# Glutathione Peroxidase-Like Activity of Simple Selenium Compounds. Peroxides and the Heterocyclic N-Oxide Resazurin Acting as O-Atom Donors

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Selenite and selenocystamine [$(CyaSe)_2$] efficiently activate the decomposition of $H_2O_2$ by GSH and by other thiols, as demonstrated using a leuco crystal violet POD-based $H_2O_2$ assay which is applicable (unlike other assays) also in presence of thiols. The GPx-like activities were estimated to be 3.6 and 2.7 μmol $H_2O_2$/min per μmol $SeO_3^{2-}$ and $(CyaSe)_2$, respectively. Both selenium compounds also activate reduction of the heterocyclic N-oxide resazurin (RN→O) to resorufin (RN) by GSH; $H_2O_2$ competes with reduction of this dye. GSSeH and CyaSeH, formed by interaction of GSH with $SeO_3^{2-}$ and $(CyaSe)_2$, respectively, are likely to be the active reductants. CyaSeH, generated γ-radiolytically from $(CyaSe)_2$, exhibits an absorption peak at 243 nm and is removed by $H_2O_2$ with a rate constant of $9.7\times10^2$ $M^{-1}$ $s^{-1}$, and slightly slower by hydroperoxides. We have no evidence for one-electron interactions between GSSeH or CyaSeH and $H_2O_2$, with formation of free radical intermediates, as previously proposed in the case of selenium-activated reduction of cytochrome *c* by GSH (Levander *et al.,* Biochemistry **23**, 4591–4595 (1973)). Our results can be explained by O-atom transfer from the substrate to the active selenol group, RSeH + $H_2O_2$ (RN→O) → RSeOH + $H_2O$ (RN), and recycling of RSeOH to RSeH (+ $H_2O$) by GSH, analogous to the selenenic acid pathway of GPx. The substrate specificity appears to be different, however, in that GPx is unable to catalyse RN→O reduction, and GSSeH hardly catalyses the decomposition of cumene- or *t*-butyl-hydroperoxide; CyaSeH, on the other hand, is active also with the hydroperoxides. RN→O is reduced to RN also by certain oxidizing free radicals, e.g. by the thiyl CyaS·; O-atom transfer may in this case lead to the generation of reactive oxyl radicals.

## Introduction

Selenium, though very toxic at high levels (Painter, 1941; Moxon and Rhian, 1943; Martin, 1973; Shamberger, 1983a), is an essential trace element, used for instance as functional group (selenocysteine) in the antioxidative enzyme glutathione peroxidase (Rotruck *et al.*, 1973; Flohé, 1982) which catalyses the reaction (1):

$$2\,GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2\,H_2O. \quad (1)$$

It is the two-electron covalent chemistry of selenium which enables O-atom transfer from the peroxide (Fraústo and Williams, 1991), without formation of free radicals as in the deleterious Fenton reaction [$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + \cdot OH$]. Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one), a seleno-organic drug with GPx-like activity, has been studied extensively in the last decade as a promising non-toxic anti-oxidant with anti-inflammatory properties (Sies, 1994; Schewe, 1995). Great interest is currently also paid to the possible anticarcinogenic effects of selenium (Shamberger, 1983b) and its chemopreventive role in carcinogenesis (Ip, 1986; Vadhanavikit *et al.,* 1993). Regarding biologically important reactions involving selenium, there is however still a need of extensive studies of the mechanisms of such reactions in simple model systems (Kice *et al.,* 1980; Kice, 1981; Douglas, 1987).

Reactions of selenite and alkyl-diselenides (RSeSeR) with thiols efficiently activate catalytic electron transfer to acceptors like cytochrome *c* (Levander *et al.*, 1973), methylene blue (Rhead and Schrauzer, 1974) and methemoglobin (Masukawa and Iwata, 1977), and reduction of oxygen (Tsen and Tappel, 1958; Seko *et al.*, 1989). Thus it appears possible that reduction of $H_2O_2$, as in reaction (1), can be activated also by these com-

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pounds. Reaction of selenite with thiols leads to seleno-trisulfides which react further, particularly at pH ≈ 7, to form unstable selenopersulfide derivatives, e.g. of glutathione (Ganther, 1968 and 1971):

$$4\,GSH + SeO_3^{2-} \rightarrow GSSG + GSSeSG + 2\,OH^- + H_2O \quad (2)$$

$$GSH + GSSeSG \rightleftarrows GSSG + GSSeH \quad (3)$$

$$GSSeH \rightleftarrows GSH + Se^\circ. \quad (4)$$

$GSSe^-$ has been proposed to be the active electron transfer catalyst in the case of cytochrome *c* reduction by GSH (Levander *et al.*, 1973):

$$GSSe^- + Fe^{III}Cytc \rightleftarrows GSSe^\cdot + Fe^{II}Cytc \quad (5)$$

$$GSH + 2\,GSSe^\cdot \rightarrow GSSe^- + H^+ + GSSeSG. \quad (6)$$

This reaction scheme is analogous to that for reduction of cytochrome *c* by GSH with GSSSG as catalyst (Massey *et al.*, 1971), i.e. GSSSG and $GSS^-$ substituted for GSSeSG and $GSSe^-$ in the reaction chain (5)-(6)-(3). As yet there is no proof however for the generation of free radical intermediates ($GSSe^\cdot$ or $GSS^\cdot$) in reactions catalysed by $GSSe^-$ or $GSS^-$; it is difficult for instance to explain why the reaction of $O_2$ with $GSS^\cdot$ fails to inhibit the recycling of persulfide *via* reaction (6) in the case of $GSS^-$-catalysed reduction of cytochrome *c* (Prütz, 1993).

An alternative reaction scheme, which involves GSSeH as chain carrier but avoids the 'free radical problem', has recently been proposed to explain catalytic reduction of the heterocyclic N-oxide resazurin (RN→O) to resorufin (RN) in GSH/$SeO_3^{2-}$ systems, i.e. the reactions cycle (7)-(8)-(3) (Prütz, 1994):

$$GSSeH + RN{\rightarrow}O \rightarrow GSSeOH + RN \quad (7)$$

$$GSH + GSSeOH \rightarrow GSSeSG + H_2O. \quad (8)$$

$H_2O_2$ inhibited RN→O reduction, suggesting a competitive chain reaction (9)-(8)-(3),

$$GSSeH + H_2O_2 \rightarrow GSSeOH + H_2O \quad (9)$$

the sum of which matches the GPx-reaction (1). We now present further evidence that catalytic reduction of $H_2O_2$ and hydroperoxides by GSH can be activated both by selenite and selenocystamine.

## Materials and Methods

### *Chemicals*

The following commercial products were used as received: selenocystamine·2HCl ($(CyaSe)_2$), cysteamine·HCl (CyaSH), DL-dithiothreitol (DTT), DNA (from salmon testes), glutathione peroxidase (GPx, from bovine erythrocytes, EC 1.11.1.9) resorufin (RN = 7-hydroxy-3*H*-phenoxazine-3-on Na-salt), and deferoxamine mesylate from Sigma Chemie; *t*-butyl-hydroperoxide, cumene-hydroperoxide, cystamine·2HCl, leuco crystal violet (CVH = Tris-(4-dimethylamino-phenyl)-methane), crystal violet ($CV^+$ = Tris-(4-dimethylamino-phenyl)-carboniumchloride) from Aldrich; bovine serum albumine (BSA), EDTA-Na-salt, Gly–Gly–Gly, GSH, GSSG, peroxidase (POD, from horseradish, EC 1.11.1.7), N-acetyl-L-cysteine (NACySH), resazurin (RN→O = 7-hydroxy-3*H*-phenoxazine-3-on-N-oxide Na-salt), NADH, dodecylsulfate-Na-salt (SDS) and Tris from Serva Feinbiochemica; $H_2O_2$ 30% (perhydrol, stabilized with ammonium nitrate) from Fluka; KI, HCOONa and $SeO_3Na_2 \cdot 5H_2O$ from Merck. All solutions were prepared with redistilled water.

### *Stopped-flow experiments*

Stopped-flow measurements were performed with a SFA–12 "Rapid Kinetics Stopped-Flow-Accessory" (Hi-Tech Scientific Ltd.) using a flow-through mixing cell of 1 cm optical path, coupled with a UV-Vis spectrophotometer (Shimadzu Corp.).

Conditions for investigating interactions of selenium compounds with GSH in absence or presence of $H_2O_2$ are given in Scheme 1. Solutions were freshly prepared for each experiment, and anaerobic conditions were obtained (if desired) by flushing the components gently with high purity $N_2$, before filling the (gastight) syringes of the stopped-flow system. Stopped-flow was also applied to investigate reduction of RN→O, and reactions of the selenol CyaSeH with $H_2O_2$ and RN→O.

### *Determination of $H_2O_2$*

Iodometry was used to calibrate $H_2O_2$ stock solutions. Consumption of $H_2O_2$ on incubating the

| Component A | Component B |
|---|---|
| 0.2 mM $SeO_3^{2-}$ or $(CyaSe)_2$<br>10–50 mM Buffer<br>± $H_2O_2$ and/or RN→O<br>± Additive | 20 mM GSH<br>10–50 mM Buffer<br>± Chelator[a] |

Scheme 1. Typical conditions used to initiate Gpx-like reactions of simple selenium compounds. The components **A** and **B** were air-saturated (unless otherwise stated) and mixed 1:1 (v/v) under thermostatically controlled conditions at 20 °C. [a] Tests were made ± chelators, EDTA (20 mM) or deferoxamine (1 mM), to assure that catalysis was not due to transition metal impurities; $Cu^{2+}$ for instance, an efficient catalyst of cytochrome *c* reduction by GSH (Prütz *et al.*, 1994), was found to be unable to catalyse RN→O reduction by GSH.

mixed components A and B (Scheme 1) could, however, not be determined with common spectroscopic $H_2O_2$ assays such as permanganate, iodometry or 4-amino-antipyrin/POD, due to interference by GSH. We have applied, with slight modification, the POD leuco crystal violet (CVH) assay (Mottola *et al.*, 1970), which was found to be applicable also in presence of GSH. After incubation, 20 µl of the mixture [A+B] (Scheme 1) was quickly combined with 600 µl CVH [0.5 mM in 10 mM HCl] and 380 µl POD [200 U/ml] to give the blue protonated dye $CVH^{2+}$ (within seconds); 2 ml buffer + solubilizer [100 mM phosphate (pH 6.8) + 0.1 M SDS] was than added to give the stable violet form $CV^+$, the absorption of which was measured at 590 nm. Calibration, i.e. the above procedure without $SeO_3^{2-}$ in the component A, gave an absorbance of $A_{590} = 0.266$ (1 cm cell) per mM $H_2O_2$ in component A (which may be converted to a virtual absorption coefficient of $\varepsilon_{590} = 80000\ cm^{-1}\ M^{-1}$ of $H_2O_2$ in the final mixture). The calibration was linear in $[H_2O_2]$ up to about 5 mM $H_2O_2$ in component A; comparison with the absorption of authentic $CV^+$ indicated that the CVH assay involves a simple 1:1 $CV^+$ per $H_2O_2$ stoichiometry.

### *γ-Radiolysis*

γ-Radiolysis was applied to initiate free radical reactions by the water radiolysis products. Equation (10) shows the primary free radical species and radiolytic yields [µM/Gy]:

$$H_2O \xrightarrow{\text{radiation}} {}^{\cdot}OH[0.28] + H^{\cdot}[0.06] + e_{aq}^{-}[0.28]. \quad (10)$$

The reducing radical anions $O_2^{\cdot -}$ and $^{\cdot}CO_2^{-}$ were generated by γ-irradiation of aerated or $N_2O$-saturated formate (40–200 mM) solutions, where all products of reaction (10) are converted into the respective radical anion (see e.g. Prütz, 1993; Prütz *et al.*, 1994):

$$N_2O + H^+ + e_{aq}^{-} \rightarrow {}^{\cdot}OH + N_2 \quad (11)$$

$$HCOO^- + {}^{\cdot}OH\ (H^{\cdot}) \rightarrow {}^{\cdot}CO_2^{-} + H_2O\ (H_2) \quad (12)$$

$$O_2 + e_{aq}^{-} \rightarrow O_2^{\cdot -}. \quad (13)$$

The selenyl radical CyaSe·, for example, was generated by reducing $(CyaSe)_2$ with $e_{aq}^{-}$ (Tamba and Badiello, 1973):

$$(CyaSe)_2 + e_{aq}^{-} \rightarrow (CyaSe)_2^{\cdot -} \quad (14)$$

$$(CyaSe)_2^{\cdot -} \rightleftarrows CyaSe^- + CyaSe^{\cdot}. \quad (15)$$

Since CyaSe·, in absence of further additives, can be assumed to recombine to $(CyaSe)_2$, the reactions (14) and (15) also provide a convenient means of generating the selenol CyaSeH. A $^{60}Co$-γ-source (Atomic Energy of Canada Ltd.) producing a dose rate of 11 Gy/min was applied.

## Results

### *Reaction of GSH with $SeO_3^{2-}$ in absence and presence of $H_2O_2$*

GSSeSG, the product of reaction (2), has been shown to be remarkably stable at initial concentrations $GSH/SeO_3^{2-} < 4$ (Ganther, 1968 and 1971). The sequential stages of reaction (2) have not been resolved in detail in the case of GSH; it is likely that a complex, pH-dependent three-stage mechanism applies as observed with 1-butanethiol (Kice *et al.*, 1980, Kice, 1981). The scenario becomes even more complicated under catalytic conditions ($GSH/SeO_3^{2-} \gg 4$) applied in this study. Fig. 1 gives, as an example, absorption spectra at various stages, taken from stopped-flow time profiles at $GSH/SeO_3^{2-} = 100$ and pH 6.5, in absence of $H_2O_2$. Four consecutive processes are discernible, particularly from the trace at 270 nm (inset). The primary spectrum (at 6.7 s) can be attributed to GSSeSG and its precursors (e.g. GSSe(O)SG and GSSeOH), the secondary spectrum (at 28 s) is in our opinion mainly due to GSSeH formed in reaction (3). GSSeH than eliminates elemental

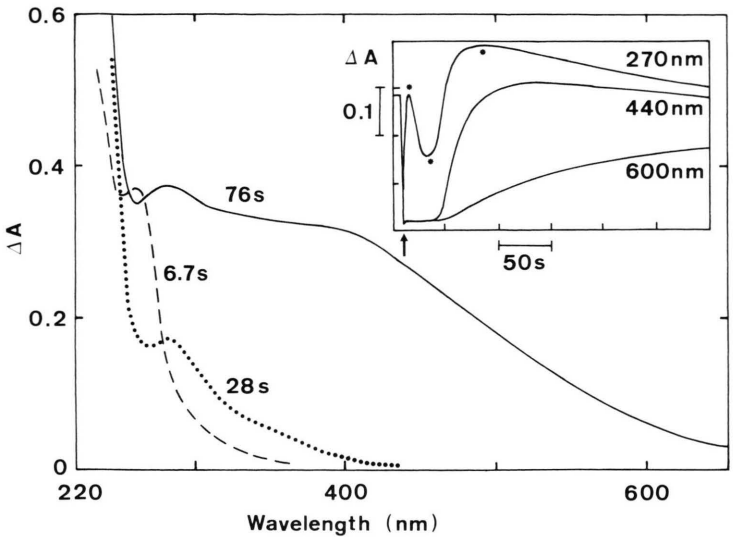


Fig. 1. Stopped-flow spectra and time profiles of absorbance changes induced by interaction between selenite and glutathione. The results were obtained for the conditions given in Scheme 1 in absence of $H_2O_2$, using 50 mM phosphate (pH 6.5), 50 mM NaCl (as additive) and 25 mM EDTA. Spectra at 6.7, 28 and 76 sec were taken from stopped-flow ( ↑ ) time profiles, such as those shown in the inset, at the times marked (*).

Se°, reaction (4), with a broad visible absorption (spectrum at 76 s) which continues to change in a time scale of minutes (see 270 and 600 nm traces) due to colloid formation. We have confirmed that the kinetics change dramatically when going to acid solutions (Ganther, 1971). The elimination of Se° was most pronounced around pH 6.5, and efficiently inhibited at pH > 7, probably due to deprotonation promoting the reverse reaction (4).

In a first attempt to demonstrate reaction (9) we have inspected its competition with the Se°-eliminating reaction (4). Fig. 2 shows that Se°-elimination at pH 6.4, detected by the build-up of 400 nm absorption, is progressively retarded on increasing the $H_2O_2$ concentration. According to our model, reaction (4) is suppressed in presence of $H_2O_2$ due to redox cycling of GSSeH through reactions (9),(8) and (3); the delayed onset of Se°-elimination marks the depletion of $H_2O_2$. From the time dependence (Fig. 2) we conclude that roughly 1 minute is required to remove 0.5 mM $H_2O_2$, thus the activity could be in the order of 5 μmol $H_2O_2$/min per μmol $SeO_3^{2-}$ applied to initiate catalysis. More accurate estimates are given below.

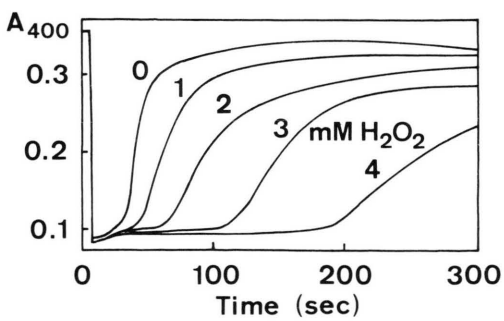


Fig. 2. Stopped-flow time profiles of 400 nm absorbance changes showing that $H_2O_2$ competes with Se° elimination upon interaction of $SeO_3^{2-}$ with GSH. The results refer to the conditions given in Scheme 1, using 50 mM phosphate (pH 6.4) and without chelator in component **B**. Initial $H_2O_2$ concentrations in component **A** are indicated.

### *Interactions of resazurin and $H_2O_2$ with selenium compounds*

Resazurin (RN→O) changes colour from blue (absorption peaks at 600 and 380 mn) to pink (absorption peak at 565 nm) upon reduction to resorufin (RN) (DeBaum and de Stevens, 1951) [spectra and the structure of RN→O are shown below, Fig. 6]. Heterocyclic N-oxides like RN→O are of interest also because they may interact with selenium compounds like peroxides, by O-atom transfer (Prütz, 1994). The data presented in Fig. 3 show that reduction of RN→O by GSH is activated both by $SeO_3^{2-}$ and $(CyaSe)_2$ and is inhibited in both cases by $H_2O_2$. $(CyaSe)_2$-activated reduction of RN→O by GSH was inhibited with similar efficiency also by *t*-butyl- and cumene-hydroperoxide, but $SeO_3^{2-}$-activated reduction was hardly inhibited by these two hydroperoxides. In

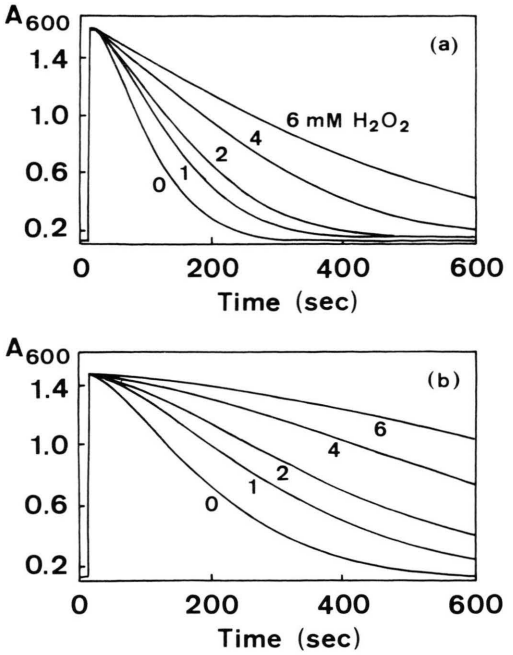


Fig. 3. Effect of $H_2O_2$ on the reduction of resazurin by GSH in presence of $SeO_3^{2-}$ (a), or selenocystamine (b). Conditions as in Scheme 1, but component **A** containing both $H_2O_2$ and 100 μM RN→O, and (a) 50 μM $SeO_3^{2-}$ in 50 mM Tris (pH 7.2), (b) 100 μM $(CyaSe)_2$ in 50 mM phosphate (pH 6.8) without chelator in component **B**; $H_2O_2$ concentrations (in component **A**) are indicated. Reduction of RN→O to RN is monitored by the absortion change at 600 nm (Prütz, 1994; spectra are shown in Fig. 6). In the time scale of 10 min there was no RN→O reduction by GSH in absence of the Se-compounds, and no reduction by the Se-compounds in absence of GSH.

the context of specificity we have recognized that GPx is unable catalyse reduction of RN→O by GSH.

The inhibitory effect of $H_2O_2$ on the selenium-catalysed reduction of RN→O by GSH (Fig. 3) might tentatively be explained by reoxidation (RN + $H_2O_2$ → RN→O + $H_2O$). Addition of $H_2O_2$ to solutions of authentic RN gave however no evidence for such a reaction. The interaction of $SeO_3^{2-}$ with GSH under aerobic conditions has been proposed to generate the $O_2^{\cdot-}$ radical (Seko *et al.*, 1989), a reducing entity which would be removed by $H_2O_2$. However, also this mechanism cannot explain the results in Fig. 3, because $O_2^{\cdot-}$ is unable to reduce RN→O; this was established by γ-radiolytic generation of $O_2^{\cdot-}$ (Materials and Methods) in the presence of RN→O.

When $SeO_3^{2-}$ was preincubated with GSH for a few minutes before addition of RN→O (in absence of $H_2O_2$), the reduction of RN→O became much slower. This is probably due a to loss of the reductant GSSeH, proposed to act in reaction (7), by the reactions (4) and (16),

$$GSSeH + GSH \rightleftarrows GSSG + H_2Se. \qquad (16)$$

If $H_2Se$ was the reducing entity, as has been suggested in the case of reduction of methylene blue (Rhead and Schrauzer, 1974) and oxygen (Seko *et al.*, 1989) in presence of $SeO_3^{2-}$ and GSH, one would have expected faster rather than slower reduction of RN→O after preincubation of $SeO_3^{-2}$ with GSH. This provides a further argument in favour of the reaction (7).

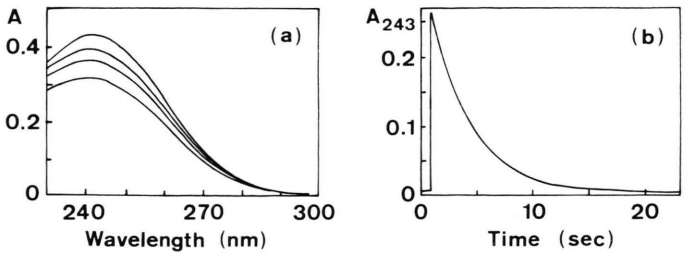


Fig. 4. Spectrum of CyaSeH (a), and kinetics of removal of CyaSeH by $H_2O_2$ (b). The results were obtained after 160 Gy γ-irradiation of a deaerated ($N_2$-saturated) solution containing 400 μM $(CyaSe)_2$, 20 mM phosphate (pH 7.5), 0.2 M HCOONa and 400 μM EDTA (see Materials and Methods). The spectra (a), recorded against the unirradiated solution, were taken (downward) at 0.5, 20, 40 and 60 min after γ-irradiation. The stopped-flow time profile (b) was obtained by rapid mixing of the above system with a deaerated 600 μM $H_2O_2$ solution, within 4 min after γ-radiolysis.

When $(CyaSe)_2$ was preincubated with GSH for a few minutes before addition of RN→O (in absence of $H_2O_2$), the reduction of RN→O became faster. Generation of the selenol CyaSeH, which is considered to be the active reductant in this system, is apparently relatively slow. In order to demonstrate the reaction of CyaSeH with RN→O and $H_2O_2$ we have generated CyaSeH γ-radiolytically (Materials and Methods). CyaSeH, with an absorption peak at 243 nm, is fairly stable under anaerobic conditions, as shown in Fig. 4a. This result is consistent with previous data obtained with selenocystine after incubation with GSH (Dickson and Tappel, 1969). In air-saturated solution CyaSeH is decomposed within about 10 minutes, with formation of secondary products absorbing in the UV. The stopped-flow time profile in Fig. 4b shows the fast removal of CyaSeH by $H_2O_2$ under anaerobic conditions. The reaction kinetics were mono-exponential and first-order in $[H_2O_2]$, and similar results were obtained upon mixing CyaSeH with *t*-butyl- or cumene-hydroperoxide solutions. Rate constants are given in Table I, they are notably higher than the rate of reaction of the ebselen selenol with $H_2O_2$, $k = 0.47\times10^2$ M$^{-1}$ s$^{-1}$ (Morgenstern *et al.*, 1992). RN→O was also reduced by CyaSeH, however, the reaction was slow (10 min time scale), and due to the natural decay of CyaSeH it was not possible to give an accurate estimate of the rate constant.

### *The catalytic activity of simple selenium compounds*

The results presented in Fig. 5 show the decomposition of $H_2O_2$ by GSH in presence of $SeO_3^{2-}$ and $(CyaSe)_2$, respectively, as detected with the CVH/POD assay (Materials and Methods). Catalytic activities, estimated from initial slopes in Fig. 4 are collected in Table II, together with val-

Table I. Rate constants of reaction of CyaSeH with $H_2O_2$ and hydroperoxides.

| Peroxide | $k$(CyaSeH + ROOH)[a] (M$^{-1}$ s$^{-1}$) |
|---|---|
| $H_2O_2$ | $9.7\times10^2$ |
| *t*-Butyl–OOH | $1.2\times10^2$ |
| Cumene–OOH | $2.5\times10^2$ |

[a] Rate constants estimated from time profiles as in Fig. 4b under anaerobic conditions.

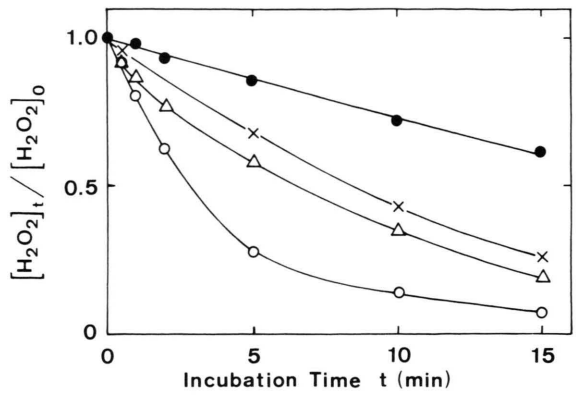


Fig. 5 Decomposition of $H_2O_2$ by incubation with GSH in presence of $SeO_3^{2-}$ or selenocystamine. The results refer to incubation at 20 °C of the components shown in Scheme 1, using 4 mM $H_2O_2$, 10 mM phosphate (pH 6.8), 0.1 M NaCl (as additive), without chelator in component **B**. $H_2O_2$ was determined by the CVH/POD assay (Materials and Methods): (●) control without Se-compound, (○) 200 μM $SeO_3^{2-}$; (×) as (○) but $SeO_3^{2-}$ added to component B (Scheme 1) and incubated 10 min before mixing with $H_2O_2$ (component A without $SeO_3^{2-}$); (△) 200 μM $(CyaSe)_2$.

Table II. Catalytic decomposition of $H_2O_2$ by thiols in presence of selenium activators.

| No. | Activator (200 μM)[a] | Thiol (20 mM) | Additive | Activity[b] |
|---|---|---|---|---|
| 1 | $(CyaSe)_2$ | GSH | – | 2.6 |
| 2 | $(CyaSe)_2$ | GSH | deaerated | 2.8 |
| 3 | $SeO_3^{-2}$ | GSH | – | 3.6 |
| 4 | $SeO_3^{-2}$ | GSH | deaerated | 3.4 |
| 5 | $SeO_3^{-2}$ | GSH | 1 mM GSSG | 3.4 |
| 6 | $SeO_3^{-2}$ | GSH | 1 mM NADH | 3.5 |
| 7 | $SeO_3^{-2}$ | GSH | 0.1 g/L DNA | 3.6 |
| 8 | $SeO_3^{-2}$ | GSH | 0.1 g/L BSA | 3.9 |
| 9 | $SeO_3^{-2}$ | GSH | 0.2 mM $Hg^{2+}$ | 1.3 |
| 10 | $SeO_3^{-2}$ | GSH | 0.2 mM $Cu^{2+}$ | 1.1[c] |
| 11 | $SeO_3^{-2}$ | DDT | – | 17.2 |
| 12 | $SeO_3^{-2}$ | CyaSH | – | ≈15[d] |

[a] 20 μM in nos. 11 and 12.

[b] Activities were estimated from initial rates (conditions as in Fig. 5), subtracting the rate of spontaneous $H_2O_2$ decomposition by the thiol alone. Numbers given are μmol $H_2O_2$/min per μmol activator applied to initiate catalysis. Stated are concentrations before mixing components A and B (Scheme 1).

[c] $Cu^{2+}$ activates spontaneous $H_2O_2$ decomposition by GSH, but reduces the activity of selenite.

[d] Spontaneous $H_2O_2$ decomposition by cysteamine is relatively fast.

ues obtained in various environments and with other thiols. In the absence of GSH there was no detectable loss of $H_2O_2$ upon addition of $SeO_3^{2-}$ or $(CyaSe)_2$, even at concentrations equal to $[H_2O_2]$. Decomposition of $H_2O_2$ by GSH in presence of selenocystine has previously been proposed to involve an O-atom transfer from $H_2O_2$ to the diselenide (Caldwell and Tappel, 1965), our results are however not consistent with such a reaction in the case of $(CyaSe)_2$, and they also exclude a direct interaction between $H_2O_2$ and $SeO_3^{2-}$. The CVH/POD assay is not applicable to hydroperoxides; the effects of hydroperoxides on RN→O reduction (see above) certainly indicate differences in the specificity of $SeO_3^{2-}$- and $(CyaSe)_2$-activated reduction of hydroperoxides, but unfortunately we cannot present numbers for the activities.

The GPx-catalysed decomposition of $H_2O_2$ by GSH, when examined with the CVH/POD assay at conditions similar to those in Table II, gave an activity of about 9000 μmol $H_2O_2$/min per μmol Se provided by the protein. This demonstrates the remarkably high turnover rate of GPx as compared to the data in Table II. The GPx-like activity induced by $(CyaSe)_2$ (Table II, no. 1) is actually similar to that of diphenyl diselenide but higher than that of ebselen (Wilson *et al.*, 1989), previously estimated from GSSG formation rates using the glutathione reductase-NADPH assay; ebselen may however be more specific with hydroperoxides formed in biomembranes and lipoproteins (Schewe, 1995). A comparison of the activities induced by $(CyaSe)_2$ and $SeO_3^{2-}$ (Table II, nos. 1 and 3) indicates that the latter is the more powerful system. It must be emphasized, however, that GSSeH is less stable than CyaSeH. The GPx-like activity induced by selenite was in fact much lower when $SeO_3^{2-}$ was preincubated few minutes with GSH prior to addition of $H_2O_2$ (cf. Fig. 5 (×) and (○)), resembling the behaviour found with RN→O as substrate (see above). This leads to the conclusion that also $H_2O_2$ decomposition does not involve Se° and $H_2Se$, the products of reactions (4) and (16). Addition of GSSG, which should reverse reaction (16), had no effect on the activity of the GSH/$SeO_3^{2-}$ system (Table II, no. 5).

Selenium-activated reduction of $H_2O_2$ is unaffected by oxygen (Table II, nos. 1 and 3 compared with 2 and 4), as previously recognized also in the case of $SeO_3^{2-}$-activated reduction of RN→O (Prütz, 1994). Interactions of $O_2$ with selenium derivatives of thiols described in literature (Tsen and Tappel, 1958; Dickson and Tappel, 1969; Seko *et al.*, 1989) are apparently much slower than the catalytic reactions under discussion. As already mentioned, GSH has been proposed to interact with $SeO_3^{2-}$ under aerobic conditions to generate $O_2^{\cdot-}$ (Seko *et al.*, 1989). However, since $H_2O_2$ removal in the present systems is oxygen-independent it appears unlikely that $H_2O_2$ decomposition was due to the Haber-Weiss reaction, $H_2O_2 + O_2^{\cdot-} \rightarrow O_2 + OH^- + {\cdot}OH$. Further arguments against free radical mechanisms, as alternatives to the O-atom transfer reactions (7) and (9) are presented below (Discussion).

Decomposition of $H_2O_2$ in the GSH/$SeO_3^{2-}$ system is not impeded by additives like NADH, DNA or BSA (Table II, nos. 6 to 8), but is inhibited by $Cu^{2+}$ and $Hg^{2+}$ ions (Table II, nos. 9 and 10); both metal ions also inhibited reduction of RN→O. The inhibitory effect of $Cu^{2+}$ is in stark contrast to its stimulatory effect on selenium catalysed reduction of methylene blue (MB) (Rhead and Schrauzer, 1974). The reason for this difference may be that the reduction proceedes by O-atom transfer in the case of $H_2O_2$ and RN→O, and by electron transfer in the case of MB. $Hg^{2+}$ is commonly known to be an antagonist of selenium catalysed reactions (Levander *et al.*, 1973; Rhead and Schrauzer, 1974; Masukawa and Iwata, 1977; Shamberger, 1983c), probably due to formation of inactive selenium-mercury complexes. Mixing of the components A and B (Scheme 1) in the presence of $SeO_3^{2-}$ and $Hg^{2+}$ (without $H_2O_2$) actually led to immediate formation of stable tawny compounds, indicating that $Hg^{2+}$ rapidly interacts with the products of reactions (2) to (4).

Of the few thiols tested, DTT is the most efficient donor of $SeO_3^{2-}$-activated $H_2O_2$ decomposition (Table II, no. 11). Also CyaSH, a poor donor for GPx (Flohé, 1982), is more efficient than GSH in the $SeO_3^{2-}$ system (Table II, no. 12).

### *Free radical interactions with resazurin*

In the context of the O-atom transfer reaction (7), we have considered the possibility that RN→O may behave as an O-atom donor also in reactions with certain free radicals. γ-Radiolysis of

a $N_2$-saturated solution containing RN→O and formate in presence of either $(CyaSe)_2$ or $(CyaS)_2$ revealed a stark contrast in the behaviour of these two compounds. The selenyl radical CyaSe· generated upon irradiation (Materials and Methods) did not react with RN→O. The thiyl radical CyaS·, on the other hand, generated analogous to reactions (14) and (15) by reduction with $e_{aq}^-$ and $\cdot CO_2^-$ (Adams *et al.*, 1967; Willson, 1970), interacted extensively with RN→O, as shown in Fig. 6a. The loss of 380 nm absorption of RN→O with increasing dose corresponds to the removal of more than 1.5 RN→O per CyaS·, and it is particularly interesting to note that CyaS·, though it is an oxidant

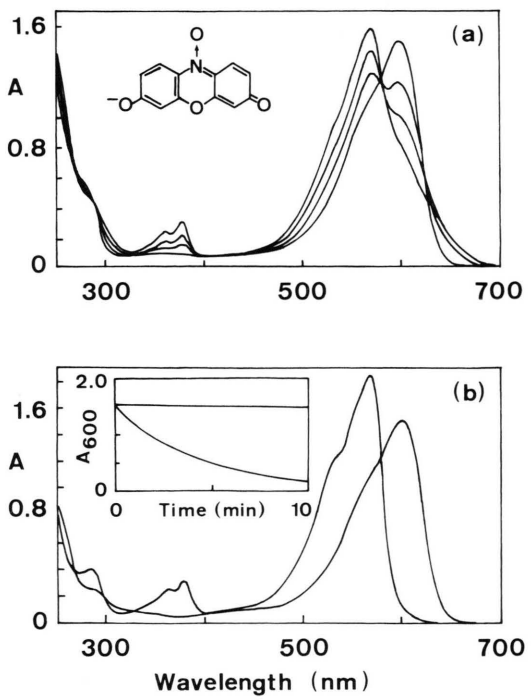


Fig. 6 Absorption spectra of RN→O, and of products formed by interaction (a) with CyaS·, and (b) with $(CyaSe)_2$/NACySH. (a) A $N_2$-saturated solution containing 2 mM $(CyaS)_2$, 40 mM HCOONa, 50 μM RN→O and 10 mM phosphate (pH 6.8) was irradiated with 0, 20, 40 and 60 Gy; in this order the absorption of RN→O (380 and 600 nm peaks) is bleached, due to reaction of CyaS·, with formation of RN (565 nm peak) and other products absorbing above 650 nm. The structure of RN→O is shown. (b) 50 μM RN→O was incubated with 50 μM $(CyaSe)_2$ and 10 mM NACySH in 50 mM phosphate (pH 6.8) to give complete reduction to RN after 15 min, shown by the 565 nm absorption which is identical to authentic RN. Insert: time profiles at 600 nm in presence and absence (upper curve) of $(CyaSe)_2$.

(Forni and Willson, 1986), generates the characteristic 565 nm absorption band of the reduced dye. Fig. 6b shows for comparison the spectra of RN→O, and of RN after reduction by $(CyaSe)_2$/NACySH, a system resembling the $(CyaSe)_2$/GSH system (Fig. 3b). Additional products with absorptions extending up to 700 nm are seen in Fig. 6a. Our results can be explained by assuming that RN→O reacts with CyaS· by O-atom transfer to form a reactive thiyl-oxyl (sulfinyl) radical which further interacts with RN→O:

$$RN{\rightarrow}O + CyaS^{\cdot} \rightarrow RN + CyaSO^{\cdot} \quad (17)$$

$$RN{\rightarrow}O + CyaSO^{\cdot} \rightarrow \text{products.} \quad (18)$$

It was recognized that RN→O readily interacts with a variety of radiolytically generated radicals; that the CyaSe· radical is inert with RN→O appears almost as an exception. Free radicals generated by reaction of ·OH with Gly–Gly–Gly, for instance, led to RN→O reduction at a yield of 2 RN per peptide-radical, suggesting that these radicals can accept two O-atoms from RN→O (possibly to form peroxy radicals); oxygen completely inhibited RN→O reduction by ·OH-products of Gly–Gly–Gly. Pulse radiolysis techniques will be required to investigate in further detail the mechanisms of such interactions of resazurin with various free radicals. Also peroxides have a tendency to interact with free radicals by O-atom transfer; the reaction of ·OH with $H_2O_2$ to yield $HO_2^{\cdot}$ and $H_2O$ (Buxton *et al.*, 1988) is a classical example.

## Discussion

$SeO_3^{2-}$-activated reduction of RN→O and $H_2O_2$ by GSH has been proposed to involve O-atom transfer from these substrates to GSSeH, reactions (7) and (9), and recycling of GSSeOH to GSSeH *via* reactions (8) and (3) (Prütz, 1994). The present results support this mechanism. Particularly we have shown that: (a) $H_2O_2$ competes with Se°-elimination from GSSeH by reaction (4) (Fig. 2), (b) inhibition of RN→O reduction by $H_2O_2$ (Fig. 3a) cannot be explained by reoxidation (RN + $H_2O_2$ → RN→O + $H_2O$) or by $O_2^{\cdot -}$-scavenging ($O_2^{\cdot -}$ + $H_2O_2$ → $O_2$ + $OH^-$ + ·OH) and is therefore likely to be due to competition between the reactions (9) and (7), (c) the effect of preincubation of $SeO_3^{2-}$ with GSH before addition of

$RN \rightarrow O$ or $H_2O_2$ is also consistent with a loss of the reducing entity GSSeH by reactions (4) and (16), (d) $H_2O_2$ is in fact decomposed catalytically in the $SeO_3^{2-}$/GSH system (Fig. 5a, Table II). The possibility of electron transfer, as opposed to O-atom transfer, is discussed below.

The $(CyaSe)_2$/GSH system bears analogies to the $SeO_3^{2-}$/GSH system (Figs. 3 and 5). It is long known that $H_2O_2$-induced oxidation of GSH can be accelerated by selenocystine. However, this was tentatively explained (Caldwell and Tappel, 1965) by a mechanism which is not catalytic, and we have already pointed out that the proposed O-atom transfer from $H_2O_2$ to diselenide is not detectable in the case of $(CyaSe)_2$. Interaction of seleno-cystine with thiols was later found to yield selenol (Dickson and Tappel, 1969), and we adopt this reaction scheme for the $(CyaSe)_2$ system:

$$GSH + (CyaSe)_2 \rightleftarrows GSSeCya + CyaSeH \quad (19)$$

$$GSH + GSSeCya \rightleftarrows GSSG + CyaSeH. \quad (20)$$

In the present paper it is shown that CyaSeH reacts fairly fast with $H_2O_2$ (Fig. 4b, Table I). The results in Fig. 3b can again be explained by an O-atom transfer mechanism with competition between $RN \rightarrow O$ and $H_2O_2$, i.e. reactions (21) and (22),

$$CyaSeH + RN \rightarrow O \rightleftarrows CyaSeOH + RN \quad (21)$$

$$CyaSeH + H_2O_2 \rightarrow CyaSeOH + H_2O \quad (22)$$

followed by the reactions (23) and (20),

$$GSH + CyaSeOH \rightarrow GSSeCya + H_2O. \quad (23)$$

The reaction cycle (22)-(23)-(20), involving formation of a selenenic acid transient, CyaSeOH, actually corresponds to the reaction mechanism proposed for the seleno-enzyme GPx (Wendel, 1980; Flohé, 1982). In adopting the selenenic acid pathway of GPx in the above reaction cycle we do not exclude seleninic acid pathways (Wendel, 1980; Kice, 1981; Flohé, 1982), i.e. $H_2O_2$ or $RN \rightarrow O$ reacting by O-atom transfer with CyaSeOH to form CyaSe(O)OH which then interacts with two GSH to regenerate CyaSeOH (+ $H_2O$ and GSSG). Selenite-activated reduction, proposed to involve the reaction cycle (9)-(8)-(3), might alternatively also be explained by O-atom transfer from $H_2O_2$ (or $RN \rightarrow O$) to GSSeOH, and recycling of GSSe(O)OH by GSH.

The rate constant $k_{22} = 9.7 \times 10^2$ $M^{-1}$ $s^{-1}$ (Table I) means that CyaSeH removal at 2 mM $H_2O_2$ (used in Fig. 5) would proceed with a half-life of about 0.35 s. The rate-determining step in the relatively slow decomposition of $H_2O_2$ ($t_{1/2}$ ~ 7 min, see Fig. 5) is therefore certainly not the reaction (22), but rather the recycling reaction sequence (23)-(20). The high turnover rate of GPx, as compared to the selenium compounds tested in this study, is obviously due to a much faster recycling of the active selenol group.

As shown by reaction (5), $GSSe^-$ has been assumed to react with cytochrome *c* by one-electron transfer to generate free radicals ($GSSe^{\cdot}$). Also other Se-derivatives have been suggested to interact with electron acceptors to produce free radicals (Levander *et al.*, 1973; Rhead and Schrauzer, 1974; Seko *et al.*, 1989). If the reactions (9) and (22) were to proceed by one-electron transfer steps, one might even expect that the primary step involves generation of ·OH radicals, as in the Fenton reaction (see Introduction):

$$GSSe^- + H_2O_2 \rightarrow GSSe^{\cdot} + OH^- + {\cdot}OH \quad (9a)$$

$$CyaSe^- + H_2O_2 \rightarrow CyaSe^{\cdot} + OH^- + {\cdot}OH. \quad (22a)$$

We have no evidence, however, for the occurrence of reactions (9a) and (22a). As shown in Table II (no. 6), the addition of NADH to the GSH/selenite system has no effect on $H_2O_2$ decomposition. Particularly, there was no indication of NADH oxidation when $SeO_3^{2-}$ or $(CyaSe)_2$ systems were incubated as shown in Scheme 1 in presence of $H_2O_2$ (1 mM) and NADH (100 to 500 µM, data not shown). If ·OH had been generated in reactions (9a) and (22a) it would either have oxidized NADH directly, or it would have interacted with GSH (present in excess) to form the $GS^{\cdot}$ radical which is known to oxidize NADH (Forni and Willson, 1986). Little is known about reactions of Se-containing free radicals, with exception of the selenite radical $SeO_3^{\cdot -}$ which is a powerful oxidant (Tamba and Badiello, 1985; Neta *et al.*, 1988) and reactions of some organic selenyl radicals of more chemical interest (Deryagina *et al.*, 1993). By analogy with sulfur-centered radicals (Chatgilialoglu and Asmus, 1990), one might expect that $RSe^{\cdot}$ and $RSSe^{\cdot}$ species are fairly strong oxidants, thus reaction (5) would rather proceed to the left (Prütz, 1993). We are unable,

though, to offer an alternative to the proposed electron transfer between selenium centres and cytochrome *c*.

RN→O, as mentioned above, is not reduced by $O_2^{\cdot -}$. The inference is that the one-electron reduction potential of the RN→O/(RN→O)$^{\cdot -}$ couple is likely to be more negative than that of oxygen, $E^\circ(O_2/O_2^{\cdot -}) = -155$ mV. The additional experience that reduction of RN→O by GSSeH is not impeded by $O_2$ (Prütz, 1994) seems to imply that GSSeH is not removed by reducing $O_2$ to $O_2^{\cdot -}$. Since one-electron reduction of RN→O is even less feasible than of $O_2$, we conclude that also reduction of RN→O by GSSeH is not a two-step electron transfer process, with formation of free radical intermediates, but rather an O-atom transfer process as depicted by reaction (7). RN→O seems on the other hand capable of interacting with certain free radicals to form oxyl radicals, e.g. reaction (17). Heterocyclic N-oxides like benzotriazine-di-N-oxides are hypoxic cell toxins (Laderoute *et al.*, 1988; Butler and Hoey, 1993), and nitro-quinoline-N-oxide, as another example, is a potent mutagen and carcinogen which might be activated by ascorbate (Bielski, 1982). The deleterious effects of these N-oxides have been proposed to be mediated by the generation of one-electron reduction products of the parent compounds; the biological effects of N-oxides might, on the other hand, be related also to their ability to acts as O-atom donors. Further investigations are required to test this concept.

The catalytic reduction of $H_2O_2$ by simple selenium compounds in combination with thiols is an interesting example of their antioxidative capability. In biological systems the instability of these compounds, and formation of inactive metabolites such as Se° and trimethylselenonium ions (Martin, 1973; Vadhanavikit *et al.*, 1993; Groeger and Ganther, 1994) may limit their activity. Proteins appear to provide specific stabilizing interactions for the intermediates of selenium-catalysed reactions, not only in the case of GPx, but also with GPx-mimic proteins such as subtilisin modified by incorporation of a selenol group (Wu and Hilvert, 1990). The question arises whether the SSeH group, which appears to be more active than the SeH group, at least with $H_2O_2$ (Table II), can be stabilized in proteins or by certain carrier molecules.

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