# Thiyl Free Radicals: Direct Observations of Electron Transfer Reactions with Phenothiazines and Ascorbate

**Luigi G. Forni, Jörg Mönig, Victor O. Mora-Arellano, and Robin L. Willson** *
*Biochemistry Department, Brunel University, Uxbridge, Middlesex UB8 3PH*

Absolute rate constants for the reaction of the thiyl radicals derived from cysteamine, cysteine, glutathione, penicillamine, and 2-mercaptopropionylglycine (Thiola) with the phenothiazines, promethazine and chlorpromazine and with ascorbate ion, have been measured by pulse radiolysis and have been found to lie in the range 0.03—1.3 × $10^9$ l $mol^{-1}$ $s^{-1}$. The reactions are considered to occur by an electron transfer mechanism. The possibility that, in biochemical systems, glutathione can act as a catalyst linking hydrogen atom and electron transfer reactions is suggested.

Phenothiazines are widely used in psychiatric therapy. However, in spite of their considerable success, they may present several undesirable side effects, such as skin photosensitisation and visual disturbance.[1,2] *In vitro* studies have shown that the radical-cation formed by one-electron oxidation of chlorpromazine ($CZ^{\bullet +}$) is a potent inhibitor of the enzymes ATPase and uridine diphosphate glucose $NAD^+$ oxidoreductase.[3,4]

Free radical species derived from phenothiazines can be conveniently studied in aqueous solution by pulse radiolysis. Studies have shown, for example, that metiazinic acid, promethazine, and chlorpromazine react rapidly with the hydroxyl radical, several peroxyl free radicals, and a number of related electrophilic species.[5-14] Furthermore, the radical-cations formed in these reactions can react rapidly with α-tocopherol (vitamin E) and ascorbate (vitamin C).[15-17]

Recently, during pulse radiolysis studies of the reactions of electrophilic radicals with the peroxidase reagent, 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonate (ABTS), it was found, unexpectedly, that a variety of thiyl radicals derived from thiol-containing compounds reacted rapidly with the reagent.[18] The reactions of some thiyl radicals with a number of organic compounds [19] and with phosphite free radicals [20] have been reported. As far as we are aware, however, the biological importance of such reactions has not been previously considered.[21] Pulse radiolysis studies of the reaction of thiyl free radicals with promethazine and chlorpromazine have now been undertaken. Also, since ABTS and the phenothiazines have been shown to react rapidly with ascorbic acid it was considered of particular biochemical interest to investigate whether thiyl radicals could react with the vitamin directly.

## Experimental

Propan-2-ol, acetone, and potassium bromide (AnalaR) were supplied by B.D.H. Ltd. Chlorpromazine (1) and promethazine (2), supplied as the hydrochlorides, were kindly given by May and Baker Ltd., and 2-mercaptopropionylglycine (Thiola, 2-MPG) (9) was a gift from Santen Pharmaceuticals. Ascorbic acid (7), cysteine (3) (hydrochloride), cysteamine (4) (hydrochloride), penicillamine (5), glutathione (6), and dithiothreitol (8) were obtained from Sigma U.K. Ltd., and were of the purest grade available.

The pulse radiolysis experiments were undertaken on the Brunel 4 MeV linear accelerator (200 ns electron pulse).[22,23] Doses of up to 10 J $kg^{-1}$ were used as determined by thiocyanate dosimetry.[24] All solutions were prepared just before experimentation, using Millipore-filtered distilled water. Solutions were saturated with nitrogen and nitrous oxide using the syringe bubbling technique,[25] and were exposed to the minimum of light.

For the studies with phenothiazines, thiyl radicals were generated by radiolysis of deaerated aqueous solutions (pH 3, adjusted with HCl) containing the sulphur compound (RSH; 50mM) and propan-2-ol and acetone (1M) [reactions (1)—(5)]. For studies with ascorbate, solutions were saturated with nitrous oxide (British Oxygen Company). Under these conditions thiyl radicals are formed from the reaction of $OH^\bullet$ with the sulphur compounds according to reactions (1), (6), and (7).[26,27]

$$H_2O \xrightarrow{\text{radn.}} OH^\bullet\ (45\%) + e_{(aq)}(45\%) + H^\bullet\ (10\%) \qquad (1)$$

$$OH^\bullet\ (\text{or } H^\bullet) + (CH_3)_2CHOH \longrightarrow H_2O\ (\text{or } H_2) + (CH_3)_2COH^\bullet \qquad (2)$$

$$e_{(aq)} + (CH_3)_2CO \longrightarrow (CH_3)_2CO^{\bullet -} \qquad (3)$$

$$(CH_3)_2CO^{\bullet -} + H^+ \longrightarrow (CH_3)_2COH^\bullet \qquad (4)$$

$$(CH_3)_2COH^\bullet + RSH \longrightarrow (CH_3)_2CHOH + RS^\bullet \qquad (5)$$

$$e_{(aq)} + N_2O \longrightarrow OH^\bullet + OH^- + N_2 \qquad (6)$$

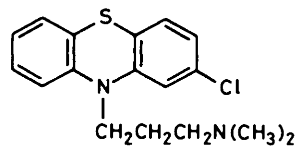
$$OH^\bullet\ (\text{or } H^\bullet) + RSH \longrightarrow H_2O\ (\text{or } H_2) + RS^\bullet \qquad (7)$$

## Results

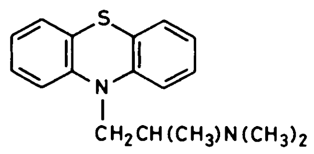
(a) *Thiyl Radicals and Phenothiazines.*—On pulse radiolysis of a nitrogen-saturated solution (pH 3) containing propan-2-ol and acetone (1M), 2-mercaptopropionylglycine (50mM), and promethazine (1mM) a strong transient absorption ($\lambda_{max.}$ 510 nm) was observed (Figure 1). The absorption decayed slowly, with a first half-life >1.5 ms. No absorption was observed when similar solutions were irradiated in the absence of 2-MPG (Figure 1). When 2-MPG was replaced by cysteine, a similar absorption was observed. This absorption was also similar to that observed on pulse radiolysis of a nitrous oxide-saturated solution of promethazine containing excess of bromide (100mM) and previously assigned [5,7,11,14] to the radical cation ($PZ^{\bullet +}$) formed according to reaction (8) (Figure 2).

$$Br_2^{\bullet -} + PZ \longrightarrow 2Br^- + PZ^{\bullet +} \qquad (8)$$

In the presence of cysteine the formation of the transient absorption occurred exponentially with time with the rate of formation increasing with increasing phenothiazine concentration. Similar observations were found when promethazine was replaced by chlorpromazine and also when studies using



(1)



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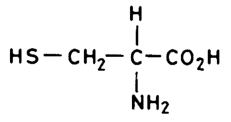
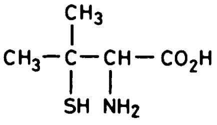


$HS-CH_2-CH(NH_2)-CO_2H$

(3)

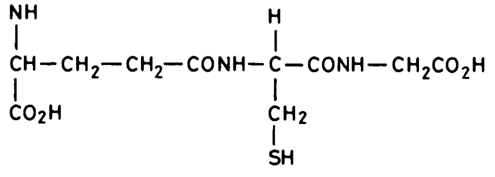
$HS-CH_2-CH_2-NH_2$

(4)



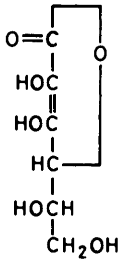
$CH_3-C(CH_3)(SH)-CH(NH_2)-CO_2H$

(5)

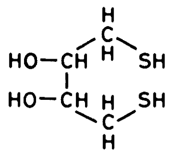


$HO_2C-CH(NH_2)-CH_2-CH_2-CONH-CH(CH_2SH)-CONH-CH_2CO_2H$

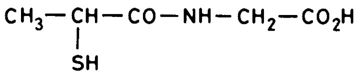
(6)



(7)



(8)



$CH_3-CH(SH)-CO-NH-CH_2-CO_2H$

(9)

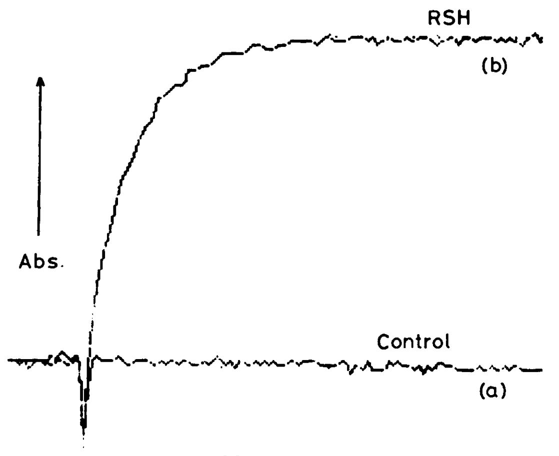


Figure 1. Changes in absorbance with time on pulse radiolysis of solutions containing propan-2-ol and acetone (1M) and promethazine (1mM) in the presence (b) and absence (a) of 2-mercaptopropionylglycine (50mM)

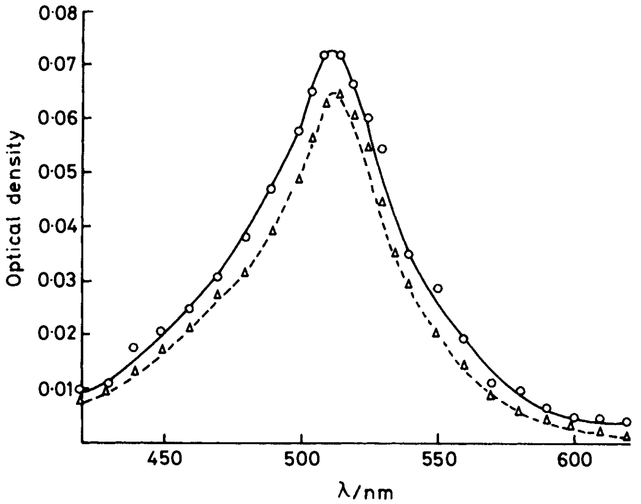


Figure 2. Transient absorption spectrum recorded 50 μs after the pulse radiolysis of (△) nitrogen-saturated solutions of promethazine (2.5mM) at pH 3, containing propan-2-ol and acetone (1M) and cysteine (50mM), and (○) nitrous oxide-saturated solutions containing potassium bromide (0.1M) and promethazine (2.5mM) at pH 3

other thiol compounds were undertaken. This is attributed to reaction (9).

$$RS^{\cdot} + PZ \longrightarrow RS^- + PZ^{\cdot +} \qquad (9)$$

Previous studies [14] have shown that in the case of the bromine free radical the yield of the promethazine and chlorpromazine radical-cations are 85% and 78% of that anticipated if the reaction occurred stoicheiometrically. For $CZ^{\cdot +}$ and $PZ^{\cdot +}$ the molar decadic extinction coefficients, 12 000 at 510 and 9 500 l mol$^{-1}$ cm$^{-1}$ at 505 nm, respectively, have been derived.[14] Taking these values, the maximum yield of radical-cation observed from thiyl radicals as a percentage of the anticipated yields have been calculated (Table), assuming the yields of thiyl radical are equal to the yields of bromide radicals in all instances. The slightly reduced magnitude of the maximum absorption observed from the reactions of most of the thiyl radicals suggests that other minor radical reactions also take place. The yields of the radical-cations obtained and the

First-order bimolecular rate constants derived for the system phenothiazine plus thiol (units are $10^8$ l mol$^{-1}$ s$^{-1}$). The yields for the promethazine and chlorpromazine radical-cations are calculated assuming a yield of 0.6 μmol J$^{-1}$ in propan-2-ol and acetone (1M)

| | Promethazine | | Chloropromazine | | Ascorbate |
|---|---|---|---|---|---|
| Thiol | *k* | Yield (%) | *k* | Yield (%) | *k* |
| Glutathione | 0.33 | 91 | 1.4 | 91 | 6.0 |
| Penicillamine | 0.27 | 95 | 1.2 | 67 | 4.9 |
| Cysteamine | 0.65 | 100 | 2.7 | 67 | 13.0 |
| Cysteine | 0.87 | 73 | 3.0 | 82 | 12.0 |
| Thiola | 3.2 | 80 | 10.0 | 77 | 7.5 |

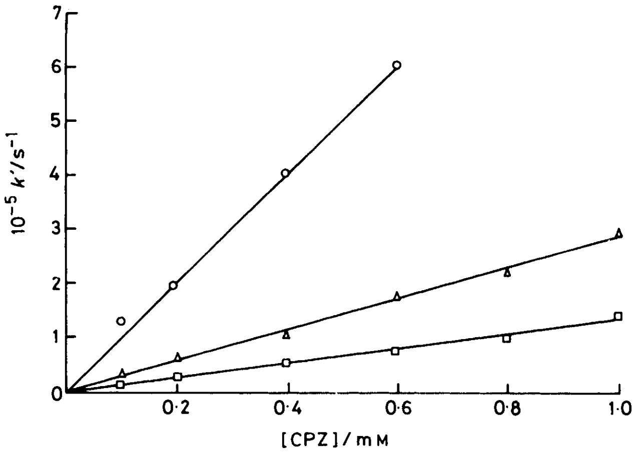


**Figure 3.** Plot of first-order rate constant for the formation of the absorption at 525 nm observed on pulse radiolysis of nitrogen-saturated solutions of chloropromazine (pH 3) containing propan-2-ol and acetone (1M); ○, Thiola; △, cysteine; and □, glutathione

absolute rate constants of the corresponding reactions derived from plots of the first-order rate constants against phenothiazine concentration are shown (Figure 3 and Table). Interestingly, when studies with the dithiol, dithiothreitol, were undertaken, the magnitude of the absorption at 515 nm was considerably reduced.

(b) *Thiyl Radicals and Ascorbate.*—On pulse radiolysis of nitrous oxide-saturated solutions containing cysteine (20mM) and ascorbate (50μM; pH 6.5 adjusted with 20mM-phosphate buffer), the formation of the characteristic absorption [28-30] of the ascorbyl radical ($A^{\cdot-}$) was observed. The rates of formation of the absorption were exponential in all cases and first order in ascorbate concentration, attributable to the corresponding reaction (10). The doses used in these studies

$$RS^{\cdot} + AH^{-} \xrightarrow{k_{10}} RS^{-} + A^{\cdot-} + H^{+} \qquad (10)$$

were in the range 3—4 J kg$^{-1}$ and the ascorbate concentration was 10—80μM. Taking for the ascorbyl radical $E_{360}$ 3 300 l mol$^{-1}$ cm$^{-1}$, the magnitude of the absorption observed with higher ascorbate concentrations corresponded to a yield of 0.50—0.58 μmol J$^{-1}$. The absolute rate constants determined for $k_{10}$ are shown (Table). In the case of glutathione and 2-MPG, similar values for the respective rate constants were obtained (*k* 4.8 and 7.5 × $10^8$ l mol$^{-1}$ s$^{-1}$) when the thiyl radicals were generated in nitrogen-saturated solutions containing excess of propan-2-ol and acetone (1M). When, in the case of glutathione, a similar system was investigated at pH 4, *k* 2.9 × $10^8$ l mol$^{-1}$ s$^{-1}$ was obtained (Figure 4).

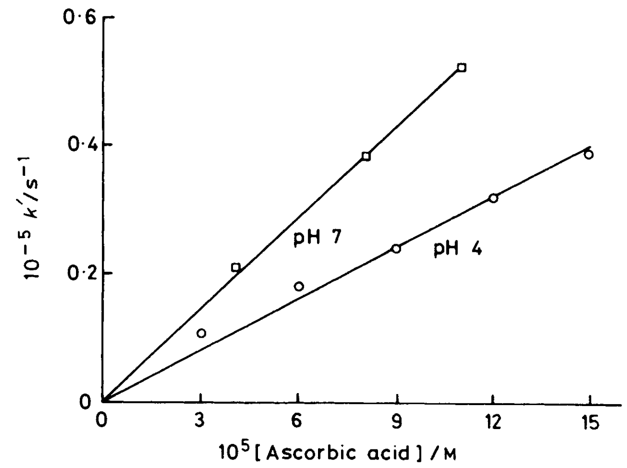


**Figure 4.** Plot of first-order rate constant for the formation of the absorption at 360 nm observed on pulse radiolysis of nitrogen-saturated solutions containing propan-2-ol and acetone (1M), glutathione (20mM), and ascorbic acid at pH 4 and 7

## Discussion

The present results clearly indicate that thiyl radicals derived from a number of biologically important sulphur-containing compounds can readily accept an electron from the phenothiazines, promethazine and chlorpromazine according to the general equation (9). Furthermore, at pH 3 this reaction goes essentially to completion. Previous studies have shown that the phenothiazine radical-cations can react with ascorbic acid, and several phenols in alkaline solution. In these instances it has been assumed that the radical-cations are acting as electron acceptors. It might be anticipated therefore, that since thiol anions are strong nucleophiles, the reverse reaction (11) may also take place under suitable conditions.

$$RS^{-} + PZ^{\cdot+} \longrightarrow RS^{\cdot} + PZ \qquad (11)$$

The acid dissociation constants (p$K_a$) of the sulphur compounds used in this study lie in the range 8—9, and at pH 3 little reaction between the thiol anions and the radical-cations is expected. At a higher pH however, the reaction may well be favoured. Unfortunately, these reactions are difficult to study experimentally, due to the problems associated with maintaining promethazine and chlorpromazine in solution in an alkaline medium. Further studies with other more soluble phenothiazines are being undertaken.

Although the absolute rates of reaction of the thiyl radicals with chlorpromazine are approximately one order of magnitude greater than with promethazine, the relative rates of reaction of the different thiyl radicals with the phenothiazines are in broad agreement with those obtained with ascorbate and with the nucleophile ABTS (Table). For chlorpromazine and promethazine, penicillamine < glutathione < cysteamine < cysteine < Thiola; for ABTS, penicillamine < glutathione < cysteine < Thiola < cysteamine. Whilst this order of reactivity may reflect to some extent the different redox properties of the corresponding thiol groups, other kinetic parameters clearly must be taken into account.

The yields of phenothiazine radical-cations resulting from these reactions have been derived from measurements obtained with the highest phenothiazine concentration used, when the yield no longer increases substantially with increasing phenothiazine concentration. At these concentrations and with the radiation dose rate used it is assumed that any lowering of the stoicheiometric yield of the radical-cations due to thiyl radical–radical interactions is small. Moreover, in the case of ascorbic acid, where studies were undertaken at neutral pH, it is assumed that under the experimental conditions used, establishment of equilibrium (12) is rapid compared with the rates of the reactions observed. It should be pointed out however, that if it is assumed that all the thiyl radicals (RS$^\cdot$) react stoicheiometrically with a particular phenothiazine then the actual value of the rate constants given (Table 1) refer to the rate constant for the overall reaction of type (13). The actual rate constant for the fraction of thiyl radicals reacting by an electron transfer mechanism and forming radical-cations is obtained by taking into account the percentage of radical-cation formed. For example, for the reaction of the glutathione thiyl radical with promethazine the rate constant $k_{(\mathrm{overall})}$ is $0.33 \times 10^8$ l mol$^{-1}$ s$^{-1}$, the yield of the radical-cation is 91%, and therefore for the electron transfer reaction (14) $k_{\mathrm{ET}} = k_{(\mathrm{overall})} \times 0.91 = 0.3 \times 10^8$ l mol$^{-1}$ s$^{-1}$.

$$\mathrm{RS^{\cdot} + RS^{-} \rightleftharpoons RSSR^{\cdot -}} \qquad (12)$$

$$\mathrm{RS^{\cdot} + PZ \longrightarrow RS^{-} + PZ^{\cdot +} + products} \qquad (13)$$

$$\mathrm{GS^{\cdot} + PZ \longrightarrow GS^{-} + PZ^{\cdot +}} \qquad (14)$$

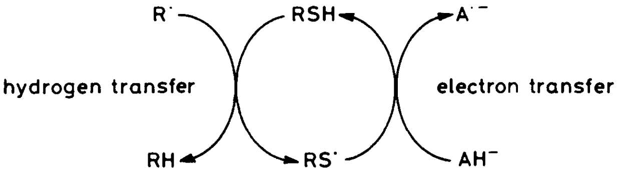
The fact that in the case of dithiothreitol (DTT) the yield of the transient absorption is considerably reduced, can be attributed to a rapid internal cyclisation of the thiyl radical with the additional thiol group on the molecule.[31,32] Previous pulse radiolysis studies have pointed to the p$K_a$ of this radical being *ca.* 5.5. The decreased absorption observed in these studies therefore implies that the protonated radical RSSRH$^\cdot$ reacts only slowly, if at all, with promethazine and chlorpromazine. Interestingly, with ABTS, the appearance of the radical-cation was observed with DTT.[33] Clearly, the monothiyl radical reacts much more rapidly with ABTS and this reaction competes effectively with the internal cyclisation reaction. Alternatively the RSSRH$^\cdot$ radical reacts rapidly with ABTS but not with the phenothiazines.

Finally, the observation that the thiyl radicals react with the phenothiazines and ascorbic acid is particularly intriguing in that it illustrates how thiols may act as a link between hydrogen atom transfer and electron transfer reactions. Such reactions may not only be of importance in general biochemistry but are also relevant to the mechanistic role of glutathione and other sulphur compounds in protecting against free radical-mediated injury. In the experiments with propan-2-ol and acetone thiyl radicals were generated by way of a hydrogen atom transfer reaction. Ascorbyl radicals can be generated similarly [reactions (15) and (16)]. The rate of reaction of ascorbic acid with the propan-2-ol radical was previously determined as $k_{15}$ $1.2 \times 10^6$ l mol$^{-1}$ s$^{-1}$, the reaction of the same radical with glutathione is $k_{16}$ $1.8 \times 10^8$ l mol$^{-1}$ s$^{-1}$, and of the glutathione radical with ascorbic acid $k_{17}$, $6 \times 10^8$ l mol$^{-1}$ s$^{-1}$ at pH 7.

$$\mathrm{CH_3COHCH_3^{\cdot} + AH^{-} \xrightarrow{k_{15}} CH_3CHOHCH_3 + A^{\cdot -}} \qquad (15)$$

$$\mathrm{CH_3COHCH_3^{\cdot} + GSH \xrightarrow{k_{16}} CH_3CHOHCH_3 + GS^{\cdot}} \qquad (16)$$

$$\mathrm{GS^{\cdot} + AH^{-} \xrightarrow{k_{17}} GS^{-} + A^{\cdot -} + H^{+}} \qquad (17)$$



**Scheme.**

Thus glutathione can be considered as a catalyst for the repair of the organic radical by a compound which more readily enters into electron rather than hydrogen transfer reactions (Scheme).

These findings have prompted studies into the possibility that radicals from compounds such as glutathione could also react with other biological molecules. Preliminary results show that this is indeed the case; the glutathione radical, for example, reacts rapidly with reduced nicotinamide dinucleotide (NADH) and also with reduced cytochrome C, *k ca.* $3 \times 10^8$ and *ca.* $1 \times 10^8$ l mol$^{-1}$ s$^{-1}$ respectively. These results, and their implications will be reported in detail elsewhere.

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