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## Studies on the Reactions of Ferric Iron with Glutathione and Some Related Thiols.

### Part III. A Study of the Iron Catalyzed Oxidation of Glutathione by Molecular Oxygen

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*Complexes formed by mixing iron(III) and reduced glutathione were prepared anaerobically and mixed with oxygen. Rapid kinetic measurements indicated the production of a transient red species, the rate of production of which was oxygen concentration dependent and required one oxygen per two iron atoms. pH studies implicated S<sup>-</sup> anion as a necessary prerequisite.*

*Mössbauer data indicated that this red complex contained iron(III) whereas the initial complex, prior to oxygenation, contained only iron(II).*

*A scheme for the iron catalyzed oxidation of glutathione by molecular oxygen is presented.*

#### Introduction

Since the discovery of glutathione (GSH) by Hopkins and the recognition that this thiol could play a role in electron transfer [1–3], there have been many studies of the autocatalytic oxidation of this biologically important compound [1–4] and other related thiols [5–11]. The very low auto-oxidation of cysteine is accelerated by small quantities of iron [5]. And it is interesting to note that iron is always associated with cysteine and glutathione [1, 2, 5, 6, 12, 13] in biological systems. Warburg and Sauma [6] demonstrated that cysteine in aqueous solutions was not oxidized by atmospheric oxygen in the absence of heavy metals and Harrison [2] showed that this was true also for glutathione. The effects of traces of heavy metals like Cu<sup>2+</sup> and Fe<sup>3+</sup> on the oxidation of cysteine, glutathione, and other thiols have been reported in other studies [1, 3].

Once the need for the metal catalyst was confirmed [1], attempts were made to derive a mecha-

nism for this catalytic process [7–11, 14, 15]. These were based on reduction potentials, reduction capacity and kinetic data. The steps common to these schemes are [10, 11, 14]:

(a) The formation of a ferrous complex of the thiol acid.

(b) An oxidation of this complex by means of oxygen to a ferric complex.

(c) An autoreduction of this ferric complex in which the iron is reduced to a divalent state while the thiol sulphur is oxidized to disulphide.

Step (b) was considered by Schubert [15] as being the cause of the red or violet colour observed in the catalytic process of thioglycol and cysteine respectively. He also related step (c) to a simultaneous breaking up of the complex and fading of the colour.

When mixed with traces of ferrous salts, and shaken with atmospheric oxygen at pH's 8–9, the colours fade with time [1, 15, 16]. These colours can be regenerated by addition of more oxygen until all the ligand has been oxidized.

In kinetic studies of thioglycol oxidation in the presence of iron(III) [16], the bleaching of the red colour was studied at 550 nm and a mechanism proposed. The pH dependence was also discussed and an optimum pH value of 4.9 was estimated for this process. The effect of pH changes on the rate of oxidation was studied [1–3, 16, 17] and it was found, for example, that copper catalysis of thioglycolic acid oxidation has an optimum at pH 6.0, whereas in glutathione oxidation the rate increased in direct proportion to the pH value [1, 17]. Hemin catalysis [1–3, 18] of glutathione oxidation by oxygen was found to have an optimum at pH 8.0. The explanation was that in glutathione the rate of oxidation depends on the degree of protonation of the sulphhydryl group. It was also suggested that the catalytic action of heavy metals can be modified by the formation of metal complexes [1, 3]. The

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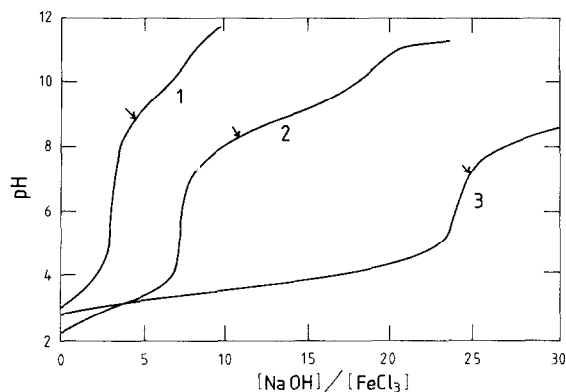


Fig. 1. pH titration curves for iron(III) chloride/GSH (under a nitrogen atmosphere), (1) 1:5, (2) 1:10, (3) 1:50 iron to glutathione proportions. The total volume in (1) and (2) was 50 ml and  $[\text{FeCl}_3] = 1 \times 10^{-3} \text{ M}$ . In (3)  $[\text{FeCl}_3] = 2 \times 10^{-3} \text{ M}$ ,  $[\text{GSH}] = 0.1 \text{ M}$  in a total volume of 25 ml. The arrows indicate the pH value at which the red colour first appears upon exposure to air.

effect of iron salts on the autocatalytic oxidation of glutathione was considered to be very small compared to the effects of  $\text{Cu}^{2+}$  and hemin [18–21].

In most of the previous reports [1–11] the kinetics of oxidation was measured by continuously monitoring oxygen uptake. Various mechanisms for the cysteine–iron autoxidation have been discussed in several of these studies. Mechanisms of oxidation of other thiols were also proposed [7, 8, 10, 11, 15, 16]. The most recent of all, by Astanina *et al.* [4, 7, 8] used kinetic data and isotope effects.

In this paper we extend our earlier acid pH studies [22, 23] on the iron–GSH system to the alkaline region. We report here the results of rapid kinetic and Mössbauer investigations of the reactions of iron–GSH complexes with molecular oxygen.

The rates and stoichiometries of reactions are presented together with spectroscopic data on the nature of intermediates in the reaction pathway and of the final products. An overall mechanism for the oxidation of glutathione by molecular oxygen catalysed by iron is proposed.

## Results and Discussion

### pH Titrations

Glutathione (GSH) and other thiols were found to reduce iron(III) to iron(II) anaerobically in acidic solution [22–23].

Under alkaline conditions glutathione–iron solutions give an intense red colour when shaken with oxygen in a similar manner to the cysteine–iron [15, 26] and thioglycolic acid–iron systems [16].

Titration data obtained under anaerobic conditions (Fig. 1) are difficult to analyse quantitatively

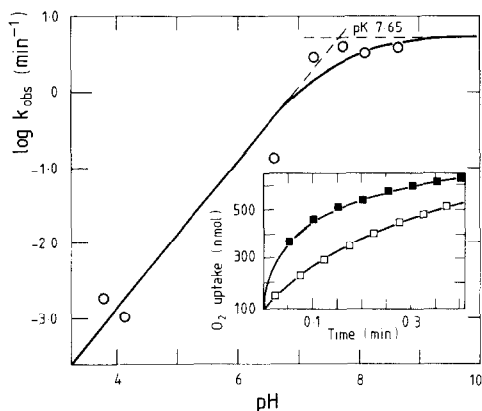


Fig. 2. The solid line represents the theoretical plot of  $\log k_{\text{obs}}$  versus pH calculated from equation (2) for the reaction of  $\text{FeCl}_3/\text{GSH}$  with oxygen. The experimental points for  $\log k_{\text{obs}}$  at different pH values are presented as (○), conditions are  $[\text{FeCl}_3] = 4 \times 10^{-3} \text{ M}$  and  $[\text{GSH}] = 0.4 \text{ M}$ . Temperature  $25^\circ \text{C}$ . The insert shows illustrative progress curves for oxygen consumption at pH 7.26 (□), and 8.6 (■).

above pH 6 because of the overlap of at least three processes, *viz.*: deprotonation of the SH and  $\text{NH}_3^+$  groups of the glutathione and the hydrolysis of the Fe(II) at pH values above 8 in these titrations [23]. Nevertheless, two points can be made; firstly there is a clear decrease in the apparent  $\text{pK}_a$  value of GSH in the presence of iron, indicating binding of the metal to the deprotonated form (probably the  $\text{S}^-$ ) and secondly that on exposure to oxygen the red colour was observed only at pH's where substantial amounts of deprotonated sulphhydryl groups might be expected to exist (Fig. 1) thus indicating that a deprotonated sulphhydryl group is a pre-condition for the production of the red intermediate [3]. Evidence in support of this interpretation will be presented below in the Mössbauer studies.

### Oxygen uptake measurements

The rate constants of the oxygen uptake for the iron(II)–GSH system at different pH values as measured by the oxygen electrode are presented in Fig. 2.

The system was found to absorb oxygen at all the pH values under study (pH 4 to 9); the insert shows some representative data. The first order rate constants from such experiments increased with increasing pH. The dramatic increase in the rate constant with pH can be related to the number of deprotonated SH groups in solution [1, 3]. A plot of  $\log k_{\text{obs}}$  (obtained from the oxygen absorption measurements) versus pH yields a slope of  $\sim 1$  below pH 7 and approaches a limiting value thereafter (see Fig. 2). This plot is consistent with the

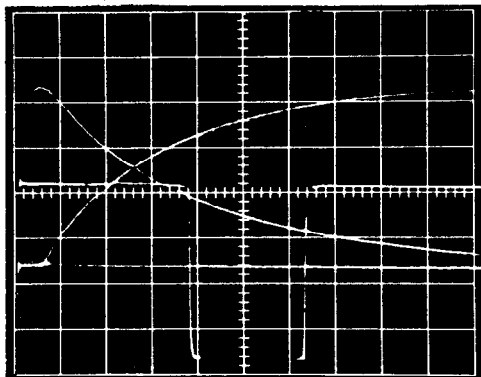
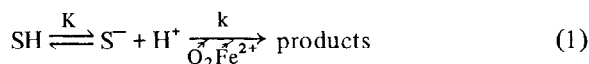


Fig. 3. Progress curves for the reaction of  $\text{FeCl}_3/\text{GSH}$  solution with oxygen. Transmittance (as a negative quality) is presented as a function of time at pH 9.0 and 25 °C,  $[\text{GSH}] = 1 \times 10^{-2} \text{ M}$ ,  $[\text{FeCl}_3] = 1.2 \times 10^{-4} \text{ M}$ , and  $[\text{O}_2] = 60 \mu\text{M}$  after mixing. The monitoring wavelength was 495 nm. The sweep times are 200 m. seconds (ascending), *i.e.* increase in absorbance, and 5 seconds (descending) per horizontal division. The maximum extinction change is 0.29.

dependence of the oxygen uptake on the deprotonation of the SH group as follows:



If

$$k_{\text{obs}} = k[\text{S}^-]$$

Then

$$k_{\text{obs}} = \frac{K}{K + [\text{H}^+]} kG_{\text{T}}$$

and

$$\log k_{\text{obs}} = \log \left( \frac{K}{K + [\text{H}^+]} \right) + \log (G_{\text{T}}k) \quad (2)$$

where  $K$  is the dissociation constant of the SH group and where  $G_{\text{T}} = ([\text{S}^-] + [\text{SH}])$  is the total glutathione concentration,  $k$  is the true rate constant for the catalytic process, and  $k_{\text{obs}}$  is the observed rate constant for oxygen uptake.

In equation (2), if  $[\text{H}^+] \gg K$ , *i.e.*  $\text{pH} \ll \text{p}K_{\text{SH}}$  the value of  $\log k_{\text{obs}}$  will be very small and dependent on the pH.

$$\log k_{\text{obs}} = \log(KG_{\text{T}}k) + \text{pH}$$

However, if  $K \gg [\text{H}^+]$ , *i.e.*  $\text{pH} \gg \text{p}K_{\text{SH}}$  then

$$\log k_{\text{obs}} = \log(G_{\text{T}}k)$$

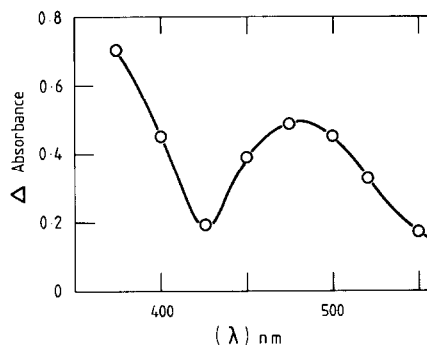


Fig. 4. The calculated absorbance spectrum of the red complex formed by the reaction of Fe-GSH with oxygen. The spectrum was calculated from progress curves of the type presented in Fig. 3  $[\text{FeCl}_3] = 1.2 \times 10^{-4} \text{ M}$  and  $[\text{O}_2] = 120 \mu\text{M}$  after mixing. The temperature was 25 °C, and pH = 9.0.

A plot of  $\log k_{\text{obs}}$  versus pH calculated from eqn. (2) gives a reasonable fit to the experimental points using a value of 7.65 for  $\text{p}K_{\text{SH}}$  and 0.74 for  $(G_{\text{T}}k)$ . The value of  $k$  obtained from this plot and with  $[G_{\text{T}}] = 0.4 \text{ mol dm}^{-3}$  (defined by experimental conditions) was  $13.7 \text{ min}^{-1}$  (Fig. 2).

#### Stopped-flow kinetics

##### Formation of the red complex

In order to examine the properties of intermediates and obtain rate data to elucidate the mechanism, rapid-mixing experiments were performed in a stopped-flow apparatus. On mixing oxygen containing solutions with iron(II)-GSH solutions at  $\text{pH} \sim 9$  a rapid increase in absorbance in the visible spectrum was observed. This indicated the rapid formation of the red complex, which then decayed in a slower process to a colourless product. A typical progress curve of these processes is represented in Fig. 3.

By performing this experiment at different wavelengths it was possible to build up the absorption spectrum of the red intermediate. Fig. 4 shows the spectrum of that red intermediate, with a maximum absorption at 495 nm. Assuming all the iron in solution is involved in the formation of the red complex it is possible to calculate its extinction coefficient as  $\epsilon_{495} \sim 2000 \text{ cm}^{-1} \text{ M}^{-1}$ .

The rate of rapid formation of the red colour was found to depend on the oxygen concentration (Fig. 5). This behaviour is consistent with a mechanism in which iron(II)-GS complex and oxygen combine in a second order reaction leading to the formation of the red complex. Fig. 5 allows the estimation of the value of the second order rate constant  $k_1$  as  $1.5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$  (from the slope of the linear portion).

TABLE I. Mössbauer Parameters at 80 K of Iron(III) and Iron(II) Reduced Glutathione Frozen Aqueous Solutions at pHs above 7.

No.	Mixture	pH	$\delta$ mm s <sup>-1</sup>	$\Delta$ mm s <sup>-1</sup>	$\Gamma$ mm s <sup>-1</sup>	%Absn. area
(1)	FeCl <sub>3</sub> /3GSH (anaerobic) contains green Fe(OH) <sub>2</sub>	8.2	0.69(2) 1.15(1)	3.35(2) 3.14(2)	0.15(2) 0.21(2)	47(2) 53(5)
(2)	FeCl <sub>2</sub> ·4H <sub>2</sub> O/3GSH (anaerobic) contains green Fe(OH) <sub>2</sub>	8.2	0.69(1) 1.08(2)	3.35(2) 3.06(2)	0.15(1) 0.33(2)	44(2) 56(4)
(3)	FeCl <sub>3</sub> /3GSH (anaerobic) contains Fe(OH) <sub>2</sub>	9.0	0.71(2) 1.18(2)	3.33(3) 3.05(2)	0.18(2) 0.24(2)	34(3) 66(4)
(4)	FeCl <sub>2</sub> ·4H <sub>2</sub> O/1.5GSSG	8.2	1.27(1)	2.95(2)	0.38(2)	100(3)
(5)	<sup>57</sup> FeCl <sub>3</sub> /10GSH (anaerobic) yellow	9.0	0.69(1)	3.32(1)	0.16(1)	100(2)
(6)	Sample (5) shaken with air (red)	9.0	0.69(1)	3.32(2)	0.16(1)	100(3)
(7)	Sample (6) shaken with more air (red)	9.0	0.69(1) 0.51(5)	3.31(1) 0.61(5)	0.17(1) 0.16(5)	93(2) 7(1)
(8)	<sup>57</sup> FeCl <sub>3</sub> /10GSH + carbon monoxide (pink)	9.0	0.68(1) 0.12(1)	3.31(1) 0.35(2)	0.18(1) 0.16(2)	70(3) 30(3)

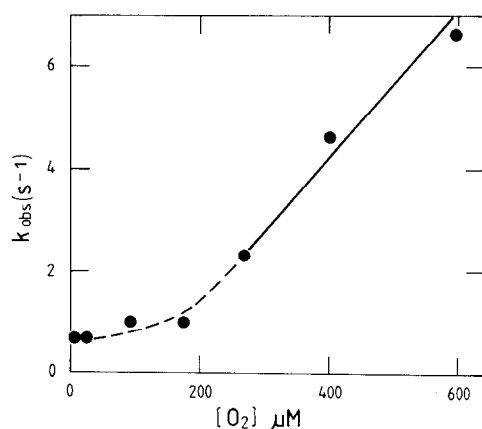


Fig. 5. Concentration dependence of  $k_{\text{obs}}$  for the formation of the red complex is shown as a function of oxygen concentration. The FeCl<sub>3</sub> concentration was  $1.2 \times 10^{-4}$  M after mixing. The straight line covers the oxygen concentration range over which the reaction is pseudo first-order. The broken line covers the concentration region where pseudo first order conditions do not apply.

The stoichiometry of oxygen to iron in the red complex was calculated from the maximum change in absorbance at varying oxygen concentrations (see Fig. 6). Taking into account the fact that we have probably underestimated the oxygen concen-

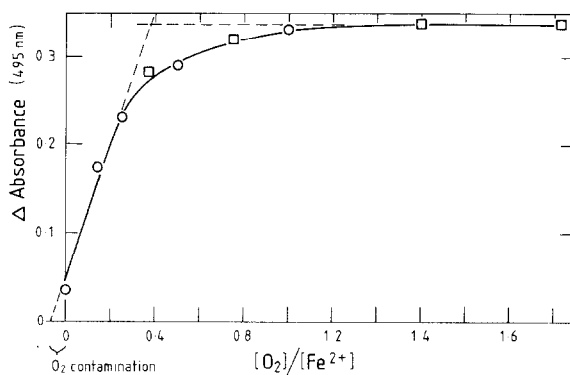


Fig. 6. Change in  $\Delta$  Absorbance at  $\lambda_{\text{max}}$  (495 nm) of the red complex at pH 9.0 as a function of oxygen-iron ratio in two different experiments, starting with (□) oxygen equilibrated water; (○) air equilibrated water. Conditions as for Fig. 3. The oxygen contamination of approximately  $5 \mu\text{M}$  is typically found for anaerobic experiments performed in a stopped-flow apparatus.

tration by approximately 5 to 10  $\mu\text{M}$  the results indicate an oxygen to iron ratio for the red complex of 0.45. We interpret this as the red complex having the formula  $(\text{Fe-GS})_2\text{-O}_2$ . The valence state of the iron in this complex is discussed below.

*The decay of the red complex*

The decay of the red complex proceeds via at least one other intermediate in a biphasic process (Fig. 3).

*Mössbauer Studies on Frozen Aqueous Solutions**Iron glutathione anaerobic samples compared with oxygen-reacted samples*

In the previous studies [22, 23] the Mössbauer spectra of anaerobically frozen solutions of iron(III) and iron(II) glutathione were reported up to pH 7 and the changes were discussed. Here we report anaerobically frozen solutions of iron(III)/GSH and iron(II)/GSH studied by Mössbauer spectroscopy before and after exposure to oxygen (Table I).

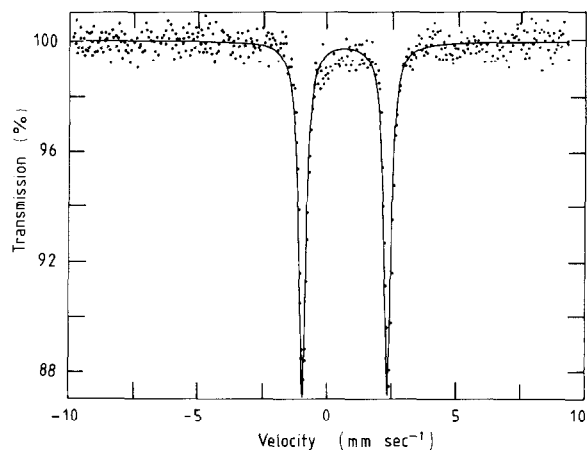
In the anaerobic iron(III)/GSH and iron(II)/GSH mixtures above pH 7.5, a green precipitate occurs [23]. The Mössbauer spectra are different to those at pH 7 in both systems [23]. The Mössbauer data for these systems (Table I) pH's 8–9, spectra 1 to 3 show evidence for two high spin iron(II) electronic environments. One of these (that associated with the green precipitate) is due to the presence of iron(II) hydrolysis products; this site has isomer shifts in the range 1.08 to 1.18 mm s<sup>-1</sup> and quadrupole splittings in the range 3.05 to 3.14 mm s<sup>-1</sup>. This site is associated with large line widths and is probably the sum of several different hydrolysis products [27–29].

The second Mössbauer site in spectra 1 to 3 has an isomer shift of 0.69 mm s<sup>-1</sup> and a quadrupole splitting of 3.33 mm s<sup>-1</sup>. Such Mössbauer parameters are similar to those found for pentacoordinate high spin iron(II) with sulphur and nitrogen containing ligands [30–32].

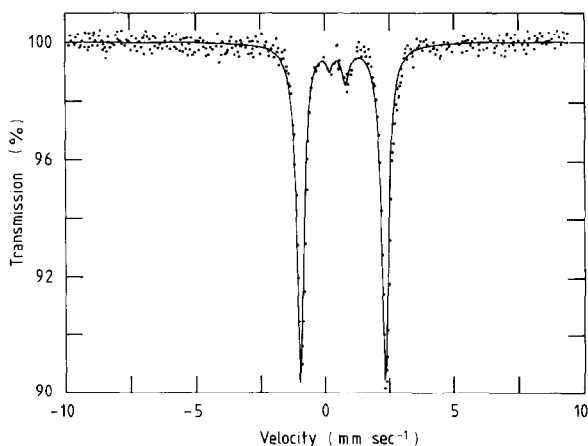
Spectrum 4 which is that resulting from a solution of FeCl<sub>2</sub>·4H<sub>2</sub>O and oxidised glutathione (GSSG) is included in the Table for comparison with the rest of the Mössbauer data presented, as all the solutions starting from iron(III) will have GSSG present as a consequence of the iron(III) to iron(II) reduction. It can be seen in this comparison that there is no site comparable to this in Table I, thus precluding an iron(II) GSSG complex as the precursor to the red complex.

This new iron(II) glutathione site was studied using 90% <sup>57</sup>Fe enriched iron and a large excess of glutathione to avoid precipitation of iron(II) hydrolysis products (see spectrum 5, Table I).

The isomer shift of the new high spin iron(II) glutathione site is in the range of those found for iron(II) sulfur containing compounds [30]. This implies that the complex present around pH 8 contains deprotonated sulphur bound to iron in addition to the peptide nitrogens and carboxylate groups. Such iron sulphur bonding would be expected here in agreement with the pK<sub>a</sub> of the SH group for glutathione [3].



(a)



(b)

Fig. 7. Mössbauer spectra at 80 K of <sup>57</sup>Fe enriched iron/10GSH frozen solution at pH 9.0. (a) after exposure to oxygen; (b) after exposing the sample which resulted in spectrum 6 to more oxygen and re-freezing <sup>57</sup>Fe = 0.2 mg/ml.

To study the iron valence state and its electronic environment in the red complex, several attempts were made to freeze the red solution after shaking with oxygen at pH 9. In the ordinary unenriched iron samples no appreciable change could be seen in the iron parameters from those of the anaerobically frozen samples, although the frozen material was intensely red in colour. This intense colour can be explained as arising from a compound with a high extinction coefficient but only a small amount of the total iron (necessary to observe the Mössbauer effect) being in this state, insufficient to produce a Mössbauer spectrum solely due to this site. The solution from spectrum 5 was thawed, exposed to air, shaken and refrozen. When only a small amount of air was introduced no great change result-

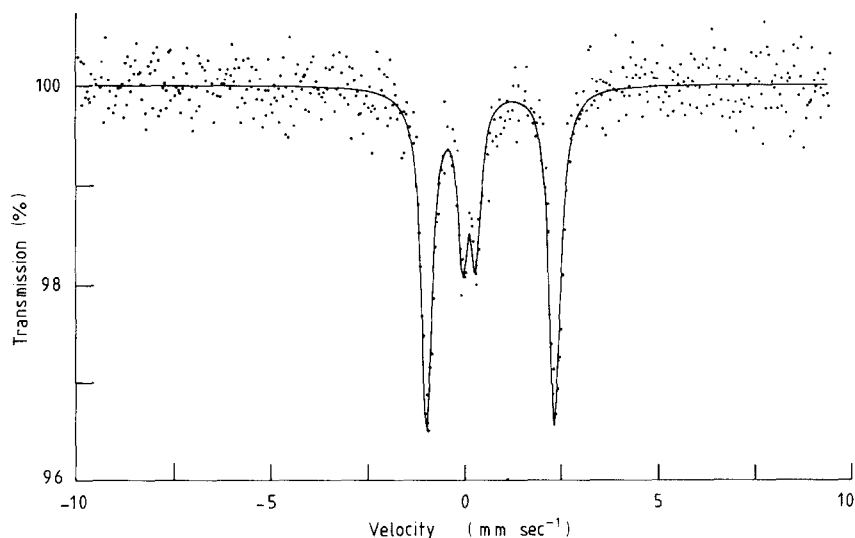


Fig. 8. Mössbauer spectrum at 80 K of frozen solution (pink) of  $^{57}\text{Fe}/10\text{GSH} + \text{carbon monoxide}$  at pH 9.0.  $^{57}\text{Fe} = 0.2 \text{ mg/ml}$ .

ed (spectrum 6) although the solution was red, but traces of iron(III) were present as can be seen in Fig. 7(a). On thawing, exposing to additional air near  $0^\circ\text{C}$ , and refreezing this solution, spectrum 7 resulted. In spectrum 7 (Fig. 7(b)) the presence of high spin iron(III) is more obvious, the material was a much more intense red colour. Obviously introduction of oxygen causes the red colour and the increase in iron(III) in the spectrum. The amount of observed iron(III) agrees with the expectation based on the stoichiometry of the reaction and the solubility of oxygen.

#### *Carbon monoxide binding of the iron(II)–glutathione complex at pH 9*

Iron(II) cysteine has been found to bind carbon monoxide in a ratio of one iron to two CO molecules [32–34]. The Mössbauer data for this material ( $\delta = 0.12$  and  $\Delta = 0.24 \text{ mm s}^{-1}$ ) are consistent with a low spin iron(II) electronic environment [34].

It has been established in this work that iron(II) glutathione solutions bind oxygen in a similar manner to iron(II) with other thiols [30] such as cysteine [33, 34]. As spectrum (5) is similar to that of iron(II) cysteine frozen solution [23] and solid [34] it seemed reasonable to expect similar behaviour with CO for this iron(II) glutathione complex. Indeed there has been a very early report of a pink complex in iron(II)–GSH solutions equilibrated with CO ( $\lambda_{\text{max}} = 490 \text{ nm}$ ) containing two CO molecules per iron [35]. Mössbauer studies on a similar sample equilibrated for 24 hrs in the dark with CO yielded spectrum 8 (Table I) (Fig. 8). This spectrum shows the presence of a low spin iron(II) site, similar to

that reported for iron(II) cysteine with CO, as well as the original high spin iron(II) electronic environment.

The presence of this new low spin site provides evidence for the ready availability of binding sites for CO (or oxygen) in the original high spin iron(II) coordination. The fact that only about 30% of the iron is converted to low spin by reaction with CO may be explained in terms of the ring sizes around the iron [30], *i.e.* the larger the ring sizes involved in the original complex before reaction with carbon monoxide, the less stable its CO complex [30].

#### **Conclusions**

The results presented in this paper are in substantial agreement with those of others working on the oxidation of cysteine and thioglycol in the presence of iron salts. Our results may be used to propose a mechanism for the autocatalytic oxidation process in which the confusion in the literature regarding the iron(III)/iron(II) cycle is clarified.

The proposal is based upon the following conclusions drawn from our experimental data.

(1) In the reduced glutathione–iron(III) system, iron(II) is produced spontaneously after mixing, through an electron transfer from the thiol to the iron(III) [22].

(2) Iron(II) forms a complex with deprotonated thiol, the formation of which is pH dependent. Favourable conditions for complex formation are

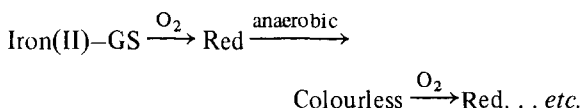
(a) a GSH iron ratio of at least five to one to prevent the hydrolysis of iron(II).

(b) pH values in the range 8–9

(3) If exposed to oxygen this iron(II)–GS complex forms a red complex which undergoes an auto-catalytic oxidation of the thiol. This process is pH dependent with  $pK = 7.65$  (Fig. 2).

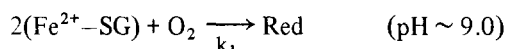
(4) The stoichiometry of the red complex is two iron atoms per oxygen molecule, *i.e.*  $(Fe-GS)_2 \cdot O_2$ .

(5) The formation of the red complex is a second order process and it decays in a biphasic process. The whole process, *i.e.* formation of the red complex with oxygen and anaerobic decay, continues as follows:



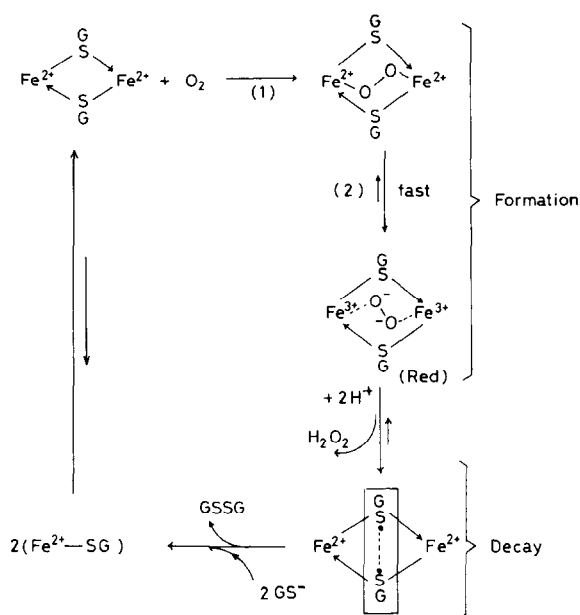
until all the ligand is oxidized.

We may apply the above information to the following reaction:

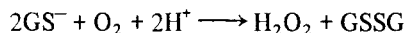


The red complex forms after the prior rapid formation of an (iron(II)–GS–O<sub>2</sub>) complex in the presence of oxygen. The change from the (iron(II)–GS–O<sub>2</sub>) complex to the iron(III) complex is not the rate limiting step. Since the frozen solution samples were trapped in about 1 second (before the red complex had decayed completely) and the Mössbauer spectrum showed the presence of iron(III), we believe the red complex is an iron(III) complex, *i.e.*  $(Fe^{III}-GS)_2 \cdot O_2^{2-}$ .

The previous discussion can be summarised in the following scheme.



The overall reaction catalyzed by this cycle is therefore



The kinetic studies are consistent with this mechanism in which the formation of the red iron(III) complex is a combination of steps (1) and (2) with step (1) being the rate limit, since the rate of formation is oxygen concentration dependent.

In common with other work on cysteine [4, 7, 8] using isotope effects,  $O_2^{2-}$  reacts with  $2H^+$  to form  $H_2O_2$ . This means the whole process involves an electron transition from one oxygen to one iron and a second electron transition from one  $GS^-$  to an iron.

### Experimental

#### pH Titrations

Iron(III) chloride ( $1 \times 10^{-3} M$ ) was titrated with different proportions of glutathione (GSH) under a nitrogen atmosphere. Small portions ( $5 \mu l$ ) of solution, were exposed to air at one pH intervals to detect the pH at which the red colour starts to appear.

#### Oxygen Absorption Measurements of Iron(II)–glutathione using an oxygen electrode

Iron(III) chloride solution ( $4 \times 10^{-3} M$ ) was mixed with  $0.4 M$  glutathione solution under anaerobic conditions in a total volume of 25 ml.

Oxygen uptake was measured using an oxygen electrode [24] (Rank Brothers, Cambridge).

The electrode was calibrated by measuring the voltage difference between air equilibrated water and anaerobic water obtained by adding a small amount of sodium dithionite to the reaction chamber (3 ml of air equilibrated water at  $25^\circ C$  contains 720 nmole  $O_2$ ).

0.5 ml iron–glutathione solution was injected into the thermostatted reaction chamber which contained 3 ml of air saturated water at the same pH and the change in oxygen concentration with time was recorded. Experiments were repeated at several pH's using this procedure.

#### Stopped-flow kinetics study

The iron–glutathione complex was prepared under a nitrogen atmosphere by mixing  $2.4 \times 10^{-4} M$  iron(III) chloride with 100-fold excess of glutathione, then adjusting the pH to 9.0. The solution was degassed, transferred under a positive pressure of nitrogen to a syringe and fitted to the stopped-flow apparatus. Another syringe containing water at pH 9.0 and oxygen at a known concentration was also fitted to the stopped-flow apparatus. Pro-



gress curves were recorded at different wavelengths after mixing the water and the complex.

Solutions of known oxygen concentration were prepared by diluting either air saturated, or oxygen saturated water with known volumes of deoxygenated water.

#### Mössbauer spectroscopy (frozen solutions)

Iron(III)–GSH and iron(II)–GSH solutions were prepared, their pH's adjusted to the required value, and the Mössbauer spectra were recorded at 80 K before and after exposure to oxygen.

The experiments were repeated using  $^{57}\text{Fe}$  enriched with excess glutathione, with the pH adjusted to 9.0. Mössbauer spectra of frozen solutions were recorded at 80 K, firstly under an inert atmosphere, secondly after bubbling with oxygen and finally after bubbling with carbon monoxide for twenty-four hours.

The Mössbauer spectra were obtained at 80 K on a spectrometer described previously [36]. The spectrometer was calibrated with a 25  $\mu\text{m}$  thick natural iron reference absorber. All isomer shifts are referred to this as zero shift.

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