positions of the phenolic ring, the model thyronines, in alkaline solution, were individually irradiated in the presence of INH. Irradiation of the T_4/INH mixture gave a pink-coloured product with maximum absorbance at about 520 nm (Fig. 3B; tracing 1). A pink-coloured product was also formed during photolysis of the T_3/INH mixture, although much less in intensity (Fig. 3B; tracing 2). As in the case of the enzymatic oxidation, irradiation of 3,5-T_2 in the presence of INH gave a non-specific light tan product with continuous absorbance (Fig. 3B; tracing 3).

Hydrazine sulphate has a detrimental effect on MPO haem as shown by Fig. 4(A). Fifteen sec after adding 100 μM hydrazine sulphate to the MPO solution at pH 7.8, a reduction of 25% in maximum haem absorbance was noted (Fig. 4A; scan 2). Thirty sec

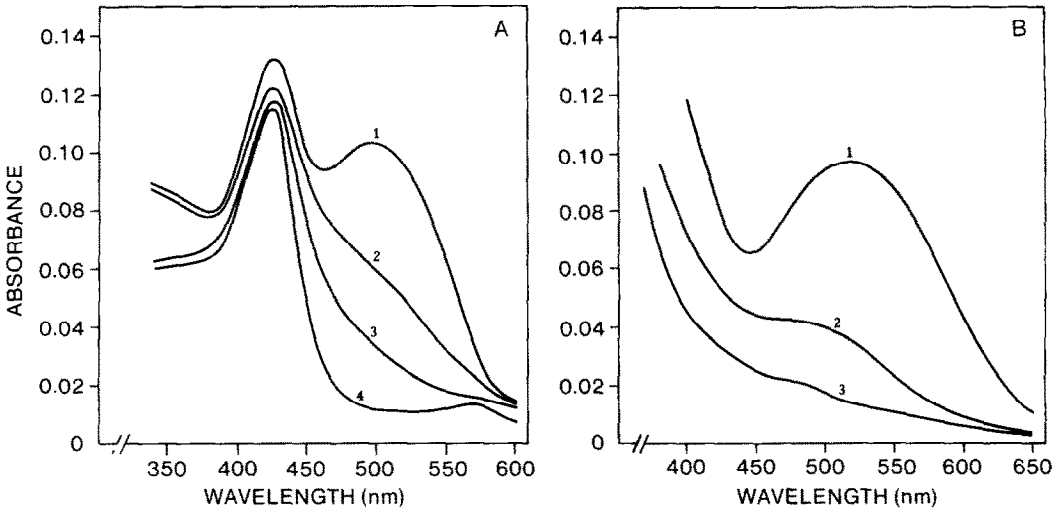

Fig. 3. Effect of the number of iodine substituents in the phenolic ring of thyronines on pigment formation. (A) MPO (1.6 μM) was dissolved in 50 mM phosphate (pH 7.8). Scan 1: 30 sec after adding 100 μM H_2O_2 to MPO + 8 μM T_4 + 60 μM INH; Scan 2: Same as in scan 1, but with 8 μM T_3; Scan 3: Same as in scan 1, but with 8 μM 3,5-T_2; Scan 4: Unreacted MPO. (B) Thyronine (1 mM) + 100 μM INH in 0.01 M NaOH was irradiated for 1 min ($\lambda > 300$ nm). Scan 1: T_4 + INH; Scan 2: T_3 + INH; Scan 3: 3,5-T_2 + INH.

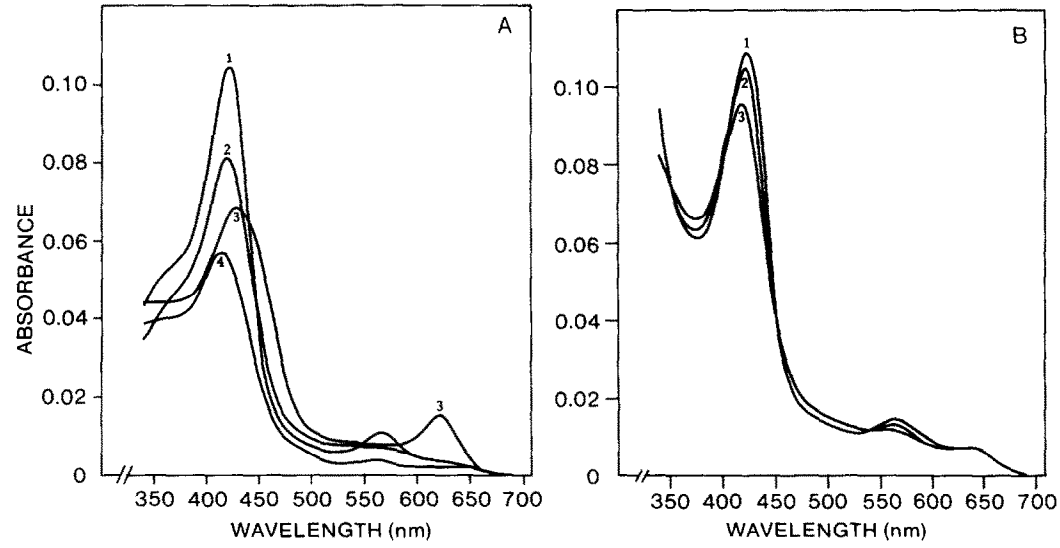

Fig. 4. Effect of hydrazine sulphate (100 μM) in 50 mM phosphate (pH 7.8) on MPO haem. (A) Scan 1: Unreacted MPO; Scan 2: 15 sec after adding hydrazine sulphate; Scan 3: 30 sec after adding 100 μM H_2O_2; Scan 4: Ascorbate added 10 min after recording scan 3. (B) Oxidation of hydrazine sulphate in the presence of 8 μM T_4. Scan 1 and scan 2: As (A); Scan 3: 10 min after adding 100 μM H_2O_2.

after adding 100 μM H_2O_2 to the system, the maximum haem absorbance decreased further to about 60% (Fig. 4A; scan 3) of the absorbance of the unreacted MPO (Fig. 4A; scan 1). Formation of compound III is also evident as indicated by the characteristic peak at 625 nm. When ascorbate was added to the system after 10 min, scan 4 was recorded which shows a reduction of about 50% in haem absorbance. These scans should be compared with those of Fig. 4(B) where 8 μM T_4 was included in the MPO–hydrazine system. When 100 μM hydrazine sulphate was added, haem loss in the presence of T_4 was negligible (Fig. 4B; scan 2). After addition of 100 μM H_2O_2, haem loss was only 15% (Fig. 4B; scan 3) compared to the original (Fig. 4B; scan 1). A similar protective action of T_4 at pH 6.5 was also noted (results not shown). In this case, however, the direct effect of hydrazine on MPO (i.e. in the absence of H_2O_2) was negligible since auto-oxidation of hydrazine will be much less in acid solution than at alkaline pH [33].

The effect of INH in the MPO–Cl^-–H_2O_2 system

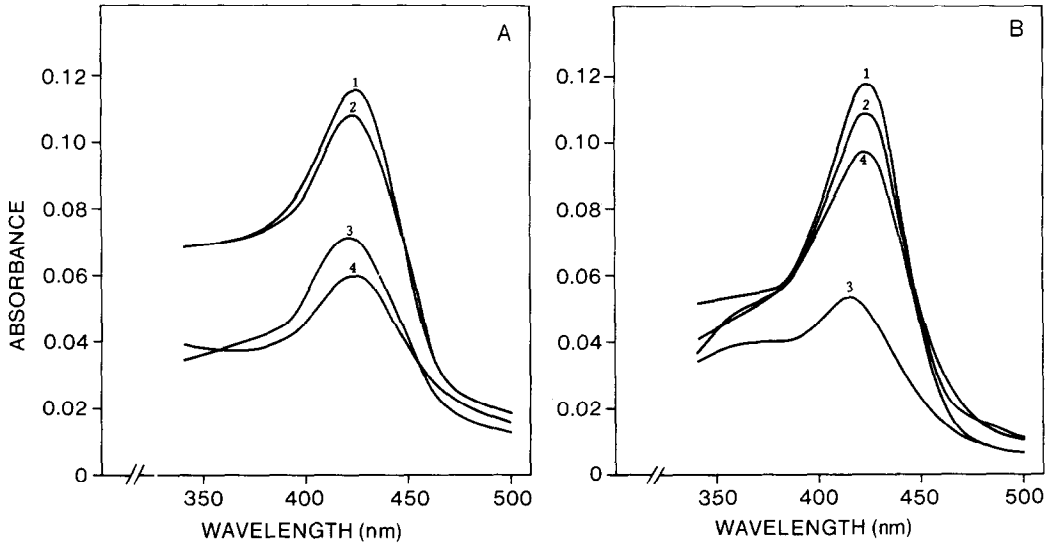

Fig. 5. Effect of chloride in the MPO–INH–H_2O_2 system. (A) MPO (1.6 μM) was dissolved in 50 mM phosphate (pH 6.5). Scan 1: Unreacted MPO; Scan 2: 10 min after adding 100 μM H_2O_2 to MPO + 0.1 M NaCl + 8 μM T_4 + 100 μM INH; Scan 3: 10 min after adding 100 μM H_2O_2 to MPO + 0.1 M NaCl + 100 μM INH; Scan 4: 10 min after adding 100 μM H_2O_2 to MPO + 0.1 M NaCl. (B) Same as (A), but reaction performed in 50 mM phosphate (pH 7.8).

on haem absorbance is demonstrated in Fig. 5. At pH 6.5 haem loss in the presence of 100 μM INH was about 40% after 10 min (Fig. 5A; scan 3). This may be compared to a loss of about 50% in the absence of INH (Fig. 5A; scan 4). The greater haem loss in the absence of INH may be a reflection of the scavenging of generated HOCl in the MPO–Cl^-–H_2O_2 system by the hydrazide. It is known that MPO can be inactivated during the respiratory burst [40, 41]. When, however, 8 μM T_4 was included in the reaction mixture containing MPO, NaCl and H_2O_2 at pH 6.5, haem loss was restricted to less than 10% (Fig. 5A; scan 2). The same experiments were repeated at pH 7.8. While haem loss at pH 7.8 in the MPO–Cl^-–H_2O_2 system was about 20% (Fig. 5B; scan 4), the presence of INH in this incubation system, resulted in a haem loss of more than 60% after 10 min (Fig. 5B; scan 3). In spite of this considerable haem damage induced by INH, the inclusion of 8 μM T_4 in the reaction mixture nevertheless inhibited haem loss dramatically to only about 7% (Fig. 5B; scan 2).

It was noted that the apparent yield of pink-coloured pigment formed during oxidation of the INH/T_4 mixture was always less in the presence of chloride than in its absence. When a mixture of INH and T_4 was oxidised in the MPO system at pH 6.5, scan 1 of Fig. 6 was recorded 30 seconds after initiation of the reaction. Two minutes later, scan 2 was recorded, which again demonstrates the instability of the pigment. Scan 4 was recorded after addition of ascorbate to the reaction mixture. In a parallel experiment, 200 μM HOCl was added 2.5 min after adding H_2O_2 to the reaction mixture. Scan 3 (Fig. 6) was then recorded which shows bleaching of the pigment by HOCl.

To determine the relationship between haem loss and irreversible peroxidase inactivation, the experiments depicted by Fig. 7 were performed. Removal of reaction products and excess reactants were achieved by passing aliquots of the reaction mixtures through Sephadex G-25. Peroxidase activity of each fraction was then determined by the guaiacol assay. In the MPO–Cl^-–INH–H_2O_2 system at pH 6.5 (Fig. 7A), peroxidase activity was decreased to about 60% of that of unreacted MPO. Comparing this result

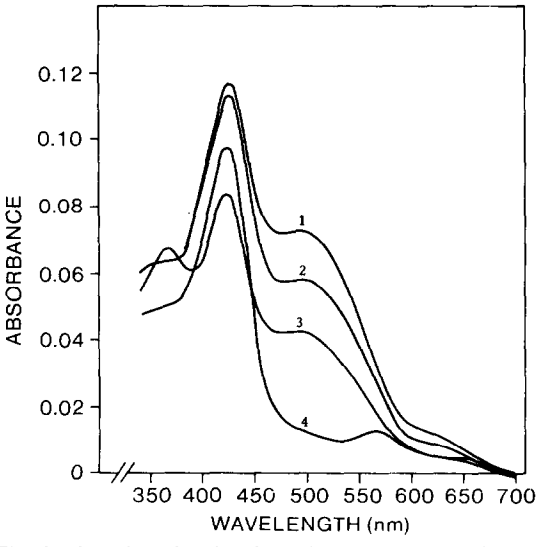

Fig. 6. Bleaching of pink-coloured pigment by HOCl. The reaction mixtures contained 1.6 μM MPO + 8 μM T_4 + 100 μM INH in 50 mM phosphate (pH 6.5). Scan 1: 30 sec after adding 100 μM H_2O_2; Scan 2: 2 min later; Scan 3: 200 μM HOCl added 2.5 min after adding 100 μM H_2O_2 to another aliquot of the reaction mixture; Scan 4: Ascorbate added after scan 2 had been recorded.

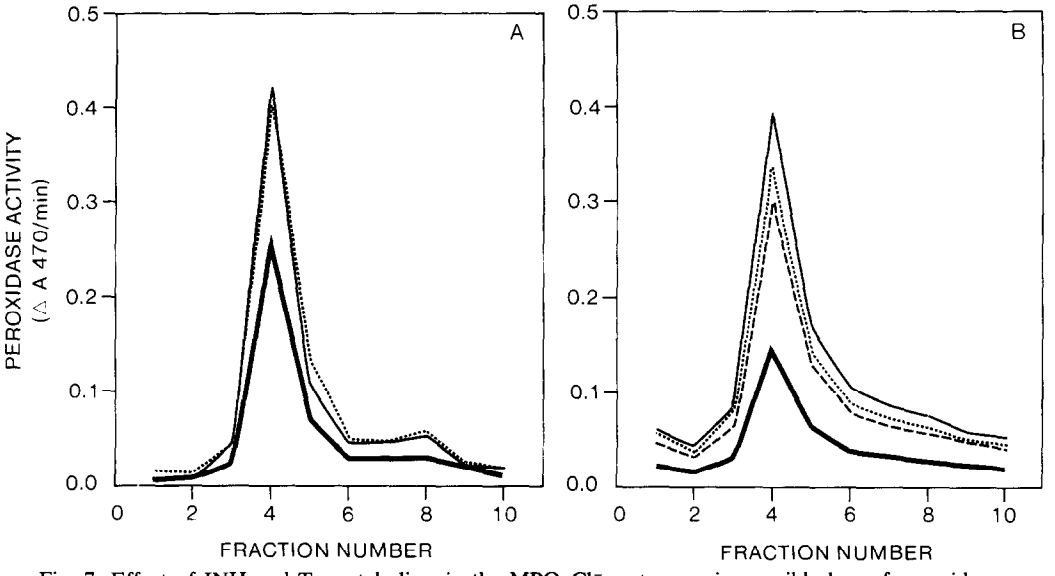

Fig. 7. Effect of INH and T_4 metabolism in the MPO–Cl^- system on irreversible loss of peroxidase activity. Aliquots (50 μl) of reaction mixtures containing 1.6 μM MPO were passed through Sephadex G-25 columns (2 × 1.3 cm) and 5-drop fractions collected. Peroxidase activity of each fraction was determined with the guaiacol assay. (A) Reactions were performed in 50 mM phosphate (pH 6.5) and the column equilibrated in the same buffer. ——, unreacted MPO; – – – –, reaction mixture contained MPO + 0.1 M NaCl + 8 μM T_4 + 100 μM INH + 100 μM H_2O_2; ——, reaction mixture contained MPO + 0.1 M NaCl + 100 μM INH + 100 μm H_2O_2. (B) Reactions were performed in 50 mM phosphate (pH 7.8) and the column equilibrated in the same buffer. ——, unreacted MPO; – – – –, reaction mixture contained MPO + 0.1 M NaCl + 8 μM T_4 + 100 μM INH + 100 μM H_2O_2; — — —, reaction mixture contained MPO + 0.1 M NaCl + 100 μM INH + 100 μm H_2O_2; ——, reaction mixture contained MPO + 0.1 M NaCl + 100 μM INH + 100 μM H_2O_2.

with that of Fig. 5A (scan 3) shows that a good correlation exists between haem loss and irreversible enzyme inactivation. Inclusion of 8 μM T_4 in this system prevented inactivation of MPO and peroxidase activity was virtually fully preserved (Fig. 7A). By contrast, at pH 7.8 (Fig. 7B), INH-induced irreversible loss of peroxidase activity in the presence of 0.1 M NaCl was more drastic. Only about a third of the original activity was still present after the reaction (Fig. 7B). Again, haem loss (Fig. 5B; scan 3) and irreversible enzyme inactivation correlated. Nevertheless, even under these conditions, T_4 could restrict loss of peroxidase activity to only about 10% when compared to the reactivity of the unreacted MPO. In the absence of Cl^- about 20% of the activity was lost during the reaction.

DISCUSSION

During the aerobic MPO-catalysed oxidation of INH at pH 7.8, a UV spectrum was recorded which had inflection points at the same wavelengths as compound III, generated during the oxidation of NADH under similar conditions. Enzymatic oxidation of INH in the presence of T_4 largely abolished compound III formation (Fig. 2C and D). This process is distinct from aerobic MPO-catalysed oxidation of NADH where compound III formation was stimulated by thyronines [25]. Compound III in the case of NADH oxidation is formed as the superoxide adduct of resting ferric MPO. Superoxide originated from the reaction of molecular oxygen with a NADH-derived free radical. The free radical may be generated by auto-oxidation and/or by a classical peroxidase reaction mechanism [42].

The oxidative metabolism of INH and other hydrazine derivates has been studied by spintrapping techniques [14]. When INH was incubated with horseradish peroxidase/H_2O_2 at pH 7.4, only hydroxyl radical could be trapped. However, in a study by Shoeb *et al.* [13], superoxide and hydroxyl radicals could not be incriminated as agents causing nitroblue tetrazolium (NBT) reduction or *p*-nitrosodimethylaniline (*p*-NDA) bleaching. These authors found that superoxide was produced only in the presence of an electron donor. In view of these considerations, the mechanism of compound III formation during INH oxidation is probably different from that of compound III formation during NADH oxidation.

During the microsomal oxidation of INH, a transient complex, which is indicative of the ferrous state of cytochrome P-450 has been detected [6, 7]. It appears likely that P-450 complexes formed during the metabolism of 1,1-disubstituted hydrazines, and possibly acyl hydrazines are aminonitrene-iron complexes. Oxidation of the hydrazine to the required aminonitrenes is readily rationalised by stepwise electron removal from the hydrazine [6, 43]. This may also apply to INH–MPO interaction. Stepwise removal of electrons from the hydrazide moiety may cause direct reduction of the adjacent haem iron,

converting it into the ferrous form. Combination of ferrous haem with molecular oxygen can also generate compound III [44]. On decomposition of compound III, O_2^- and H_2O_2 are released [45]. The presence of these reactive oxygen species together with iron released from damaged haem will create favourable conditions for production of the destructive hydroxyl radical via the Fenton reaction [46].

When INH was oxidised in the MPO–H_2O_2 system (without Cl^-), compound III was rapidly formed (Fig. 2A and B). Inclusion of T_4 in the reaction mixture prevented compound III formation and protected the haem against degradation (Fig. 2C and D). Hydrazine, a hydrolysed product of INH also has a destructive effect on haem [5, 7]. When hydrazine sulphate was oxidised in the MPO–H_2O_2 system, haem loss was also found to be considerable (Fig. 4A). Again, T_4 could protect the haem against degradation (Fig. 4B). In the oxidation of both INH and hydrazine, diimide is an intermediate product, with nitrogen as the ultimate metabolite [8]. The spin-trap studies of Noda *et al.* [47] showed that a hydrazine radical is formed as precursor of diimide. It is conceivable that this radical may contribute to haem destruction. Our results on the metabolism of INH (in the absence of Cl^-) suggest a correlation between compound III formation and haem loss. Such a relationship has also been indicated by studies on lactoperoxidase-catalysed H_2O_2 metabolism [48].

Schonbaum [28] made an extensive thermodynamic study on complex formation of peroxidases with hydroxamic acids, hydrazides and amides. He concluded that binding of these compounds and peroxidase hydrogen donors such as phenols and aromatic amines was fully competitive. This suggests the proximity of the hydrazide binding site to the prosthetic group of peroxidase. Since the phenolic compounds, T_4 (or T_3) can bind to the prosthetic group of haemoproteins [26, 27], it is proposed that T_4 competes effectively with INH (or hydrazine) for the same binding locus near the MPO haem iron. T_4 will thus prevent reduction of the resting ferric enzyme to the ferrous state and thereby limit haem damage.

While only OH$^\cdot$ could be trapped by Sinha [14] during anaerobic oxidation of INH in the horseradish peroxidase/H_2O_2 system at pH 7.4, two carbon-centred radicals (R$\dot{C}$=O, R$^\cdot$; R = 4-pyridinyl) were trapped at pH 10. The same R$^\cdot$ was also formed in the prostaglandin/arachidonic acid system at pH 7.6 [14]. Formation of carbonyl radicals is followed by carboxylate and peroxy radicals [33] which may mediate NBT reduction and *p*-NDA bleaching [13]. It is generally believed that *in vivo* activation of hydrazines to free radical intermediates may provide the ultimate toxins [49]. The precursor of carbonyl radical (R$\dot{C}$=O), is a carbanion which seems to be a rate-determining step in the oxidation pathway of INH [13].

Oxidation of chloride by the MPO–H_2O_2 system leads to the formation of chlorinating agents [17], which are also oxidising agents. With INH also included in the MPO system, hydrazide oxidation could be accelerated (i.e. via nonenzymatic HOCl interaction at acid pH). Scavenging of HOCl could, however, also be detrimental, since it will deprive the neutrophil of this important antimicrobial agent and inflammation may be aggravated [50].

Extensive haem loss was experienced when INH was oxidised in the MPO system in the presence of 0.1 M NaCl. No compound III, however, could be observed in this system (Fig. 5A and B). Resonance Raman studies strongly suggest the direct coordination of chloride to the iron atom in MPO haem [51]. If this were the case, then Cl^- and O_2^- will compete directly for binding to haem iron and consequently compound III formation will be inhibited. Furthermore, reaction products formed during the oxidation of chloride may also scavenge superoxide [52, 53]. A factor which could intensify haem loss at pH 7.8 (Fig. 5B) may be an increased production of INH-derived carbon-centred radicals due to nonenzymatic oxidation by chlorinating agents generated in the MPO reaction system. Other species such as chloramines (formed by reaction of HOCl with nitrogen-containing compounds [54]) and OH$^\cdot$ and singlet oxygen, which may be formed as products of the reaction of O_2^- and HOCl [52, 53], may also cause haem loss. Chlorinating agents generated at acid pH (mostly HOCl [17]) are much more destructive to haem (Fig. 5A) than those generated at pH 7.8 (Fig. 5B). This destructive effect of chlorinating agents or its products may be balanced or even exceed the effects exerted by additional reactive species generated in the presence of INH. Whatever the mechanism leading to haem destruction might be, it is clear that T_4 can also afford protection against inactivation of MPO in the presence of both INH and chloride.

Deiodination is the most important degradative pathway of T_4 and accounts for up to 85% of the disposal of the hormone [36]. This can be achieved by either reductive or oxidative mechanisms. Monodeiodination is a reductive enzymatic process which results in the nonrandom removal of iodine atoms from both the rings of T_4 and replacement by hydrogen atoms. Deiodination under oxidative conditions is a process distinct from monodeiodination since it does not lead to the formation of the lesser iodinated thyronines.

Ether link cleavage of T_4 during human leukocytosis was demonstrated to be a major degradative pathway of the hormone, since up to 50% of all T_4 degraded was converted to DIT. The metabolism of the phenolic ring, however, remains uncertain, but iodide is likely to be an end product of its degradation [36]. Photoactivated flavin-induced degradation studies of T_4 were also reported [55, 39]. According to the reaction mechanism proposed, deiodination of a quinoid-free radical of T_4 is followed by etherbond splitting and the release of DIT. Irradiation of T_4 with light (wavelength cut-off 300 nm) in hydrogen-donating solvents led to stepwise removal of iodine atoms, while irradiation in water at alkaline pH took an entirely different course and virtually no known metabolites of T_4 were present in the photolysates [56]. Irradiation of bromophenols in water can yield dihydroxy-benzenes which derive from carbon–bromine bond cleavage and reaction with water [57]. It is conceivable that irradiation of T_4 in alkaline solution with light of wavelength above 300 nm will effect homolytic scission of the C—I bonds predominantly

in the phenolic ring [56]. The phenyl radicals thus formed may react with water to yield 3′,5′-dihydroxy-3,5-diiodo-thyronine. Hydrolysis of this compound in alkaline solution may lead to the formation of 2,6-dihydroxy-*p*-quinone and/or 4,6-dihydroxy-*o*-quinone. Lissitzky and Bouchilloux as cited by Wynn and Gibbs [38] suggested that hydroxylation of the phenolic ring of thyronines labilises the diphenyl ether, a proposal also favoured by Plaskett [37]. Consequently, 2-hydroxy-*p*-benzoquinone and 4-hydroxy-*o*-benzoquinone could be isolated as products of the spontaneous hydrolysis of 3′-hydroxythyronine [38]. Similar products may be formed during the peroxidative metabolism of T_4.

The first step of most, if not all, oxidations of phenols, is the formation of free radicals. The existence of T_4 free radicals was confirmed by the electron proton resonance (EPR) studies of Borg [58]. Just as quinols may be formed as intermediates in the formation of the diphenyl ether during T_4 synthesis [59], it is possible that quinols may be formed as degradation products [38]. In the MPO–H_2O_2 system, the oxidation of T_4 may be represented by Fig. 8. During the degradation of T_4 by rat liver microsomes, the 3′ and 5′ iodines are removed virtually simultaneously and the diphenyl ether is split to yield 3,5-diiodotyrosine [37, 38], Quinol ethers are known to be unstable, especially those with iodines in the 3 and 5 positions [59]. An end product of the phenolic ring may be a hydroxyquinone [38, 39, 60], similar to that postulated for the photolytic decomposition of T_4 in water at alkaline pH. The hydroxyquinone as a possible product of T_4 oxidation may serve as an additional electron carrier and may thus facilitate redox reactions in the active centre of MPO.* Correspondence between the photolytic and enzymatic oxidations of T_4 was also noted when INH was included in the reaction mixtures. In both cases, pink-coloured pigments were formed which could be bleached by ascorbate. The pigments were somewhat more stable at pH 7.8 than at pH 6.5.

During the chemiluminescent base-catalysed autooxidation of linear hydrazides [33] or their enzymatic oxidation [12], an acyl anion is formed which will be resonance-stabilised in the case of INH [13, 33]. Substituents on quinones may be displaced by other substituents [61]. Thus, hydroxyl groups may be displaced by anilino groups or the sulphonate group by the amino group as in the Folin reaction. This displacement reaction has been used as a system for determination of proline and hydroxy-proline, both of which form 1,2-naphto-quinone derivatives possessing characteristic absorption spectra. It is also known that *p*-quinones can react with pyridinyl compounds to form *N*-substituted betaines [61]. Such replacement or addition reactions are possible between oxidised INH and quinones formed during the oxidative or photolytic decomposition of T_4. However, since the phenolic ring iodines of T_4 are good leaving groups, it is more likely that coupled products in which a nitrogen moiety (i.e. the 4-pyridinyl nitrogen of INH) is substituted for the leaving group, are formed [8] as is indicated in Fig. 8. When T_4 in alkaline solution was irradiated for 1 min ($\lambda > 300$ nm), INH then added, and irradiation continued, the intensity of the pink colour was considerably less than when a mixture of T_4 and INH was irradiated from the beginning. Similarly, when T_4 was oxidised in the MPO–H_2O_2 systems and INH then added, together with additional H_2O_2, no pigment formation could be observed. These results imply that during the oxidative metabolism of INH, reaction with pre-oxidised T_4 does not proceed readily.

When T_3 was substituted for T_4 in either the photolytic or the enzymatic reactions, the absorbance of the pigment formed was lower. This effect was coupled with a lower efficiency of T_3 to protect the MPO haem. Since T_3 possesses only one iodine in the phenolic ring, it should have theoretically only 50% of the potency of T_4 to scavenge potentially harmful INH-derived intermediates. It is likely that the INH-derived acyl ion may be the reactive intermediate in its reaction with T_4, rather than a further oxidation product of INH, i.e. carbonyl radical [33]. When INH was oxidised in a horseradish peroxidase–H_2O_2 system at pH 7.4, no radicals besides $OH^{\cdot}$ could be spin-trapped [14]. Carbon-centred radicals were generated only under more extreme conditions (pH 10) in this peroxidase system [14]. It is therefore unlikely that carbon-centred radicals will be generated in significant amounts in our MPO–H_2O_2 system at pH 7.8 and even more unlikely at pH 6.5. Furthermore, T_4 was less effective in protecting MPO haem during phenylhydrazine oxidation, while it could protect against hydrazine sulphate or intermediates formed during oxidation of INH or its meta isomer, nicotinic acid hydrazide. Oxidation of phenylhydrazine must proceed via different intermediates, since no equivalent anion formation is possible as in the case of INH or nicotinic acid hydrazide. Instead, phenyl radical is formed directly from the phenyldiazine intermediate [9]. These considerations suggest that the phenyl radical must be involved in haem destruction. No pigment formation was also observed during the oxidation of mixtures containing hydrazine sulphate and T_4 or phenylhydrazine and T_4.

Attempts to characterise the presumed addition products formed during INH/T_4 oxidation were unsuccessful. The pink-coloured pigment is unstable, especially at acid pH where HPLC analyses were performed. Furthermore, the photolytic and enzymatic INH/T_4 reaction mixtures tend to change colour gradually with time, from an intense pink to a light brown pigment which had continuous absorbance. This may be indicative of a polymerisation process [61]. It should also be noted that differences in products formed during the photolytic and enzymatic processes may exist. The iodines in the non-phenolic ring of T_4 remain stable during enzymatic oxidation [36–39], while in the photolytic reaction, homolytic iodine splitting from the non-phenolic ring, although limited, cannot be excluded under our experimental conditions [56]. Additional adducts with activated INH may thus result. This may explain, at least in part, the difference in the maximum absorbance of pink-coloured pigment formed

* van Zyl, Basson and van der Walt, submitted for publication.

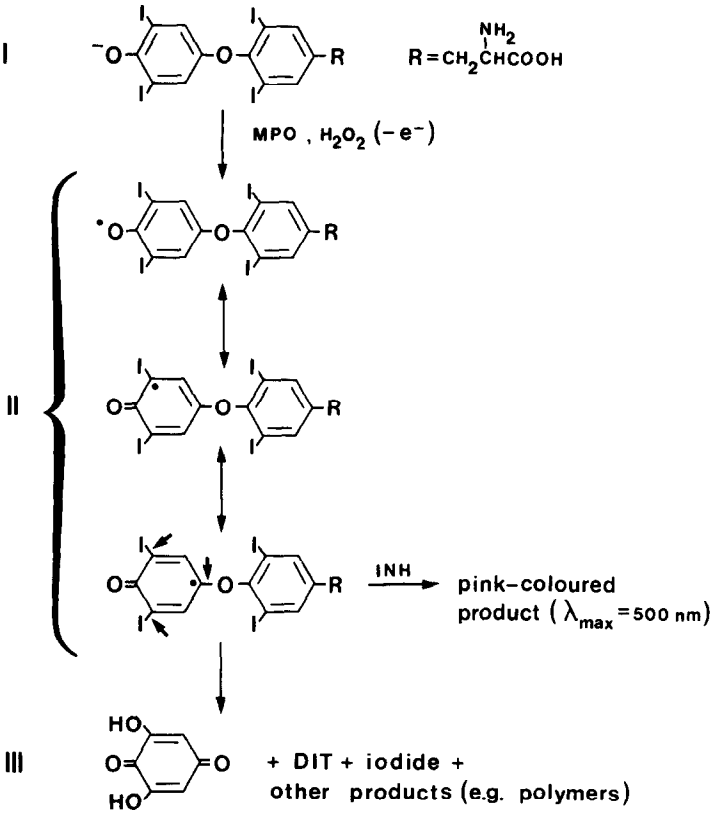

Fig. 8. Proposed scheme for the MPO-catalysed oxidation of T_4 and its reaction with oxidised INH. The MPO system removes an electron from T_4 (I) to produce free radical (II), the quinol ether form. Quinol ether formation labilises the phenolic ring C—I bonds and the ether bond. Consequently a dihydroxy-*p*-quinone (III) may be formed. In the presence of INH an unstable pink-coloured product with maximum absorbance at 500 nm, presumably a *N*-substituted betaine, is formed. The 4-piridinyl nitrogen of oxidised INH can couple at the positions of the phenolic ring iodide leaving groups, while ether bond splitting may occur simultaneously.

during the enzymatic (500 nm) and the photolytic (520 nm) reactions, respectively.

In conclusion, the mechanism by which T_4 can provide protection against haem loss during MPO-catalysed INH oxidation may be as follows:

(1) T_4 competes effectively with INH (or hydrazine sulphate) for the same binding locus near the prosthetic group of MPO. This will inhibit direct reduction of the ferric haem by the hydrazine to form the ferrous state and consequently compound III formation in the presence of O_2. The ferro-peroxidase/compound III states are associated with irreversible enzyme inactivation by cleavage of the haem moiety and liberation of iron [48].

(2) Oxidation of INH in a MPO–Cl^-–H_2O_2 system at pH 7.8, resulted in more haem damage and loss of peroxidase activity than in the absence of Cl^-. Chlorinating agents, formed as a result of Cl^- oxidation, may oxidise or chlorinate the hydrazide group of INH to form chloramines [54] or may form increased concentrations of potentially harmful free radicals. HOCl generated at pH 6.5 also caused considerable haem loss and may also react further to produce toxic agents such as OH· and singlet oxygen [52, 53]. Although T_4 can stimulate the chlorinating activity of MPO, it nevertheless provided protection against haem loss.

(3) When INH was oxidised in the presence of T_4, a pink-coloured product was formed as a result of the scavenging of potentially harmful intermediates. It has been demonstrated by Hill and Thornalley [62] that spintrapping agents, which decrease the level of reactive radicals, inhibit haemolysis and haemoglobin oxidation caused by phenylhydrazine. In the presence of Cl^-, less pigment was apparently formed than in its absence. This may be explained, at least in part, by the fact that HOCl can bleach the pigment when added after the reaction had taken place. Hypochlorous acid formed in the MPO–Cl^-–H_2O_2 system can thus be scavenged, not only by the hydrazide, but also by the INH/T_4-derived addition product. The non-phenolic ring product of T_4 oxidation, DIT, is incapable of protecting the enzyme and no pigment was formed during the enzymatic oxidation of INH in its presence.

The substrate of MPO, hydrogen peroxide, is generated by an electron transport chain which includes the haemoprotein, cytochrome *b*-245 (for a review see [63]). Since hydrazines bind to haemoproteins and thereby inhibit the action of MPO, it is possible that the inhibition of MPO-mediated activity could be dependent also upon the inhibition of the gen-

eration of superoxide which can dismutase to form H_2O_2.

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