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GENERATION OF SUPEROXIDE RADICAL AND HYDROGEN PEROXIDE BY 2,3,6-TRIAMINOPYRIDINE, A METABOLITE OF THE URINARY TRACT ANALGESIC, PHENAZOPYRIDINE

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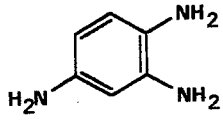
2,3,6-Triaminopyridine, a metabolite of the widely-used drug phenazopyridine, has been shown to autoxidize at neutral pH, generating superoxide radical and hydrogen peroxide. Hydrogen peroxide was also detected in erythrocytes exposed to this substance, and these cells suffered oxidative damage, as reflected by methaemoglobin formation and glutathione depletion. The *in vitro* effects of 2,3,6-triaminopyridine are closely similar to those of the structurally-related compound, 1,2,4-triaminobenzene. The latter substance is known to be highly toxic *in vivo* by mechanisms which may involve free radical production and oxidative stress. It is possible, therefore, that triaminopyridine may be similarly toxic in animals and that this metabolite could be responsible for some of the harmful side-effects associated with phenazopyridine use.

KEY WORDS: Phenazopyridine, 2,3,6-triaminopyridine, hydrogen peroxide, superoxide radical, oxidative damage.

INTRODUCTION

The azo dyes, Chrysoidine, Direct Black 38, Prontosil rubrum and Brown FK are known to induce mutations in bacterial systems and to cause necrosis of skeletal and cardiac muscle in animals[1-5]. Although of diverse chemical structure, all these compounds undergo reductive cleavage *in vivo*[2,6,7], yielding the aromatic polyamine, 1,2,4-triaminobenzene (I). The latter compound is strongly mutagenic[1,3,8] and myotoxic[9], and it is likely that this metabolite is responsible for the toxic effects of the parent azo dyes.



(I)

1,2,4-Triaminobenzene readily autoxidizes *in vitro*, generating superoxide radical and hydrogen peroxide[10]. It also undergoes one-electron oxidation via the

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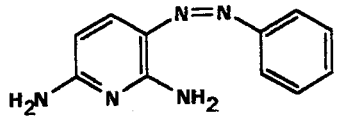
cytochrome c/cytochrome oxidase system of the respiratory chain[11] and it has been suggested that amine radicals and/or "active oxygen" species formed from this substance may be responsible for both its genotoxicity and its selective toxicity to muscle[10–12].

The drug phenazopyridine (II) (2,6-diamino-3-[phenylazo]pyridine) is used, in the form of its hydrochloride salt, as a urinary tract analgesic (Pyridium®, Parke-Davis). This compound is registered for use in many countries, and the world production may be as high as 150 tonnes/year[13]. Like the azo compounds mentioned above, phenazopyridine undergoes reduction in animals, yielding 2,3,6-triaminopyridine (III) and aniline (IV) as primary metabolites[14].



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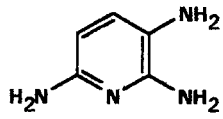

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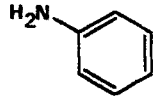


(IV)

In susceptible individuals, or after excessive doses, phenazopyridine consumption is associated with haemolytic anaemia[15,16], nephrotoxicity[17] and muscle damage[18]. The effects on blood have been attributed to aniline, a known haemolytic agent[14], but the possible involvement of 2,3,6-triaminopyridine in the adverse side-effects of phenazopyridine has not been considered.

In view of the structural similarity between 1,2,4-traminobenzene and 2,3,6-triaminopyridine, analogous chemical and toxicological properties are to be expected. As a first stage in the investigation of the toxicology of 2,3,6-triaminopyridine, the generation of superoxide radical and hydrogen peroxide during the autoxidation of this substance has been investigated, together with its ability to generate hydrogen peroxide and to cause oxidative damage in a model cellular system. The results are described in the present communication.

MATERIALS AND METHODS

2,3,6-Triaminopyridine dihydrochloride was synthesized by the method of Albert and Barlin[19]. Catalase (from bovine liver) was from Boehringer, superoxide dismutase (from bovine erythrocytes) was from Sigma. Chelex-100 was purchased from BioRad Laboratories and equilibrated to pH 7.0 before use.

The rate of oxidation of 2,3,6-triaminopyridine was determined by measurement of rate of oxygen consumption, using a Yellow Springs Instruments Model 53 oxygen monitor. The buffer was Chelex-treated 0.05 M phosphate (pH 7.0), the temperature was maintained at 25°C and the concentration of 2,3,6-triaminopyridine was 500 μM. In experiments in which the effects of catalase and superoxide dismutase were investigated, the enzymes were added before the triaminopyridine.

For preparation of erythrocyte suspensions, rat blood, containing EDTA as anticoagulant, was centrifuged and the plasma and buffy coat discarded. The cells were washed 3 times with isotonic phosphate buffer (pH 7.4) and resuspended in the same medium to a haematocrit of 5%. The cells were incubated at 37°C in a shaking water bath. After equilibration, 2,3,6-triaminopyridine, as a solution in isotonic saline, was added to give final concentrations of 200, 100, 50, 25 and 12.5 μM. Control suspensions received saline alone. Aliquots were withdrawn for assay at various time intervals.

Hydrogen peroxide generation in erythrocytes was detected by the method of Cohen and Hochstein[20] in which H_2O_2 production is reflected by inhibition of endogenous catalase in the presence of 3-amino-1,2,4-triazole. Methaemoglobin was measured in cell lysates by the technique of Evelyn and Malloy[21] and erythrocytic glutathione concentrations were determined by the method of Beutler, Duron and Kelly[22]. Cell lysis during incubation with triaminopyridine was assessed by comparison of the total haemoglobin content (measured as cyanmethaemoglobin[21]) of centrifuged cell suspensions with that of similar suspensions which had been lysed with saponin.

RESULTS

Rate of autoxidation of 2,3,6-triaminopyridine

At pH 7.0, a 500 μM solution of 2,3,6-triaminopyridine absorbed oxygen at a rate of 2.27 ± 0.11 μmoles/l/min (mean ± SEM, $n = 9$). The initial rate of oxygen uptake was decreased in a concentration-dependent manner by superoxide dismutase (Figure 1) and by catalase (Figure 2). The maximum inhibition induced by both enzymes was close to 50%.

Hydrogen peroxide formation and oxidative damage in erythrocytes exposed to 2,3,6-triaminopyridine

Hydrogen peroxide was generated in erythrocytes incubated with 2,3,6-triaminopyridine, as reflected by a dose-dependent inhibition of endogenous catalase in the presence of 3-amino-1,2,4-triazole (Table 1).

Haemoglobin was oxidized to methaemoglobin in cells exposed to triaminopyridine while cellular levels of glutathione declined. The severity of these effects was again dependent upon the amine concentration, with a significant effect on both parameters being recorded at 25 μM. Triaminopyridine did not, however, induce cell lysis; the percentage lysis was no greater in cells exposed to the amine than in control erythrocytes (Table 1).

DISCUSSION

2,3,6-Triaminopyridine readily autoxidized at neutral pH, as reflected by oxygen uptake from solution. As discussed in detail for 1,2,4-triaminobenzene[10] and N,N,N′,N′-tetramethyl-p-phenylenediamine[23], the autoxidation of aromatic polyamines is initiated by electron transfer from amine to oxygen (Reaction 1); superoxide formed in this process may oxidize more amine (Reaction 2).

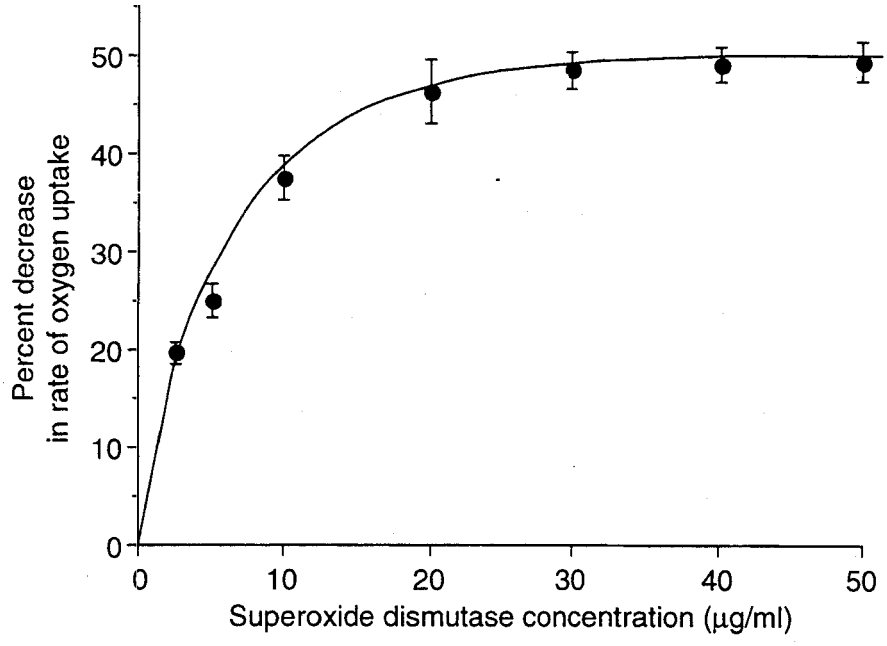


FIGURE 1 Inhibition by superoxide dismutase of oxygen uptake during the autoxidation of 2,3,6-triaminopyridine. Assays were conducted as described in Materials and Methods; results shown are the means and SEM of four separate determinations.

TABLE 1
Hydrogen peroxide generation, methaemoglobin formation, glutathione depletion and cellular lysis in erythrocytes exposed to 2,3,6-triaminopyridine

Concentration of 2,3,6-triaminopyridine (μM)	Hydrogen peroxide generation[a]	Methaemoglobin formation[b]	Glutathione depletion[c]	Cellular lysis[d]
0	2.2 ± 0.3	0.7 ± 0.2	0.0	2.3 ± 0.2
12.5	16.1 ± 1.9	0.3 ± 0.1	5.6 ± 1.8	2.1 ± 0.2
25	55.5 ± 1.4	2.2 ± 0.2	16.2 ± 2.5	2.7 ± 0.4
50	79.4 ± 1.1	3.9 ± 0.2	45.3 ± 1.0	2.1 ± 0.1
100	94.8 ± 0.8	11.0 ± 0.6	79.5 ± 2.3	2.4 ± 0.2
200	98.5 ± 0.2	25.4 ● 1.8	96.5 ± 1.3	2.2 ± 0.1

Rat erythrocytes (5% haematocrit) were incubated at 37°C with triaminopyridine at the concentrations shown. Assays were conducted as described in Materials and Methods; results shown are the means and SEM of four determinations.

[a]Measured as percentage inhibition of endogenous catalase after incubation with triaminopyridine in the presence of 3-amino-1,2,4-triazole for 2h.

[b]Expressed as a percentage of total haemoglobin. Methaemoglobin levels were determined after incubation with triaminopyridine for 4h.

[c]Percentage decrease in glutathione level after incubation with triaminopyridine for 4h. The initial concentration of reduced glutathione in the erythrocytes was 2.83 mmoles/l of packed cells.

[d]Percentage of lysed cells after incubation with triaminopyridine for 4h.

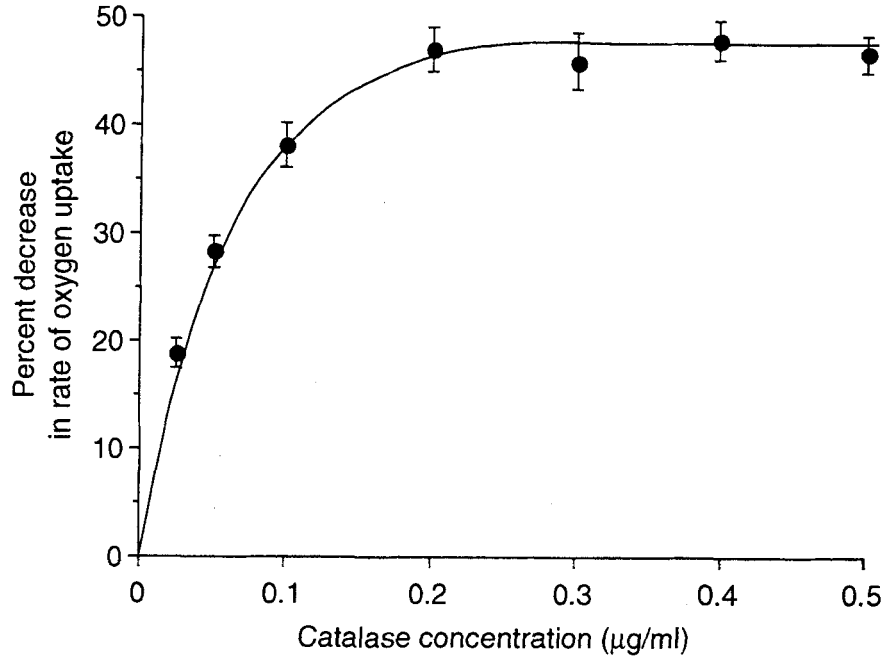
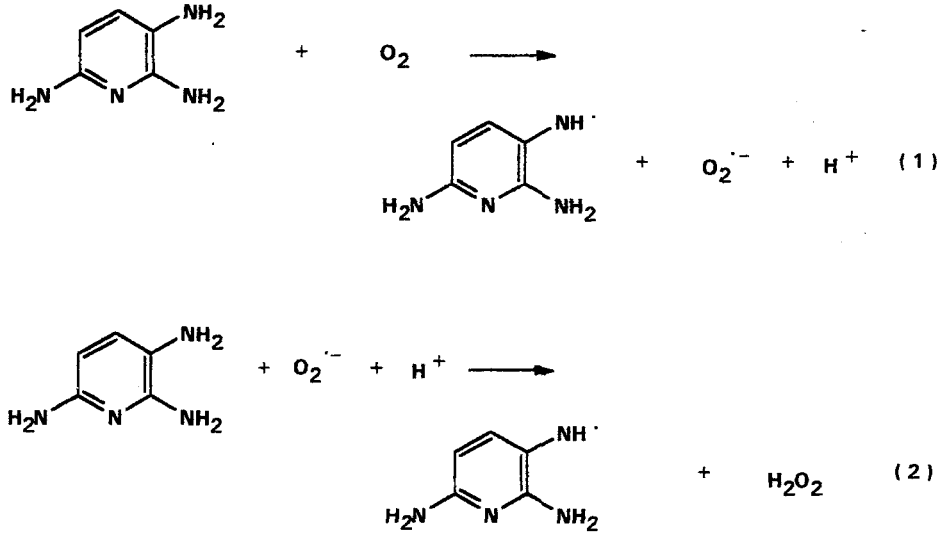


FIGURE 2 Inhibition by catalase of oxygen uptake during the autoxidation of 2,3,6-triaminopyridine. Assays were conducted as described in Materials and Methods; results shown are the means and SEM of five separate determinations.



Such a mechanism, with Reaction 1 being rate-limiting and irreversible, would be consistent with the effects of superoxide dismutase and catalase; both enzymes inhibited autoxidation to a degree close to the 50% expected for destruction of $O_2^{\cdot -}$ and H_2O_2 via reactions 3 and 4.

$$2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (3)$$

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \quad (4)$$

Erythrocytes provide a useful model system for study of intracellular generation of "active oxygen" species. Hydrogen peroxide was detected in such cells incubated with 2,3,6-triaminopyridine and oxidative damage occurred, as indicated by methaemoglobin formation and glutathione depletion. Similar changes have been observed in erythrocytes exposed to other aromatic polyamines[10,23,24]. In accord with data on other oxidizing agents[25], the oxidative damage induced in erythrocytes by triaminopyridine was not associated with cellular lysis.

2,3,6-Triaminopyridine is thus able to generate "active oxygen" species under physiological conditions and to cause oxidative damage to cells *in vitro*. In these respects, it closely resembles 1,2,4-triaminobenzene, suggesting that triaminopyridine may similarly be toxic to animals, and that this substance may be involved in the toxic side-effects of phenazopyridine. Of particular interest is the observation of muscle damage following an overdose of the drug[18]. Muscle is a comparatively rare target site of poisonous chemicals, but myotoxicity is a characteristic feature of autoxidizable polyaminobenzenes[9,24,26]. A study of the *in vivo* effects of 2,3,6-triaminopyridine would be of interest.

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