Myeloperoxidase–oxidase oxidation of cysteamine

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Cysteamine oxidation was shown to be catalysed by nanomolar concentrations of myeloperoxidase in a peroxidase–oxidase reaction, i.e. an O_2 -consuming oxidation of a compound catalysed by peroxidase without H_2O_2 addition. When auto-oxidation of the thiol was prevented by the metal-ion chelator diethylenetriaminepenta-acetic acid, native, but not heat-inactivated, myeloperoxidase induced changes in the u.v.-light-absorption spectrum of cysteamine. These changes were consistent with disulphide (cystamine) formation. Concomitantly, O_2 was consumed and superoxide radical anion formation could be detected by Nitro Blue Tetrazolium reduction. Both superoxide dismutase and catalase inhibited the reaction, whereas the hydroxyl-radical scavengers mannitol and ethanol did not. O_2 consumption increased with increasing pH (between pH 6.0 and 8.0), and 50 % inhibition was exhibited by about 3 mM-NaCl at pH 7.0 and by about 100 mM-NaCl at pH 8.0. Cysteamine was about 5 times as active (in terms of increased O_2 consumption at pH 7.5) as the previously reported peroxidase–oxidase substrates NADPH, dihydroxyfumaric acid and indol-3-ylacetic acid. A possible reaction pathway for the myeloperoxidase–oxidase oxidation of cysteamine is discussed. These results indicate that cysteamine is a very useful substrate for studies on myeloperoxidase–oxidase activity.

INTRODUCTION

Since the discovery of myeloperoxidase (MPO) by Agner (1941), three aspects of its catalytic activity have been studied. Initially, mainly the peroxidase reaction (i.e. H_2O_2 -dependent oxidation of compounds) was investigated with various organic compounds as substrates. Reactive oxidation products of this reaction were suggested by Agner (1947, 1950) to participate in detoxification of bacterial toxins. MPO gained increased interest when pseudohalides and halides were recognized as peroxidase substrates (Klebanoff *et al.*, 1966; Klebanoff, 1967, 1968; Zgliczyński *et al.*, 1971; Agner, 1972). The MPO/ H_2O_2 /halide system is a very powerful antimicrobial and toxic system and has been extensively studied (reviewed by Klebanoff & Clark, 1978, pp. 410–434). These catalytic activities mentioned above require the addition of H_2O_2 and therefore can only be expressed *in vivo* where H_2O_2 is generated, for example in connection with the oxidative burst of leucocytes.

In contrast with the peroxidase reactions of MPO, the peroxidase–oxidase reaction [i.e. O_2 -consuming oxidation of compounds catalysed by peroxidase without H_2O_2 addition (reviewed by Yamazaki & Yokota, 1973)] may occur without leucocyte activation. However, only fragmentary reports concerning this type of reaction have appeared. In his first report on MPO, Agner (1941) reported that dihydroxymaleic acid was a peroxidase–oxidase substrate for MPO. Later, NADH and NADPH were found to be oxidized by MPO (without H_2O_2 addition) if certain cofactors such as 2,4-dichlorophenol, thyroxine, ergothioneine or $MnCl_2$ were included in the reaction mixture (Klebanoff, 1959, 1962; Odajima & Yamazaki, 1970; Odajima, 1971; Takanaka & O'Brien, 1975). In this reaction superoxide radical anion is generated, and therefore MPO was once even considered as a candidate for the primary oxidase, responsible for

the respiratory burst of neutrophils (Kakinuma & Chance, 1977; Klebanoff & Clark, 1978, pp. 352–355).

Most studies on the peroxidase–oxidase reaction have been carried out with plant peroxidases, mainly horseradish peroxidase. Swedin & Theorell (1940) first reported the oxidation of dihydroxymaleic acid by plant peroxidase. In later studies with plant peroxidases, some additional peroxidase–oxidase substrates were found, such as indol-3-ylacetic acid, triose reductone, NAD(P)H and naphthohydroquinones (reviewed by Yamazaki & Yokota, 1973).

Our interest in cysteamine began when it was found that cysteamine increased the granulocytic activity as measured by zymosan-induced iodination (S. Lindvall & K. Domeij, unpublished work). In the present paper we describe the properties of MPO-mediated cysteamine oxidation and show that cysteamine is a useful substrate for studies on MPO–oxidase activity.

EXPERIMENTAL

Chemicals

Cysteamine and its hydrochloride salt, cystamine dihydrochloride and taurine were bought from Fluka (Buchs, Switzerland). Nitro Blue Tetrazolium, diethylenetriaminepenta-acetic acid (DTPA), D-mannitol, guaiacol, NADPH, dihydroxyfumaric acid and hypotaurine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.) H_2O_2 (Perhydrol) and indol-3-ylacetic acid were obtained from Merck (Darmstadt, Germany). Desferrioxamine B methanesulphonate (Desferal) was from CIBA-GEIGY (Basle, Switzerland), NaOCl from Kebo-Lab (Spånga, Sweden) and ethanol (99.5 %) from Svensk Sprit (Stockholm, Sweden). Determination of the purity of the cysteamine base showed that it contained 30 % as its disulphide (cystamine), as analysed by its u.v.-

absorption spectrum. The impurity of the cysteamine base was taken into account when the thiol solutions were prepared for the experiments (Figs. 6 and 7). In order to prevent further auto-oxidation the thiol was dissolved in dilute H_2SO_4 (final concentration $200\ \mu\text{M}$).

Biochemicals

Chronic-myeloid-leukaemia MPO (donor: H_2O_2 oxidoreductase, EC 1.11.1.7) was kindly donated by Professor I. Olsson (Olsson *et al.*, 1972). It was further purified by CM-Sepharose CL-6B and Sephacryl S-200 column chromatography and the cation-exchange peaks II and IV were used after dialysis against distilled water (Svensson *et al.*, 1987). The absorption ratios A_{430}/A_{280} were 0.81 and 0.83 and the specific enzyme activities with guaiacol as substrate were 648 kat/mol and 677 kat/mol. Normal human MPO (cation-exchange peak IV; A_{430}/A_{280} 0.83; 749 kat/mol; isolated as previously described) was used without dialysis (Fig. 7 and Table 2). The enzyme concentration was calculated from the molar absorption coefficient ϵ_{430} $1.78 \times 10^5\ \text{M}^{-1}\cdot\text{cm}^{-1}$ (Agner, 1958). Heat inactivation was carried out by incubating $0.28\ \mu\text{M}$ -MPO in distilled water in a sealed ampoule at 95°C for 40 min. Human superoxide dismutase (SOD, $\text{O}_2^{\cdot-}:\text{O}_2^{\cdot-}$ oxidoreductase, EC 1.15.1.1), bovine catalase ($\text{H}_2\text{O}_2:\text{H}_2\text{O}_2$ oxidoreductase, EC 1.11.1.6) and bovine albumin were bought from Sigma Chemical Co. and were all dialysed against distilled water before use to minimize metal-ion contamination. The specific activities of SOD and catalase were 1800 and 31000 units/mg respectively. Since commercial catalase may contain significant amounts of SOD (Halliwell, 1973; Trotta *et al.*, 1974), the catalase was assayed for SOD activity. No activity was found, which means that $16\ \mu\text{g}$ of the catalase contained less than 1 ng of SOD. Where indicated, SOD (as a $0.1\ \text{mg/ml}$ aqueous solution in a sealed ampoule) was heated at 120°C for 20 min in an autoclave.

Methods

Cysteamine oxidation was monitored by scanning the u.v.-absorption spectrum (1200 nm/min) at time intervals at 37°C in a Beckman DU-7HS spectrophotometer equipped with a kinetics accessory. The spectral changes were always consistent with cystamine formation. The initial cysteamine oxidation rate was calculated from the absorption change at $270\ \text{nm}$ by using ϵ_{270} $91\ \text{M}^{-1}\cdot\text{cm}^{-1}$ at pH 7.0 and ϵ_{270} $51\ \text{M}^{-1}\cdot\text{cm}^{-1}$ at pH 8.0 [the difference in molar absorption coefficient between cysteamine in its oxidized form (cystamine) and reduced form].

O_2 consumption was measured with a Clark-type oxygen electrode covered with a standard Teflon membrane and a model 53 O_2 monitor equipped with a Micro field conversion kit (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.). Air-saturated reaction mixture in a total volume of $1.0\ \text{ml}$ was equilibrated at 37°C and meter-adjusted to 100% saturation. Reaction was started by the addition of a $10\ \mu\text{l}$ portion of enzyme. Under the conditions in our experiments the initial O_2 concentration was assumed to be $200\ \mu\text{M}$ (Lessler & Brierley, 1969), which was used for the calculation of the initial rate of O_2 consumption.

Nitro Blue Tetrazolium reduction was measured as previously described (Svensson *et al.*, 1987).

SOD activity was determined as described by McCord

& Fridovich (1969). One unit inhibits the reduction of cytochrome *c* by 50% under the specified conditions.

Catalase activity was determined by the addition of about 50 ng of catalase/ml to $12\ \text{mM}$ - H_2O_2 in $50\ \text{mM}$ -potassium phosphate buffer, pH 7.0, at 25°C and the initial decrease of absorption at $240\ \text{nm}$ was measured. Specific activity was calculated by using ϵ_{240} $40\ \text{M}^{-1}\cdot\text{cm}^{-1}$ (Nelson & Kiesow, 1972). One unit decomposes $1.0\ \mu\text{mol}$ of $\text{H}_2\text{O}_2/\text{min}$ under these conditions.

Specific enzyme activity of MPO was determined with guaiacol as substrate in $0.1\ \text{M}$ -potassium phosphate buffer, pH 8.0, as described by Himmelhoch *et al.* (1969). Enzyme activity was expressed as mol of H_2O_2 consumed/s (= 1 kat) by using ϵ_{470} $26.6\ \text{mM}^{-1}\cdot\text{cm}^{-1}$ for tetraguaiacol (Chance & Maehly, 1955).

NaOCl was standardized iodometrically (Custer & Natelson, 1949; Kimura *et al.*, 1969).

RESULTS

MPO-dependent cysteamine oxidation

When a solution of $400\ \mu\text{M}$ -cysteamine was incubated in $50\ \text{mM}$ -potassium phosphate buffer, pH 7.0, at 37°C , progressive changes occurred in the u.v.-absorption spectrum, with an increase of the absorption at $270\ \text{nm}$,

Fig. 1. Auto-oxidation and MPO-mediated oxidation of cysteamine

The u.v.-absorption spectrum was scanned at time intervals after cysteamine addition (A_{270} is shown in the Figure). All incubation mixtures ($1.0\ \text{ml}$) contained $400\ \mu\text{M}$ -cysteamine hydrochloride in $50\ \text{mM}$ -potassium phosphate buffer, pH 7.0. Additions: $56\ \text{nM}$ -MPO (677 kat/mol) (curve c, native enzyme; curve d, heat-inactivated enzyme) and $200\ \mu\text{M}$ -DTPA (curves b-d). Mean values and ranges of duplicate experiments (curve c, seven experiments) are shown.

which means that cysteamine auto-oxidizes during these conditions (Fig. 1, curve a). DTPA ($200\ \mu\text{M}$) effectively prevented this auto-oxidation (curve b). This is in accordance with the suggestion made by Misra (1974) that traces of transition-metal ions catalyse thiol auto-oxidation with concomitant O_2 consumption and superoxide radical anion formation. Also, in the presence of the chelator no O_2 consumption or superoxide radical anion formation could be detected (see below). In contrast, $200\ \mu\text{M}$ -desferrioxamine did not affect the changes in the u.v.-absorption spectrum of cysteamine (results not shown), which indicates that under the conditions in our experiments trace metal(s) other than iron seem(s) to promote cysteamine auto-oxidation. However, even in the presence of $200\ \mu\text{M}$ -DTPA, progressive changes occurred with time in the u.v.-absorption spectrum if $56\ \text{nM}$ -MPO was added (curve c). Increasing the DTPA concentration to $1000\ \mu\text{M}$ did not decrease the absorption changes. Native MPO was necessary for the changes to occur, since heat-inactivated enzyme induced only minute spectral changes (curve d), nor did native MPO without cysteamine show any changes (results not shown).

Comparison of the MPO-dependent changes in the u.v.-absorption spectrum of cysteamine with the spectra of potential oxidation products, i.e. the corresponding disulphide (cystamine), sulphinic acid (hypotaurine) and sulphonic acid (taurine), showed that the disulphide was formed (Fig. 2a). The spectral conversion of cysteamine into cystamine had an isosbestic point at $250\ \text{nm}$.

In the studies above, no H_2O_2 was added and the thiol

auto-oxidation was prevented by the metal-ion chelator. These results indicate that MPO might act as an oxidase when cysteamine is the substrate, since O_2 was the only possible oxidant in the reaction mixture.

This prompted us to investigate the O_2 consumption during the reaction (Fig. 3). An O_2 uptake was observed (curve a), which was dependent on the simultaneous presence of both MPO (curve b) and cysteamine (curve c). Heat-inactivated MPO could not induce O_2 consumption (curve d). The native-MPO-dependence and the concomitant O_2 consumption without H_2O_2 addition means that this cysteamine oxidation is an MPO-oxidase reaction.

The fate of the consumed O_2 was investigated with Nitro Blue Tetrazolium-reduction experiments. The results indicated that a reduction of the dye occurred during the MPO-mediated cysteamine oxidation (see Table 1). An initial rate of Nitro Blue Tetrazolium reduction could not be estimated, since the initial linear part of the curve was too short ($< 30\ \text{s}$), and therefore the absorption increase after $2.5\ \text{min}$ was used. Both cysteamine and MPO in the reaction mixture were necessary for the dye reduction to occur. These results imply that cysteamine cannot transfer electrons directly to Nitro Blue Tetrazolium under the conditions in our experiments. This has been observed with thiols at higher pH (Misra, 1974). However, the dye reduction in the peroxidase-oxidase reaction was inhibited by SOD (see below), which indicates that superoxide radical anion is responsible for the dye reduction. Also, the thiol and disulphide anion radicals, which might be formed during

Fig. 2. Changes in the u.v.-absorption spectrum of cysteamine mediated by MPO and NaOCl

(a) Incubation mixtures contained $56\ \text{nM}$ -MPO ($677\ \text{kat/mol}$)/ $200\ \mu\text{M}$ -DTPA/ $50\ \text{mM}$ -potassium phosphate buffer, pH 7.0. Cysteamine hydrochloride ($400\ \mu\text{M}$), cystamine dihydrochloride ($200\ \mu\text{M}$), hypotaurine ($400\ \mu\text{M}$) and taurine ($400\ \mu\text{M}$) were added as indicated and spectra were recorded. The extent of cysteamine oxidation was increased by the addition of MPO in two steps (half at the start and half after $29\ \text{min}$). To obtain the spectrum of unoxidized cysteamine ($0\ \text{min}$), $1\ \mu\text{g}$ of SOD/ml was included in the incubation mixture (see Table 1). (b) Cysteamine hydrochloride ($400\ \mu\text{M}$) and cystamine dihydrochloride ($200\ \mu\text{M}$) were added as indicated to $200\ \mu\text{M}$ -DTPA/ $50\ \text{mM}$ -potassium phosphate buffer, pH 7.0, and spectra were recorded. Then $50\ \mu\text{M}$ -, $100\ \mu\text{M}$ - or $200\ \mu\text{M}$ -NaOCl was added to the cysteamine mixture as indicated and spectra were recorded after $1.5\ \text{min}$.

the reaction (see the Discussion section), cannot reduce Nitro Blue Tetrazolium, since upon addition of inhibitory concentration of SOD and addition of H_2O_2 no dye reduction could be detected, even though cysteamine oxidation did occur (Table 1). These results show that univalent reduction of O_2 to superoxide radical anion occurs during the MPO-oxidase oxidation of cysteamine.

Involvement of reduced oxygen species

Since superoxide radical anion was formed in the reaction mixture, the involvement of various reduced oxygen species in MPO-mediated cysteamine oxidation was investigated by using SOD, catalase and hydroxyl-radical scavengers (Table 1). Native (but not heat-inactivated) SOD at $1 \mu\text{g}/\text{ml}$ completely inhibited cysteamine oxidation and O_2 consumption. A 50% inhibition of the initial rate of cysteamine oxidation was obtained by about $0.1 \mu\text{g}$ of SOD/ml. Also, catalase ($16 \mu\text{g}/\text{ml}$)

Fig. 3. O_2 consumption during MPO-mediated cysteamine oxidation

The complete reaction mixture contained $400 \mu\text{M}$ -cysteamine hydrochloride/ 56 nM -MPO ($677 \text{ kat}/\text{mol}$)/ $200 \mu\text{M}$ -DTPA/ 50 mM -potassium phosphate buffer, pH 7.0. Omission and substitution of reaction mixture components are indicated. Typical time traces are shown.

inhibited cysteamine oxidation and O_2 consumption completely, and 50% inhibition of cysteamine oxidation was obtained by about $1 \mu\text{g}$ of the enzyme/ml. Albumin ($100 \mu\text{g}/\text{ml}$) did not affect the cysteamine oxidation or O_2 consumption rate, which means that the inhibition of catalase was not a non-specific protein effect. The simultaneous presence of both SOD and catalase in the incubation mixture also resulted in inhibition of cysteamine oxidation. The addition sequence of SOD ($0.05 \mu\text{g}/\text{ml}$) or catalase ($1.6 \mu\text{g}/\text{ml}$) and MPO to the reaction mixture did not affect their inhibition of the cysteamine oxidation rate. Addition of SOD ($0.5 \mu\text{g}/\text{ml}$) or catalase ($16 \mu\text{g}/\text{ml}$) 3 min after cysteamine addition prevented further cysteamine oxidation. Addition of $10 \mu\text{M}$ - H_2O_2 to the reaction mixture, containing 56 nM -MPO, 1 min before cysteamine increased the initial rate of cysteamine oxidation by 40%. However, in experiments with 224 nM -MPO we found no increased oxidation rate (results not shown). The hydroxyl-radical scavengers mannitol and ethanol had no effect on cysteamine oxidation or O_2 consumption at concentrations up to 100 mM (Table 1). Only higher ethanol concentration (500 mM) resulted in a diminished cysteamine oxidation rate, i.e. about 50% inhibition (results not shown). In conclusion, the inhibition by catalase and SOD means that H_2O_2 , in addition to superoxide radical anion, is formed during the MPO-oxidase oxidation of cysteamine and also that both of these reduced oxygen species are necessary intermediates in the reaction.

Influence of cysteamine and MPO concentrations

The initial cysteamine oxidation and O_2 consumption rates were investigated at different cysteamine concentrations with a fixed MPO concentration (56 nM). Both cysteamine oxidation and O_2 consumption rates increased with increasing cysteamine concentration (Fig. 4). However, the rate did not increase linearly with cysteamine concentration. Both cysteamine oxidation and O_2 consumption seemed to increase in an exponential mode with cysteamine concentration. At a cysteamine

Table 1. Effects of SOD, catalase and hydroxyl-radical scavengers on MPO-mediated cysteamine oxidation, O_2 consumption and Nitro Blue Tetrazolium reduction

The complete reaction mixture contained $400 \mu\text{M}$ -cysteamine hydrochloride/ 56 nM -MPO ($677 \text{ kat}/\text{mol}$)/ $200 \mu\text{M}$ -DTPA/ 50 mM -potassium phosphate buffer, pH 7.0, and $25 \mu\text{M}$ -Nitro Blue Tetrazolium (in the assay of Nitro Blue Tetrazolium reduction). Additions and omissions of reaction mixture constituents are indicated.

	Initial cysteamine oxidation rate ($\mu\text{M}/\text{min}$)			Initial O_2 consumption rate ($\mu\text{M}/\text{min}$)			Nitro Blue Tetrazolium reduction ($10^4 \times \Delta A_{560}/2.5 \text{ min}$)		
	Mean	(range)	<i>n</i>	Mean	(range)	<i>n</i>	Mean	(range)	<i>n</i>
Complete	20	(19–22)	7	4.2	(3.4–5.2)	8	106	(104–110)	3
+SOD ($1 \mu\text{g}/\text{ml}$)	–1	(–2–0)	2	–0.4	(–0.6––0.2)	2	1	(1–2)	2
+SOD (heated) ($1 \mu\text{g}/\text{ml}$)	20	(17–22)	2	4.4	(4.0–4.8)	2	–	–	–
+SOD ($5 \mu\text{g}/\text{ml}$) + H_2O_2 ($10 \mu\text{M}$)	16	(14–17)	4	–	–	–	–3	(–7–1)	2
– MPO + SOD ($5 \mu\text{g}/\text{ml}$) + H_2O_2 ($10 \mu\text{M}$)	1	(1–1)	2	–	–	–	–	–	–
+Catalase ($16 \mu\text{g}/\text{ml}$)	0	(–1–1)	2	–0.4	(–0.6–0.0)	2	2	(0–4)	2
+Albumin ($100 \mu\text{g}/\text{ml}$)	20	(19–21)	2	4.2	(4.2–4.2)	2	103	(96–110)	2
+SOD ($1 \mu\text{g}/\text{ml}$) + catalase ($16 \mu\text{g}/\text{ml}$)	–1	(–2–0)	2	–	–	–	–	–	–
+Mannitol (100 mM)	19	(19–20)	2	5.0	(4.8–5.2)	2	–	–	–
+Ethanol (100 mM)	17	(15–20)	2	4.4	(4.2–4.6)	2	–	–	–

Fig. 4. Effect of cysteamine concentration on MPO-mediated cysteamine oxidation and O₂ consumption

The incubation mixtures contained 56 nM-MPO (677 kat/mol)/200 μM-DTPA/50 mM-potassium phosphate buffer, pH 7.0, and concentrations of cysteamine hydrochloride as indicated. Some cysteamine oxidation did occur with 1600 μM-cysteamine also in the absence of MPO (5% of that with MPO). Mean values and ranges of duplicate experiments are shown.

concentration of 400 μM, both cysteamine oxidation and O₂ consumption increased with increasing MPO concentration (Fig. 5) when the enzyme concentration was low (< 50 nM). Higher enzyme concentration did not result in higher activity. With 840 nM-MPO the cysteamine oxidation rate was only 20% of the activity with 112 nM-MPO, which means that excess MPO is inhibitory.

In the concentration intervals of MPO and cysteamine used in Figs. 4 and 5 we found that about 4 mol of cysteamine was oxidized/mol of O₂ consumed. Since cysteamine oxidation (to cystamine) is a one-electron oxidation process, we conclude that O₂ is ultimately reduced to water, although superoxide radical anion and H₂O₂ were detected as intermediates (see above). This means that H₂O₂ does not accumulate during the reaction with these conditions, i.e. that the further reduction of H₂O₂ is not a rate-limiting reaction step.

Effects of pH and chloride

The effect of pH on O₂ consumption is shown in Fig. 6. O₂ consumption increased with increasing pH in the pH interval investigated. This dependence on pH of activity seems to coincide with the deprotonation of cysteamine [$pK_{a,SH} = 8.6$ (Fasman, 1976)]. Also, auto-oxidation of thiols is known to increase with increasing pH. However,

Fig. 5. Effect of MPO concentration on MPO-mediated cysteamine oxidation and O₂ consumption

The incubation mixture contained 400 μM-cysteamine hydrochloride/200 μM-DTPA/50 mM-potassium phosphate buffer, pH 7.0, and concentrations of MPO (677 kat/mol) as indicated. Mean values and ranges of duplicate experiments are shown.

Fig. 6. Effect of pH on MPO-mediated O₂ consumption

Incubation mixtures contained 400 μM-cysteamine (base), 56 nM-MPO II (----, 648 kat/mol) or 56 nM-MPO IV (—, 677 kat/mol), 200 μM-DTPA and 50 mM-potassium phosphate buffer at the pH indicated. Mean values and ranges of duplicate measurements are shown.

in the presence of 200 μM-DTPA the initial O₂ consumption rate was less than 0.3 μM/min even at pH 8.0 in the absence of MPO, which means that thiol auto-oxidation is not responsible for the increased O₂ consumption at higher pH. No difference in sensitivity to pH between MPO peak II and peak IV was found. The

Fig. 7. Effect of chloride on MPO-mediated O_2 consumption

The incubation mixtures contained $400\ \mu\text{M}$ -cysteamine (base)/ $56\ \text{nM}$ -MPO ($749\ \text{kat/mol}$)/ $200\ \mu\text{M}$ -DTPA/ $50\ \text{mM}$ -potassium phosphate buffer, pH 7.0 or 8.0, and supplemented with NaCl at the concentrations indicated. Typical time traces are shown.

similarity of response to pH changes by MPO peaks II and IV gives further support for the suggestion that the different MPO forms obtained upon cation-exchange chromatography have the same enzymic properties (Svensson *et al.*, 1987). Comparison of the changes induced at pH 8.0 in the u.v.-absorption spectrum of cysteamine with that of cystamine showed that cystamine was also formed at pH 8.0. The initial cysteamine oxidation rate, as measured by the absorption increase at $270\ \text{nm}$, was $52\ (45\text{--}59)\ \mu\text{M/min}$ (mean value and range of triplicate experiments with MPO peak IV, $677\ \text{kat/mol}$). This gives $4\text{--}5\ \text{mol}$ of oxidized cysteamine/mol of O_2 consumed, which indicates that at pH 8.0 also no accumulation of H_2O_2 occurs. Also, with $50\ \text{mM}$ -Tris/HCl buffer, pH 8.0, MPO-dependent changes occurred in the u.v.-absorption spectrum of cysteamine that were consistent with cystamine formation. However, the initial rate of cysteamine oxidation was only 30% of that with phosphate buffer. This lower activity with Tris/HCl buffer may be explained by the inhibitory effect of chloride (see below).

The effect of chloride on O_2 consumption at pH 7.0 and pH 8.0 was investigated. Typical results are shown in Figs. 7(a) and 7(b). At pH 7.0 no inhibition of the initial rate of O_2 consumption was observed with chloride concentrations below $1\ \text{mM}$. At pH 7.0 a complete inhibition was obtained at $10\ \text{mM}$ -chloride, whereas only slight inhibition was obtained at pH 8.0 with the same chloride concentration. At pH 8.0 a considerable O_2 consumption occurred even with 10-fold higher concentration ($100\ \text{mM}$ -chloride). With $100\ \text{mM}$ -chloride in the absence of enzyme no O_2 consumption was observed.

To investigate the product formed with chloride in the reaction mixture we studied the changes in the u.v.-absorption spectrum of cysteamine during the reaction. With the same conditions as in Fig. 2(a), but with $3\ \text{mM}$ -NaCl in the reaction mixture, we found that the changes were still consistent with cystamine formation. The rate of changes was decreased as compared with experiments

without added NaCl (results not shown), which is analogous to the O_2 consumption experiments in Fig. 7(a).

Since H_2O_2 is generated during the MPO-oxidase oxidation of cysteamine (see above), Cl^- might be oxidized to HOCl during the conditions in Fig. 7 (Zgliczyński, 1980). To investigate the reaction between HOCl and cysteamine, we first studied the changes in the u.v.-absorption spectrum of cysteamine during the reaction. When NaOCl was added to reaction mixtures containing cysteamine, rapid changes occurred in the u.v.-absorption spectrum (essentially complete in $1.5\ \text{min}$) that were consistent with cystamine formation (Fig. 2b). When $50\text{--}200\ \mu\text{M}$ -NaOCl was substituted for MPO in the complete reaction mixture (see Table 1), no Nitro Blue Tetrazolium reduction was observed ($\Delta A_{560}/2.5\ \text{min} < 4 \times 10^{-4}$). NaOCl ($200\ \mu\text{M}$) did not oxidize the reduced Nitro Blue Tetrazolium formed in complete reaction mixtures with MPO as measured by A_{560} . Thus, although NaOCl can oxidize cysteamine to cystamine, this oxidation does not seem to be accompanied by superoxide radical anion generation.

Comparison of peroxidase-oxidase substrates

NADH and NADPH have previously been reported to be MPO-oxidase substrates. In contrast with the MPO-mediated cysteamine oxidation, NADH oxidation catalysed by horseradish peroxidase has an acidic pH optimum (Yokota & Yamazaki, 1965). At pH 7.5 and in the presence of DTPA only slight O_2 consumption was observed (Table 2). Even at 5-fold higher concentration ($2\ \text{mM}$) the initial O_2 consumption rate was less than that induced by $400\ \mu\text{M}$ -cysteamine. Only minute O_2 consumption without MPO (i.e. auto-oxidation) was observed at $2\ \text{mM}$ -NADPH.

Dihydroxyfumaric acid has also been reported to be an MPO-oxidase substrate. Some O_2 consumption was observed with $400\ \mu\text{M}$ - and $2\ \text{mM}$ -dihydroxyfumaric acid, which was dependent upon the presence of MPO in the

Table 2. Comparison of cysteamine with previously reported peroxidase-oxidase substrates

The incubation mixture contained 56 nM-MPO (749 kat/mol)/200 μ M-DTPA/50 mM-potassium phosphate buffer, pH 7.5, and peroxidase-oxidase substrate at concentration indicated. Mean values and ranges of triplicate experiments are shown.

Peroxidase-oxidase substrate	Initial O ₂ consumption rate (μ M/min)
Cysteamine hydrochloride (400 μ M)	8.4 (7.3–9.3)
NADPH (400 μ M)	1.5 (1.0–1.7)
NADPH (2 mM)	3.8 (3.3–4.3)
NADPH (2 mM) without MPO	0.3 (0.0–0.5)
Dihydroxyfumaric acid (400 μ M)*	1.3 (1.0–1.7)
Dihydroxyfumaric acid (2 mM)*	2.3 (2.0–2.7)
Dihydroxyfumaric acid (2 mM)* without MPO	0.0 (0.0–0.0)
Indol-3-ylacetic acid (400 μ M)*	1.8 (1.6–2.0)
Indol-3-ylacetic acid (400 μ M)* without MPO	0.0 (0.0–0.1)

* Incubation mixtures contained 100 mM-ethanol.

reaction mixture. However, the activity was much less than that observed with cysteamine under these conditions.

Several reports have described indol-3-ylacetic acid as a peroxidase-oxidase substrate for horseradish peroxidase. At a concentration of 400 μ M we found an MPO-dependent initial rate of O₂ consumption of 1.8 μ M/min. With 2 mM-indol-3-ylacetic acid we observed a higher initial rate of O₂ consumption. However, the O₂ consumption ceased in less than 2 min, which made it difficult to obtain accurate measurements of the initial rate. A possible explanation for this may be that generated free radicals (Mottley & Mason, 1986) destroy the enzyme at this high indol-3-ylacetic acid/MPO ratio. Ethanol, at a final concentration of 100 mM, was included in the reaction mixture to solubilize dihydroxyfumaric acid and indol-3-ylacetic acid. However, since this concentration of ethanol did not affect cysteamine oxidation (see Table 1) we assume that it does not affect the results in Table 2.

DISCUSSION

We have shown that cysteamine is an MPO-oxidase substrate at physiological pH and that this reaction can be studied under conditions where no auto-oxidation of the thiol occurs. To avoid the problem with auto-oxidation we have, throughout this study, used a metal-ion chelator at a concentration that prevents auto-oxidation.

Indications for the involvement of thiols at high concentrations in peroxidase-oxidase reactions was found by Olsen & Davis (1976) and Harman *et al.* (1984). The former researchers found an O₂ consumption and thiol oxidation when mixing 5 mM-dithiothreitol and 1.5 μ M horseradish peroxidase. The high concentrations of thiol and enzyme and the absence of chelator in the reaction mixture make it likely that auto-oxidation contributed to the activity observed. However, in the

presence of 1 mM-DTPA, Harman *et al.* (1984) detected thiyl radicals and O₂ consumption in a mixture containing 10 mM-cysteine and 2.5 μ M horseradish peroxidase.

The inhibition of SOD and catalase both when added before and 3 min after cysteamine addition indicate that both superoxide radical anion and H₂O₂ are involved in propagating steps of MPO-mediated cysteamine oxidation. The possibility that the SOD inhibition was due to H₂O₂ accumulation can be ruled out since H₂O₂ addition did not inhibit the reaction (rather, it stimulated the reaction) and since catalase also inhibited the reaction. The finding that superoxide radical anion is a necessary intermediate in MPO-mediated cysteamine oxidation may explain why only an initial brief Nitro Blue Tetrazolium reduction was obtained, since Nitro Blue Tetrazolium scavenges superoxide radical anion [$K = 6 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Bielski & Richter, 1977)]. Peroxidase-oxidase reactions with NADH and dihydroxyfumaric acid have also been found to be SOD-sensitive, whereas with indol-3-ylacetic acid the reaction has been found to be less sensitive or insensitive (Odajima, 1971; Yamazaki & Yamazaki, 1973; Smith *et al.*, 1982). However, in contrast with cysteamine oxidation, NADH oxidation was not inhibited by SOD when added halfway during the reaction (Odajima, 1971; Halliwell, 1978a), which indicates that in this case superoxide is only required for initiation. Dihydroxyfumaric acid, NADH and indol-3-ylacetic acid oxidations by horseradish peroxidase were inhibited by catalase, although higher catalase concentrations compared with the MPO-mediated cysteamine oxidation were required (Halliwell, 1977, 1978a; Smith *et al.*, 1982).

The concomitant formation of superoxide radical anion and H₂O₂ during MPO-mediated cysteamine oxidation makes the formation of hydroxyl radicals possible through a metal-ion-catalysed Haber-Weiss reaction (Van Hemmen & Meuling, 1977; Halliwell, 1978b). Since hydroxyl-radical scavengers did not inhibit the reaction, this radical does not seem to be a necessary intermediate in MPO-mediated cysteamine oxidation. This is in contrast with results of studies on thiol auto-oxidation (Misra, 1974). Halliwell (1977, 1978a) found also that hydroxyl-radical scavengers exhibited only slight inhibition on dihydroxyfumaric acid and NADH oxidation catalysed by horseradish peroxidase.

Reactions generating superoxide radical anion during MPO-oxidase oxidation of cysteamine were considered. A possible reaction might be the reduction of O₂ by disulphide radical anion [which is known to be formed from thiyl radical and thiol (Hoffman & Hayon, 1973)], yielding superoxide radical anion and disulphide (Mićić *et al.*, 1978). Alternatively, superoxide radical anion has been suggested to be formed from MPO compound III (Winterbourn *et al.*, 1985). In our reaction mixtures this would require the reduction of ferric MPO by cysteamine, yielding thiyl radical and ferro-MPO, followed by addition of O₂ to ferro-MPO yielding compound III (Odajima & Yamazaki, 1970). This reaction sequence should, however, be insensitive to catalase. Since no cysteamine oxidation, O₂ consumption or superoxide radical anion generation was observed in the presence of catalase, compound III seems to be an unlikely source of superoxide radical anion under the conditions in our experiments. A third reaction, the reduction of traces of metal ions [which are inevitably present in laboratory reagents (Wong *et al.*, 1981)] by cysteamine, followed by

reduction of O_2 to superoxide radical anion by the reduced metal ions (Misra, 1974), seems also unlikely, since we used a metal-ion chelator at a concentration where no auto-oxidation of cysteamine could be detected.

Peroxidases have in most cases been found to catalyse univalent oxidation of substrates (Nakamura *et al.*, 1985, and references cited therein) and univalent oxidation of thiols to thiyl free radicals by horseradish peroxidase/ H_2O_2 has been demonstrated by Harman *et al.* (1984, 1986). Thiyl radicals are, in turn, known to initiate chain reactions with concomitant O_2 consumption. On the basis of the work described above and the results reported in the present paper we propose a set of reactions that might occur during the MPO-oxidase oxidation of cysteamine [reactions (1)–(6)]:

The involvement of H_2O_2 as a necessary intermediate and the requirement for native MPO suggest the involvement of a peroxidatic oxidation step, i.e. the reaction sequence:

where the number in parentheses stands for the oxidizing equivalent retained in the enzyme (Yamazaki & Yokota, 1973). We propose a univalent oxidation of cysteamine (RSH) [reaction (1)], which is consistent with superoxide radical anion generation requiring the preformation of thiyl radicals ($RS\cdot$) (see above). The thiyl radical of cysteamine may then react with its thiolate anion (RS^-) to form disulphide radical anion ($RSSR^-$) [reaction (2); $K = 8 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Hoffman & Hayon, 1973)].

Hoffman & Hayon (1973) have found that the thiolate anion forms of thiols react more avidly than their protonated forms with thiyl radicals (to form disulphide radical anions). Our finding that O_2 consumption increased with increasing pH (and with increasing deprotonation of thiol; see Fig. 6) may therefore be explained if we assume that disulphide radical anion formation [reaction (2)] is a rate-limiting reaction of MPO-oxidase oxidation of cysteamine. This assumption [that reaction (2) is rate-limiting] is supported by the observed dependence on cysteamine concentration of MPO-oxidase activity (Fig. 4). Such an exponential-like dependence may be expected if the reaction pathway involves a rate-limiting step where cysteamine and/or its reaction product react with themselves.

Disulphide radical anion may then reduce O_2 to form disulphide ($RSSR$) and superoxide radical anion ($O_2^{\cdot -}$) [reaction (3); $K = 8 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Mićić *et al.*, 1978)]. The dismutation reaction [reaction (4); $K =$

$2 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Bielski & Allen, 1977)] can then regenerate H_2O_2 . The reaction sequence (1)–(4) generates, however, only 1 H_2O_2 (and consumes 1 H_2O_2), which is not consistent with the rapid burst of cysteamine oxidation, O_2 consumption and superoxide radical anion generation that we find. In addition, termination reactions, such as the dimerization of thiyl radicals [reaction (5) (Adams *et al.*, (1967))], may drain the reaction sequence of its intermediates, which also indicates that a surplus of reaction intermediates should be formed. On the basis of this and the finding that superoxide radical anion is an essential intermediate (see above), we propose that superoxide radical anion may also react with cysteamine [reaction (6)], which is analogous to the oxidation of other thiols by superoxide radical anion (Asada & Kanematsu, 1976). This reaction, in conjunction with reactions (1)–(3), generates a surplus of thiyl radical and H_2O_2 and therefore these reactions may be a possible explanation for the MPO-oxidase oxidation of cysteamine.

The inhibition of peroxidase-oxidase oxidation by excess enzyme (see Fig. 5) has previously been reported for dihydroxyfumaric acid oxidation catalysed by horseradish peroxidase (Yamazaki & Piette, 1963). These authors found that the inhibition was due to peroxidase compound III formation, since dihydroxyfumaric acid is a poor substrate for compound III. During the MPO-oxidase oxidation of cysteamine we find superoxide radical anion generation (see Table 1), and therefore MPO compound III may be formed by the reaction of superoxide radical anion and ferric MPO (Odajima, 1971). At higher MPO concentration, compound III formation may be expected to compete more efficiently with reaction (6). Thus, if we assume that cysteamine is a more sluggish substrate for compound III than for compound I and compound II [which has been found for all substrates investigated (Yamazaki & Yokota, 1973, and references cited therein)], then MPO compound III formation may be a possible explanation for the observed dependence on MPO concentration of MPO-oxidase activity.

We have shown that H_2O_2 is generated during the MPO-oxidase oxidation of cysteamine. Addition of NaCl to the reaction mixture might therefore cause HOCl generation (Zgliczyński, 1980). Although we found that NaOCl can oxidize cysteamine to cystamine, no superoxide radical anion generation was detected during this oxidation. A possible explanation for the chloride inhibition found in Fig. 7 might therefore be that the necessary regeneration of a surplus of reduced oxygen species is diminished when chloride is present in the reaction mixture. In addition, it is known that HOCl generated by the MPO/ H_2O_2 /Cl⁻ system can destroy MPO itself (Zgliczyński *et al.*, 1968). Support for these explanations is obtained from the difference in chloride-inhibition-sensitivity at pH 7.0 and 8.0, i.e. higher chloride concentrations were required for inhibition at pH 8.0 than at pH 7.0 (Figs. 7a and 7b). If we assume that the steady-state concentration of H_2O_2 is about the same at pH 7.0 and pH 8.0, then a higher chloride concentration would be required for equivalent HOCl formation at pH 8.0 than at pH 7.0 (Zgliczyński, 1980).

If occurring in biological systems, MPO-mediated cysteamine oxidation may be expected to occur in an environment with low chloride concentration and at pH

above neutrality. The MPO concentration in normal human blood has been reported to be about 1 nM (Hansen *et al.*, 1976; Olsson *et al.*, 1979; Venge *et al.*, 1984). Sufficient MPO may be present locally *in vivo*, but the cysteamine concentration in mammalian tissues has been reported to be very low (Ida *et al.*, 1984; Ziegler *et al.*, 1983). Since only slight cysteamine oxidation was observed at low cysteamine concentration (see Fig. 4), the MPO-mediated cysteamine oxidation seems to be of minor importance *in vivo* under normal conditions. However, cysteamine has been used in radiation protection (Phillips, 1977, and references cited therein) and in acetaminophen overdosage (Hughes *et al.*, 1977; Prescott *et al.*, 1977), where the higher cysteamine concentration might cause some MPO-oxidase reaction. To our knowledge the modes of action of cysteamine in these conditions have not been elucidated, nor has the mechanism of the side effects of cysteamine (Smith *et al.*, 1978, and references cited therein). Therefore a possible connection between the pharmacological effects of cysteamine and its property as a MPO-oxidase substrate cannot be excluded.

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REFERENCES

- Adams, G. E., McNaughton, G. S. & Michael, B. D. (1967) in *The Chemistry of Ionization and Excitation* (Johnson, G. R. A. & Scholes, G., eds.), pp. 281–293, Taylor and Francis, London.
- Agner, K. (1941) *Acta Chem. Scand.* **2**, Suppl. **8**, 1–62.
- Agner, K. (1947) *Nature (London)* **159**, 271–272.
- Agner, K. (1950) *J. Exp. Med.* **92**, 337–347.
- Agner, K. (1958) *Acta Chem. Scand.* **12**, 89–94.
- Agner, K. (1972) in *Structure and Function of Oxidation-Reduction Enzymes* (Åkeson, Å. & Ehrenberg, A., eds.), pp. 329–335, Pergamon Press, Oxford.
- Asada, K. & Kanematsu, S. (1976) *Agric. Biol. Chem.* **40**, 1891–1892.
- Bielski, B. H. J. & Allen, A. O. (1977) *J. Phys. Chem.* **81**, 1048–1050.
- Bielski, B. H. J. & Richter, H. W. (1977) *J. Am. Chem. Soc.* **99**, 3019–3023.
- Chance, B. & Maehly, A. C. (1955) *Methods Enzymol.* **2**, 764–775.
- Custer, J. J. & Natelson, S. (1949) *Anal. Chem.* **21**, 1005–1009.
- Fasman, G. D. (1976) *Handbook of Biochemistry and Molecular Biology: Physical and Chemical Data*, vol. 1, 3rd edn., pp. 305–351, CRC Press, Cleveland.
- Halliwel, B. (1973) *Biochem. J.* **135**, 379–381.
- Halliwel, B. (1977) *Biochem. J.* **163**, 441–448.
- Halliwel, B. (1978a) *Planta* **140**, 81–88.
- Halliwel, B. (1978b) *FEBS Lett.* **92**, 321–326.
- Hansen, N. E., Karle, H., Andersen, V., Malmquist, J. & Hoff, G. E. (1976) *Clin. Exp. Immunol.* **26**, 463–468.
- Harman, L. S., Mottley, C. & Mason, R. P. (1984) *J. Biol. Chem.* **259**, 5606–5611.
- Harman, L. S., Mottley, C. & Mason, R. P. (1986) *J. Biol. Chem.* **261**, 1642–1648.
- Himmelfoch, S. R., Evans, W. H., Mage, M. G. & Peterson, E. A. (1969) *Biochemistry* **8**, 914–921.
- Hoffman, M. Z. & Hayon, E. (1973) *J. Phys. Chem.* **77**, 990–996.
- Hughes, R. D., Gazzard, B. G., Hanid, M. A., Trewby, P. N., Murray-Lyon, I. M., Davis, M., Williams, R. & Bennet, J. R. (1977) *Br. Med. J.* **ii**, 1395.
- Ida, S., Tanaka, Y., Ohkuma, S. & Kuriyama (1984) *Anal. Biochem.* **136**, 352–356.
- Kakinuma, K. & Chance, B. (1977) *Biochim. Biophys. Acta* **480**, 96–103.
- Kimura, M., Murayama, K., Nomoto, M. & Fujita, Y. (1969) *J. Chromatogr.* **41**, 458–461.
- Klebanoff, S. J. (1959) *J. Biol. Chem.* **234**, 2480–2485.
- Klebanoff, S. J. (1962) *Biochim. Biophys. Acta* **56**, 460–469.
- Klebanoff, S. J. (1967) *J. Exp. Med.* **126**, 1063–1078.
- Klebanoff, S. J. (1968) *J. Bacteriol.* **95**, 2131–2138.
- Klebanoff, S. J. & Clark, R. A. (1978) *The Neutrophil: Function and Clinical Disorders*, North-Holland, Amsterdam.
- Klebanoff, S. J., Clem, W. H. & Luebke, R. G. (1966) *Biochim. Biophys. Acta* **117**, 63–72.
- Lessler, M. A. & Brierley, G. P. (1969) *Methods Biochem. Anal.* **17**, 1–29.
- McCord, J. M. & Fridovich, I. (1969) *J. Biol. Chem.* **244**, 6049–6055.
- Mićić, O. I., Nenadović, M. T. & Carapellucci, P. A. (1978) *J. Am. Chem. Soc.* **100**, 2209–2212.
- Misra, H. P. (1974) *J. Biol. Chem.* **249**, 2141–2145.
- Mottley, C. & Mason, R. P. (1986) *J. Biol. Chem.* **261**, 16860–16864.
- Nakamura, M., Yamazaki, I., Kotani, T. & Ohtaki, S. (1985) *J. Biol. Chem.* **260**, 13546–13552.
- Nelson, D. P. & Kiesow, L. A. (1972) *Anal. Biochem.* **49**, 474–478.
- Odajima, T. (1971) *Biochim. Biophys. Acta* **235**, 52–60.
- Odajima, T. & Yamazaki, I. (1970) *Biochim. Biophys. Acta* **206**, 71–77.
- Olsen, J. & Davis, L. (1976) *Biochim. Biophys. Acta* **445**, 324–329.
- Olsson, I., Olofsson, T. & Odeberg, H. (1972) *Scand. J. Haematol.* **9**, 483–491.
- Olsson, I., Olofsson, T., Ohlsson, K. & Gustavsson, A. (1979) *Scand. J. Haematol.* **22**, 397–406.
- Phillips, T. L. (1977) *Cancer* **39**, 987–998.
- Prescott, L. F., Park, J., Sutherland, G. R., Smith, I. J. & Proudfoot, A. T. (1977) *Lancet* **ii**, 109–113.
- Smith, A. M., Morrison, W. L. & Milham, P. J. (1982) *Biochemistry* **21**, 4414–4419.
- Smith, J. M., Roberts, W. O., Hall, S. M., White, T. A. & Gilbertson, A. A. (1978) *Br. Med. J.* **i**, 331–333.
- Svensson, B. E., Domeij, K., Lindvall, S. & Rydell, G. (1987) *Biochem. J.* **242**, 673–680.
- Swedin, B. & Theorell, H. (1940) *Nature (London)* **145**, 71–72.
- Takanaka, K. & O'Brien, P. J. (1975) *Biochem. Biophys. Res. Commun.* **62**, 966–971.
- Trotta, P. P., Pinkus, L. M. & Meister, A. (1974) *J. Biol. Chem.* **249**, 1915–1921.
- Van Hemmen, J. J. & Meuling, W. J. A. (1977) *Arch. Biochem. Biophys.* **182**, 743–748.
- Venge, P., Foucard, T., Henriksen, J., Håkansson, L. & Keuger, A. (1984) *Clin. Chim. Acta* **136**, 121–130.
- Winterbourn, C. C., Garcia, R. C. & Segal, A. W. (1985) *Biochem. J.* **228**, 583–592.
- Wong, S. F., Halliwel, B., Richmond, R. & Skowronek, W. R. J. (1981) *Inorg. Biochem.* **14**, 127–134.
- Yamazaki, H. & Yamazaki, I. (1973) *Arch. Biochem. Biophys.* **154**, 147–159.
- Yamazaki, I. & Piette, L. H. (1963) *Biochim. Biophys. Acta* **77**, 47–64.
- Yamazaki, I. & Yokota, K. (1973) *Mol. Cell. Biochem.* **2**, 39–52.

- Yokota, K. & Yamazaki, I. (1965) *Biochim. Biophys. Acta* **105**, 301–312
- Zgliczyński, J. M. (1980) in *The Reticuloendothelial System* (Sbarra, A. J. & Strauss, R. R., eds.), pp. 255–278, Plenum Press, New York
- Zgliczyński, J. M., Stelmaszyńska, T., Ostrowski, W., Naskalski, J. & Sznajd, J. (1968) *Eur. J. Biochem.* **4**, 540–547
- Zgliczyński, J. M., Stelmaszyńska, T., Domański, J. & Ostrowski, W. (1971) *Biochim. Biophys. Acta* **235**, 419–424
- Ziegler, D. M., Poulsen, L. L. & York, B. M. (1983) in *Functions of Glutathione: Biochemical, Physiological, Toxicological, and Clinical Aspects* (Larsson, A., Orrenius, S., Holmgren, A. & Mannervik, B., eds.), pp. 297–305, Raven Press, New York

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