Kinetics of the Copper- and Iron-Catalysed Oxidation of Cysteine by Dioxygen

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For the purpose of controlling levels of thiols and peroxide in biological experiments, the kinetics of 'autoxidation' of cysteine (RSH) catalysed by copper or iron ions, added to the reaction medium as Cu(II) and Fe(II) or Fe(III) salts, has been studied. The reaction may be described as proceeding in two steps, only the second of which is spontaneous:

$$2\ RSH + O_2 \rightarrow RSSR + H_2O_2 \quad \text{(i)}$$

$$2\ RSH + H_2O_2 \rightarrow RSSR + 2\ H_2O \quad \text{(ii)}$$

The Cu(II)-catalysed reaction (i) follows Michaelis–Menten kinetics with respect to RSH, and probably O_2, and is, partly at least, second order with respect to Cu. The rate of (ii) is first-order with respect to RSH and H_2O_2 and is enhanced by Fe(II) and Fe(III) but practically not at all by Cu(II). With Cu(II) as the catalyst of the overall reaction, H_2O_2 is one of the final products. With Fe(II) or Fe(III) as the catalyst, the overall reaction, which proceeds without formation of H_2O_2, is first-order with respect to RS^- and Fe(II)/Fe(III) (at least at high [Fe]). When catalysed by an FeO(OH) sol the kinetics change to zeroth-order with respect to RSH. The Cu- and Fe-catalysed rates are not additive; at certain concentrations Fe slows down the overall Cu-catalysed reaction and changes the kinetics towards zeroth order, reducing the levels of H_2O_2.

The mechanisms of the catalysis by Cu of (i) and by Fe of (i) and (ii) have not yet been fully explained. The present study indicates a possible role for binuclear complexes (Cu + Cu or Cu + Fe) and shows further the usefulness of fitting data to numerical solutions of differential equations for models describing reaction rates, in work aimed at the elucidation of mechanisms.

Introduction

In experimental systems containing biological materials such as cells or cellular constituents, it is usually difficult to maintain constant concentrations of cysteine, cysteamine or other thiols. To a great extent this is due to contamination by water, chemicals and glassware with trace metals, especially copper and iron, which catalyse the oxidation of thiols by molecular oxygen. This 'autoxidation' of thiols leads further to the formation of biologically active intermediates, especially H_2O_2 shown to be generated in the Cu-catalysed* reaction, the effects of which, or the reaction products of which, such as the ·OH radical, have to be considered specifically in studies of the radioprotective action of thiols[1-4] and, in the general context of oxidative stress, with numerous other consequences.[5,6]

Kinetic studies of the oxidation by dioxygen, catalysed by added Cu(II) or/and Fe(II)/Fe(III), of radiobiologically important thiols were undertaken with the purpose of determining rate constants useful for the design of experiments with respect to the desired stability of a thiol, and control of doses of peroxide to biological materials. With such practical purposes the studies thus aim at descriptions of the reactions in operational terms, valid under physiological conditions and at thiol and metal-ion concentrations in the ranges 10^{-4}–10^{-2} M and 10^{-7}–10^{-5} M, respectively, rather than at a clarification of mechanisms. Data for the Cu-catalysed oxidation of cysteine that are useful for such purposes have been presented by Hanaki and Kamide.[7,8] Several researchers of the Cu- and Fe-catalysed autoxidation of cysteine and other thiols have clarified the general traits of the reactions involved, but have so far been unable to arrive at an understanding of the details of the mechanisms. It is generally agreed that the Cu-catalysed reaction proceeds in two steps [eqns. (1) and (2)].†

*The 'autoxidation' of thiols by Cu or Fe has been studied by adding Cu(II), Fe(II) or Fe(III) salts to reaction media. Since the mechanisms have not yet been completely clarified, the valency state, when given, refers to that of the metal in the added salt. When not needed (or not known) the valency states are not given. [Cu] and [Fe] denote total concentration of the respective metal.

†Abbreviations: RSH, (total) cysteine; $[\ ]_0$ initial concentration; k_0 zeroth-order rate constant; $k_0' = k_0[RSH]/(K + [RSH])$; k_1 and k_1' first-order rate constants for reaction of thiolate anion, RS^-, and total thiol, respectively; k_2 and k_2' corresponding for second-order reactions; CV coefficient of variation; SS (as index) steady state; Tris, EDTA, see Experimental.

$$2\,RSH + O_2 \rightarrow RSSR + H_2O_2 \quad (1)$$
$$2\,RSH + H_2O_2 \rightarrow RSSR + 2\,H_2O \quad (2)$$
$$4\,RSH + O_2 \rightarrow 2\,RSSR + 2\,H_2O \quad (3)$$

Reaction (1), the stoichiometry of which has been shown in dilute solution,[8] is catalysed by Cu whereas reaction (2) is spontaneous, although strongly enhanced by Fe.[9] It is questionable whether (2) is catalysed by Cu under physiological conditions.[10,11] Since H_2O_2 is a final product[7,8,11–14] the stoichiometry of the overall reaction is described by eqn. (4) better than by eqn. (3) (cf. Ref. 14), where a ($0 < a < 1$) is the fraction of H_2O_2 formed which reacts further according to eqn. (2).

$$2(1+a)RSH + O_2 \rightarrow (1+a)RSSR + (1-a)H_2O_2 + 2aH_2O \quad (4)$$

It has been suggested[7,15,16] that reaction (1) is a chain reaction comprising the reduction Cu(II) → Cu(I) within a complex $Cu(SR)_n$, with formation of free thiyl radicals, RS·, which recombine to give the disulfide, RSSR. Cu(I) then is reoxidized by O_2 giving rise to superoxide anion radicals, $O_2^{\cdot-}$, that disproportionate to H_2O_2 and O_2. Zwart *et al.*[14] have interpreted kinetic data obtained in strongly alkaline solution by assuming that Cu(I)SR, although shown to be formed anaerobically,[17,18] is not an intermediate, and that RS· and $O_2^{\cdot-}$ are not liberated into the medium but remain as ligands in activated Cu(II) complexes. In the iron-catalysed reaction H_2O_2 does not appear as an end product, and the overall reaction may therefore be described by eqn. (3).

Results

1. Oxidation of cysteine by H_2O_2. Reaction (2) was found, according to standard criteria,[19] to be first-order with respect to cysteine and hydrogen peroxide. This is in agreement with earlier findings for the oxidation, by H_2O_2, of cysteine[9,20] and of *o*-mercaptophenylacetic acid in the presence of EDTA.[21] In 40 mM Tris at pH 7.2 the rate constant, k_2', calculated for the concentration of total cysteine, [RSH], of eqn. (5) was determined to be 310 M^{-1} min^{-1} at 37 °C.

$$\frac{d[RSH]}{dt} = -k_2'\,[RSH]\,[H_2O_2] \quad (5)$$

The pH dependence (Table 1) shows that the reacting species is the thiolate anion, RS^-, with $k_2 \approx 2700$ M^{-1} min^{-1} at 37 °C. The temperature dependence is relatively small, $k_2 \approx 1700$ M^{-1} min^{-1} at 25 °C, corresponding to $\Delta H^* \approx 27$ kJ mol^{-1}. Increased ionic strength (0.010–0.120 M phosphate buffer of pH 7.2, μ = 0.025–0.30) produced a slight enhancement of the reaction rate (Table 1), evidently the result of two opposing effects, viz., an increased degree of ionization of –SH[22] and a decreased activity coefficient of RS^-. The rate constant found is compatible with the value[2] (defined as $k'/2$ of the present study) at 'room temperature' as determined by Barton *et al.*[20] At pH 13.5, and T = 23 °C, the rate constant was determined to be $k_2 \approx$ 10 M^{-1} min^{-1},[11] a value two orders of magnitude less than that of the present study, extrapolated to 23 °C. This indicates that H_2O_2 rather than its anion HO_2^- (pK_a 11.6[23]) is the reactive species under physiological conditions.

Table 1. Second-order rate constants (k_2') in the reaction of cysteine with H_2O_2 at different temperatures, pH values and ionic strengths, and the calculated second-order rate constants (k_2) of the reaction of the thiolate anion.[a]

Temperature/°C	Buffer	pH	k_2'/M^{-1} min^{-1}	k_2/M^{-1} min^{-1}
25	Tris	7.2	160	1780
	40 mM	7.7	410	1810
		8.2	830	1660
		8.7	1300	1710
		9.2	1640	1800
			Mean value:	1730
37	Tris	7.2	310	2780
	40 mM	7.7	757	2700
		8.3	1584	2590
		8.7	2090	2620
			Mean value:	2700
37	Phosphate			
	10 mM	7.2	350	
	60 mM	7.2	380	
	120 mM	7.2	400	

[a] For the calculation of k_2 the concentrations of thiolate anion (RS^-) at different pH values were estimated from the dissociation constants of cysteine-SH: pK_a = 8.2 at 25 °C and pK_a = 8.1 at 37 °C (cf. Ref. 26). Due to the alternative dissociation of –SH or $-NH_3^+$ in the second ionization step,[22,27] handbooks[28] sometimes erroneously identify the dissociation of the SH group of cysteine with its pK_3 at about 10.5. The nucleophilic reactivity of cysteine, e.g. versus acrylonitrile,[27] definitely agrees with pK_{SH} about 8; cf. also arguments by Benesch and Benesch[29] and review by Cecil and McPhee.[30]

Table 2. Influence of Cu(II) and Fe(II) on the rate of the oxidation of cysteine by H_2O_2. Conditions: 40 mM Tris, pH 7.2, 37 °C.

Catalyst	Catalyst concentration/μM	k_2'/M^{-1} min^{-1}
None		310
Cu(II)	0.5–1	340
Fe(II)	0.3	735
Fe(II) + 0.3 μM EDTA		800
Fe(II) + 3 μM EDTA		310
Fe(II)	1	1600
Fe(II)	3	5500
Fe(II)	10	14000

Reaction [(2) and (5)] was found to be little, if at all, affected by added Cu(II) at concentrations around 1 μM. This is compatible with results of Pascal and Tarbell[21] and Zwart *et al.*[14] The catalytic action of Cu(II) found in some earlier studies[24] carried out under aerobic conditions might well be due to simultaneous 'autoxidation' [eqn. (1)]. In some of the experiments (Table 2) k_2' increased by about 10 % at 0.5–1 μM Cu(II). This increase was taken into account in the kinetic study of reaction (1) below. It must be stated, however, that the increase may very well be due to catalysis of a reaction between RSH and residual oxygen, $d[H_2O_2]/dt$ being much less influenced than $d[RSH]/dt$ by Cu. However, except in extreme cases, the influence of an error of 10 % in k_2' on the rate of the overall reaction (3) and on the concentration of H_2O_2 during the course of this reaction, is negligible.[25] Under conditions in which steady-state $[H_2O_2]$ develops, the influence of k_2 on $[H_2O_2]$ will be maximal; at the same time its influence on $d[RSH]/dt$ will be zero [cf. eqns. (6c), (6d)].

In contrast, reaction (2) was found to be strongly catalysed by Fe(II) (Table 2) [or by Fe(III) which, after reduction, probably acts by the same mechanism], even at concentrations so low (about 1 μM or less) that reaction (1) is

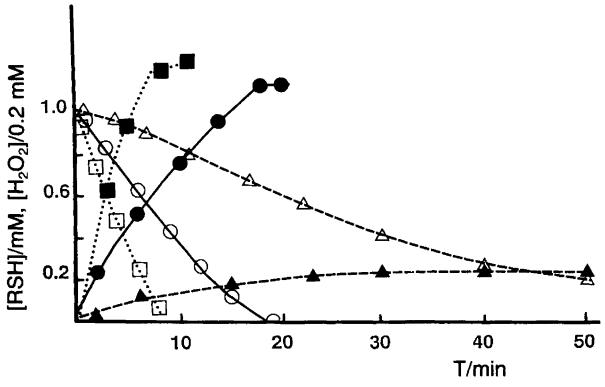

Fig. 1. Autoxidation of cysteine (1 mM) catalysed by Cu(II) at concentrations 0.3 μM (△), 1 μM (○) and 5 μM (□). Solutions of cysteine were bubbled with air at 37 °C in the presence of 40 mM Tris buffer (pH 7.2) and $CuCl_2$. The concentrations of residual cysteine (empty symbols) and generated H_2O_2 (filled symbols) were determined at given time intervals.

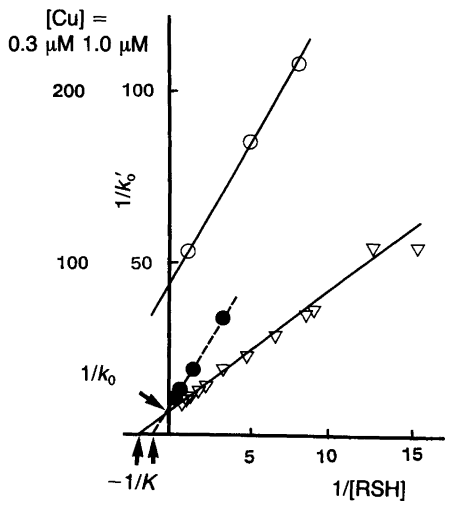

Fig. 2. Lineweaver–Burke presentation of $1/k_0'$ vs. 1/[RSH] from an experiment with [Cu(II)] = μM (▽) and 0.3 μM (○), pH 8.1, 37 °C, in O_2. Curve with filled circles: k_0' at mean values of intervals for [Cu] = 1 μM in Table 3.

hardly affected. This is in agreement with several earlier studies.[9,21,24] This catalytic action of Fe is completely inhibited by a ten-fold excess of EDTA.

2. The Cu-catalysed oxidation of cysteine by O_2. The changes in the concentrations of cysteine ([RSH]) and hydrogen peroxide ($[H_2O_2]$) following the administration of Cu(II) to solutions of the thiol are illustrated in Fig. 1.

At high [RSH] and low [Cu], the rate of disappearance of RSH, $-d[RSH]/dt$, approaches a constant value, and $[H_2O_2]$ approaches a steady-state concentration, $[H_2O_2]_{SS}$. This indicates zeroth-order kinetics of the first step [eqn. (1)] of the overall reaction [(3) or (4)], in keeping with observations of the rate of consumption of oxygen.[7,12,14,31] The slower rate of eqn. (1) at lower [RSH] (Table 3) is compatible with Michaelis–Menten kinetics as illustrated in Fig. 2. The rates of change of [RSH] and $[H_2O_2]$ may therefore be described by eqns. (6a)–(6c).

$$\frac{d[RSH]}{dt} = -k_0' - k_2'\,[RSH]\,[H_2O_2] \tag{6a}$$

$$\frac{d[H_2O_2]}{dt} = \frac{k_0'}{2} - \frac{k_2'}{2}\,[RSH]\,[H_2O_2] \tag{6b}$$

where

$$k_0' = \frac{k_0\,[RSH]}{K + [RSH]} \tag{6c}$$

At high [RSH] the concentration of H_2O_2 and the rate $d[RSH]/dt$ assume the steady-state values [eqns. (6d, e)].

The pseudo-zeroth order rate constants, k_0', of eqns. (6a, b) were calculated from

$$[H_2O_2]_{SS} = \frac{k_0'}{k_2'\,[RSH]} \tag{6d}$$

Table 3. Autoxidation of cysteine (RSH) at 37 °C in 40 mM Tris pH 7.2, in the presence of Cu(II).

$[RSH]_o$ /mM	[Cu] /μM	Concentration[a] /mM	$-\frac{d[RSH]}{dt}$ /μM min^{-1}	$\frac{d[H_2O_2]}{dt}$ /μM min^{-1}	k_0' /μM min^{-1}	$[H_2O_2]$/μM at mid interval	
						Expected	Found
30	1	30–20[b]	240	0	120	14	(15)[c]
10	1	10–5	2.3(1)10[2][d]	2.3	117(10)[d]	49	48
		5–1.7	150	2.2	90	59	70
3	1	3–1.4	148	5	80	88	92
1	1	1–0.5	70(5)[d]	15	51	80	70
1	0.5	1–0.4	32	2.5	19	52	50
1	0.25	1–0.7	14	0.6	8	24	18
0.3	1	0.3–0.2	35(1)[d]	11	29	46	44[e,f]

[a]Concentration intervals are given within which the total rate of disappearance of RSH (eqn. 6a) and the rate of formation of H_2O_2 may be presented as linear functions of time. The zeroth-order rate constant, k_0', for eqn. (6b), and the expected and found $[H_2O_2]$ are given. [b]Solution becomes turbid at about 20 mM because of precipitation of cystine. [c]From experiment with cysteamine. [d]Mean value and maximum error from three experiments. [e]Rapid rise of $[H_2O_2]$. [f]Final $[H_2O_2]$ = 104 μM, i.e. corresponding to 70 % of the amount expected to be generated (150 μM).

and, respectively,

$$\lim_{[RSH]\to\infty} \frac{d[RSH]}{dt} = -2k_0' \qquad (6e)$$

experimental determinations of $d[RSH]/dt$ and $d[H_2O_2]/dt$, using values of k_2' from section 1, in curves of the kinds given in Fig. 1, and also by an explicit solution of eqns. (6a, b).[25] Agreement between the expected and found $[H_2O_2]$ is exemplified in Table 3. The complexity of the system is illustrated in Table 4, which shows the dependence of k_0' on oxygen pressure, pH, [RSH] and [Cu(II)].

With the Michaelis–Menten function (6c) for k_0', the system of differential equations (6a, b) has no explicit solution. The parameters k_0, K and k_2' in these equations were therefore estimated using a non-linear regression with numerical solutions of the equations (see the subsection Calculations, below). A study of the data set of Table 4 generated reasonable values of the parameters. Since the values of k_2' were, by and large, compatible with the values determined separately (Table 1), the values of k_0 and K were studied further after insertion of k_2' = 310 and 1350 M^{-1} min^{-1} at pH 7.2 and 8.1, respectively. The suggested model fits well for most of the data sets without systematic residuals around the fitted curve. Certain variations in values obtained can be ascribed to the fact that the experiments were not designed with an analysis of this kind in mind; in particular, uncertainty in the time points and systematic errors in decisively important measurements at low concentrations may have increased the variance.

For data sets where the estimation algorithm failed to converge, the ratio k_0/K seemed to be constant. With reparametrization of the model given in eqn. (6c), eqn. (7) is obtained and data for $c = k_0/K$ are given in Table 5

$$\frac{k_0[RSH]}{K + [RSH]} = \frac{c[RSH]}{1 + \frac{[RSH]}{K}} \qquad (7)$$

were generated. The ratio k_0/K is the semi-first order rate constant for reaction (1) at low concentrations of RSH ($[RSH] \ll K$; under the conditions studied $K \leq 1\times10^{-3}$M).

Table 4. Influence of pH, oxygen tension and concentrations of cysteine and Cu, on k_0', determined from eqn. (6e).

P_{O_2}	pH	[RSH]/mM	k_0'/μM min^{-1} at [Cu]/μM				
			0.3	1	2.5	5	10
Air	7.2	1	12	67	85	100	118
		5	30	81	163	191	225
	8.1	1	3	48	210	720	–[a]
		5	8	60	334	800	870
O_2	7.2	1	14	107	450	–[a]	–[a]
		5	40	240	740	850	1 400
	8.1	1	5	37	≈200	≈900	–[a]
		5	9	97	≈550	≈1 950	–[a]

[a]Too fast for measurement.

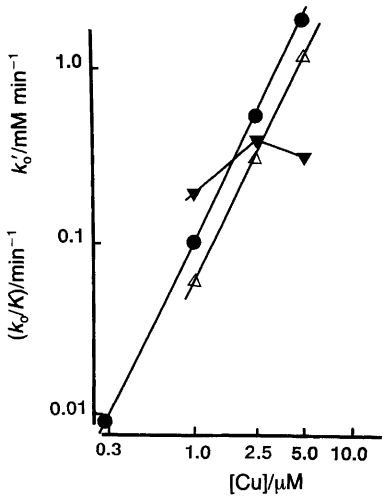

Fig. 3. Influence of [Cu(II)] on k_0' (circles; data from Table 4, pH 8.1, O_2) and on k_0/K [mean values of data from Table 4; (▼) pH 7.2, air; (△) pH 8.1, air and O_2]. Logarithmic presentation.

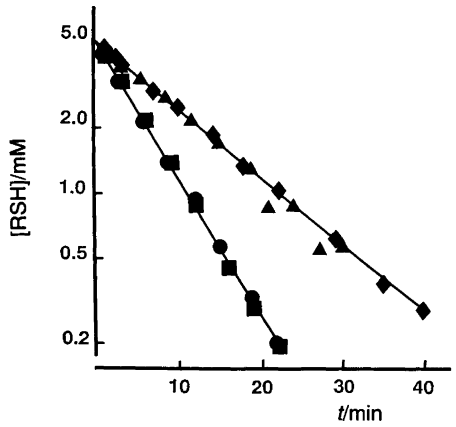

Fig. 4. Log [RSH] vs. time in autoxidation of 5 mM cysteine in 40 mM Tris catalysed by 10 μM Fe(III) added from acidic solution, pH 8.1, 29 °C, equilibrium with air (♦) and with oxygen (●). The latter also in the absence of buffer (■). (▲) shows the equivalent O_2 consumption (ΔO_2), given as (ΔO_2) final − $(\Delta O_2)_t$, under the conditions of the experiment in air. Temperature and pH were chosen to permit direct comparison with the data of Taylor *et al.*[32]

The values in Table 5 give support to the indication from Table 4 that influences exerted by the [Cu] and $[O_2]$ are different at pH 7.2 and 8.1 (Fig. 3). At pH 8.1 data in both Tables are compatible with k_0 (and k_0') being proportional to the square of the [Cu], possibly with saturation at 10 μM Cu. At pH 7.2 a saturation effect predominates at [Cu] ≥ 1 μM.

The influence of the oxygen pressure is much smaller at pH 8.1 than at pH 7.2. Assuming the effect of $[O_2]$ to follow Michaelis–Menten kinetics, the data for k_0/K in Table 5 indicate Michaelis' constants $K_{O_2} \approx 0.05$ atm at pH 8.1 and ≈ 1.4 atm at pH 7.2 (in this calculation the evidently erroneous value at pH 8.1, 2.5 μM Cu, $[RSH]_0$ = 5 mM, was omitted).

Table 5. Estimates of k_0/K, with s.e.s, from experiments in Table 4. Data at 0.3 μM Cu not analysable because the reactions were not followed to completion.

P_{O_2}	pH	[RSH]/mM	$(k_0/K)/min^{-1}$ at [Cu]/μM		
			1	2.5	5
Air	7.2	1	21(2)	43(9)	39(17)
		5	18(4)	–[a]	24(5)
	8.1	1	5(0.3)	32(1)	116(5)
		5	6(0.3)	38(1)	116(8)
O_2	7.2	1	41(5)	136(40)	–[b]
		5	41(7)	74(6)	103(17)
	8.1	1	7(1)	36(2)	157(8)
		5	7(2)	19(3)[c]	138(18)

[a]Not analysable because reaction was not followed to completion. [b]Reaction too fast for measurements. [c]This value is obviously too low by a factor ca. 2, because a reaction cannot be slower at higher P_{O_2}.

3. The Fe-catalysed oxidation of cysteine. Hydrogen peroxide is not detectable during the Fe-catalysed reaction, and the stoichiometry is therefore described by eqn. (3). It is, in fact, indicated that free H_2O_2 is not an intermediate as it is in the Cu-catalysed reaction [eqns. (1) and (2)]; this follows, i.a., from the observation that catalase, at a catalytic power ca. ten times greater than the rate of disappearance of H_2O_2 through reaction (2) at given [Fe] and [RSH] (cf. Section 1), was found not to affect the reaction rate (data not shown).

When Fe was administered as monomeric Fe(II) or Fe (III), i.e. from solutions prepared by dissolving the respective salts in 0.05 M H_2SO_4, the reaction rate, measured by $d[RSH]/dt$ or $d[O_2]/dt$, was invariably found to be first-order with respect to [RSH] (log[RSH] vs. t being strictly

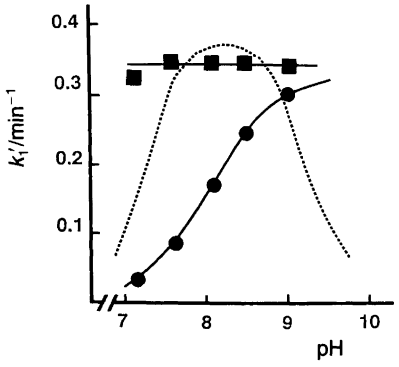

Fig. 5. pH Dependence of the first-order rate constant, k_1', for the Fe(III)-catalysed autoxidation of cysteine at 29 °C (●), and of the calculated k_1 for the thiolate anion $k_1 = k_1'\ (1 + 10^{pK_a - pH})$ (■). For comparison, the pH dependence of the zeroth-order rate constant, k_0 (– – – –), according to Taylor *et al.*[32] (see Discussion).

Table 6. First-order rate constants of the Fe(II)- and Fe(III)-catalysed autoxidation of cysteine of initial concentrations 1–5 mM, in 40 mM Tris buffer. Values are averages of 3–6 determinations during the course of six months with a maximum variation of about 10 %, where not otherwise stated.

Added catalyst	[Fe]/μM	P_{O_2}	k_2'/min^{-1} at		
			pH 8.1		pH 7.2
			29 °C	37 °C	37 °C
None	0	O_2	<0.0002	<0.0002	<0.0002
Fe(II)	1	Air	–	–	≤0.001[a]
		O_2	0.009	–	–
	10	Air	0.090	0.075	0.016[b]
		O_2	0.17[c]	0.14	0.024[b]
Fe(III)	1	Air	0.003	–	–
		O_2	0.006	–	–
	10	Air	0.065[d]	–	0.015
		O_2	0.15	0.13	0.027 (0.007)[e]

[a]Reaction order could not be determined. [b]Tendency to somewhat lower rates at [RSH] = 5 mM; cf. Table 2. [c]For unknown reasons values as low as 0.13 min^{-1} obtained occasionally. [d]Rate constant for consumption of O_2: in 40 mM Tris k_1' = 0.067 (cf. Fig. 4); in absence of buffer k_1' = 0.054 min^{-1}. [e]Mean value and maximum error.

linear) at [Fe] about 10 μM (Fig. 4). Fe(III) is nearly as effective as Fe(II) [indicating the same mechanism following the initial reduction of Fe(III)]. First-order rate constants at pH 7.2, 37 °C and at pH 8.1, 29 and 37 °C, are given in Table 6. The reaction rate increases monotonously with pH in the range studied (7.2–9.5), the rate constant being approximately proportional to [RS$^-$] (Fig. 5).

The observation of first-order kinetics is compatible with results of certain earlier studies[31] of the Fe-catalysed autoxidation of cysteine and other thiols, but it diverges from the alternative model presented by Taylor *et al.*[32] for the Fe (III)-catalysed reaction, suggested to be zeroth-order with respect to cysteine and oxygen, and two-thirds order with respect to Fe. Taylor *et al.* also found that the reaction rate exhibited a maximum at pH 8.1 (see Fig. 5). Zeroth-order kinetics with respect to cysteine were approached only when $FeNH_4(SO_4)_2$ was initially dissolved in water, and especially if the stock solution was prepared from an aged sample, judged from its yellowish colour to be partly hydrolysed, or if it was permitted to age before use (Fig. 6). Under these conditions the reaction is presumably due to surface catalysis on iron(III) oxyhydroxide, $(FeOOH)_n$, particles. In this study the rate of the reaction catalysed by monomeric Fe(II) or Fe(III) was approximately doubled by switching from air to oxygen (Table 6), corresponding to one-half-order kinetics or Michaelis–Menten kinetics. The dependence on [Fe] (Fig. 7) was found to be linear above ca. 5 μM Fe; below this [Fe] the rate is approximately proportional to [Fe]$^{1.5}$ indicating the influence of second-order kinetics, although it cannot be excluded that the effect is due to losses of catalytically active Fe, e.g. through adsorption onto glass walls.

4. Mixed Cu + Fe catalysis. Water, glassware, chemicals and biological materials often contain both Cu and Fe. Therefore, the consequences of contamination of the Cu catalyst by Fe and *vice versa* were investigated.

The zeroth-order rate constant for the oxidation of cysteine by oxygen, catalysed by colloidal FeO(OH), was –

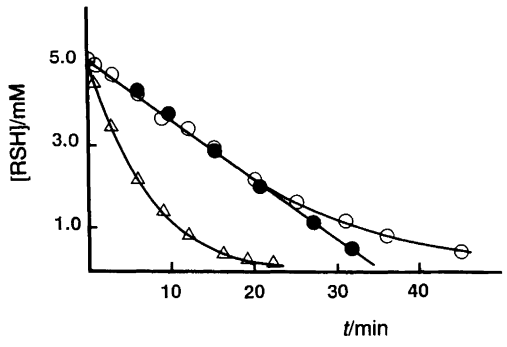

Fig. 6. Rate of autoxidation of 5 mM cysteine catalysed by 10 μM Fe(III) at pH 8.1, 29 °C, equilibrium with oxygen (*a*) (△) ionic Fe(III), 40 mM Tris; (*b*) (○) aged $FeNH_4(SO_4)_2$ (see text) dissolved in water, reaction mixture with 40 mM Tris; (*c*) (●) same without buffer (final pH 8.4).

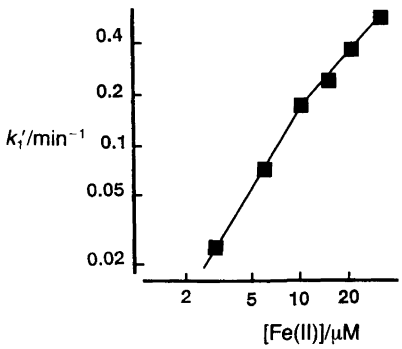

Fig. 7. Logarithmic presentation of k_1' of the Fe(II)-catalysed autoxidation of cysteine as a function of [Fe(II)].

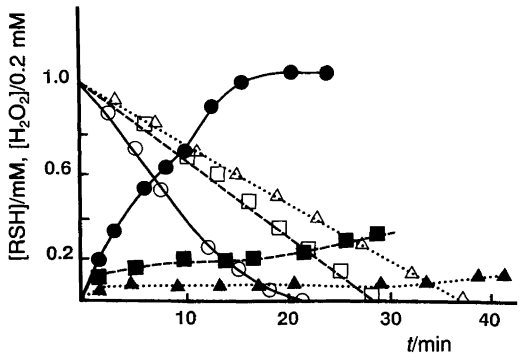

Fig. 8. Concentrations of cysteine (open symbols) and H_2O_2 (filled symbols) during the autoxidation at 37 °C of 1 mM cysteine in equilibrium with air, catalysed by 1 μM Cu (○); 1 μM Cu + 0.1 μM Fe(II) (□); 1 μM Cu + 0.3 μM Fe(II) (△). Fe alone at these concentrations has no influence on [RSH]. 40 mM Tris pH 7.2.

independently of the age of the stock solution – some 2.5 times lower than that determined by Taylor *et al.*[32] under identical conditions (10 μM Fe, pH 8.1, 29 °C). It was investigated whether contamination of the Fe catalyst with Cu (0.1–1 μM) would enhance the reaction; in fact, a certain decrease in the rate was found.

In studies of the Cu-catalysed reaction addition of Fe was found to have conspicuous effects (Fig. 8): a slowing down of the reaction, change of d[RSH]/d*t* to strict zeroth-order, and decrease of the concentration of or elimination of H_2O_2 (see also Table 7). This reduction of $[H_2O_2]$ cannot simply be ascribed to an Fe-catalysis of the oxidation of RSH by H_2O_2 [eqn. (2)], which would have led to a net increase in −d[RSH]/d*t*. Addition of 0.1–1 μM Fe(II) – the catalytic power of which is very weak (cf. Fig. 7) – to 1 μM Cu(II) caused, in fact, a reduction of k_o' and k_o, indicating a fundamental change in the mechanism. It is possible that no free H_2O_2 is formed in this mixed catalysis. Indications that the Cu-catalysed reaction (1) follows second-order kinetics with respect to Cu (see Fig. 3 and following section) might imply the involvement of binuclear complexes, or collisions between two mononuclear complexes,[14] in the catalytic action of Cu. It is tempting to suggest specific action of dissimilar metal atoms in such binuclear complexes or collisions. Chains such as Cu(I) + Fe(III) → Cu(II) + Fe(II) have been described.[33]

Discussion

1. Mechanisms. Owing to the great qualitative and quantitative similarity between cysteine and cysteamine with regard to the kinetics of the Cu- and the Fe-catalysed oxidation by O_2 (to be published), the following discussion concerns both thiols. Only with respect to the mixed Cu/Fe-catalysis are certain differences observed between the two compounds.

Cu catalysis. Despite several studies the mechanisms of the copper-catalysed oxidation of RSH [eqn. (1)] are not yet understood in detail, and the reactive intermediates have not been identified with certainty.[18]

An interpretation of the mechanism has to account for the complex dependence of the reaction rate on experimental variables. (a) [RSH]: Michaelis–Menten kinetics in both neutral (Fig. 2) and alkaline[14] solution. Hence, the overall reaction [eqn. (4)] deviates from Michaelis–Menten kinetics, as shown by Hanaki and Kamide;[8] their data are, however, compatible with the reaction [eqn. (1)] following such kinetics. (b) $[O_2]$: evidence for Michaelis–Menten kinetics for the reaction [eqn. (4)] has been presented;[8] in alkaline solution, dependence on $[O_2]^{1/2}$ was shown, however.[14] The variation of k_o' and of k_o/K with respect to air and pure oxygen in the present study (Tables 4 and 5) is compatible with the former but not with the latter possibility. (c) [Cu]: observations in the present study are, by and large, compatible with dependence on the square of [Cu], and Hanaki and Kamide[8] have presented data showing that the overall reaction [eqn. (4)] approaches a similar dependence on $[Cu]^2$ (if the contribution from reaction [eqn. (2)] is considered to decrease with increasing rate, i.e. with increasing [Cu]). At higher [Cu] saturation is observed. In

Table 7. Effects of Fe(II) on the rate of disappearance of cysteine, −d[RSH]/d*t*, and on the concentration of H_2O_2 $[H_2O_2]t_{\frac{1}{2}}$, after half completion of the oxidation of RSH, in the copper-catalysed autoxidation of cysteine. Equilibrium with air, 40 mM Tris pH 7.2 (in one experiment 8.1).

$[RSH]_o$ /mM	[Cu] /μM	k_o' /μM min^{-1}	(−d[RSH]/d*t*)/μM min^{-1} at [Fe]/μM =					$[H_2O_2]t_{\frac{1}{2}}$/μM at [Fe]/μm =		
			0	0.1	0.3	1	10	0	0.1	0.3[d]
0.3	1	35	42	–	37	42	–	56	–	0
1[b]	0.2	6.3	12	–	6.5	6.2	–	40	–	0
1[a]	0.86	45	63	40	34	39	88	130	44	14
1[b]	5	102	137	–	160	170	500	156	–	0
5	1	90	145	100	63	50	180	85	–	–
1(pH 8.1)	1	18	34	16	12	–	–	17	8	0
1[b,c]	1	56	72	34	28	28	–	116	32	0

[a]Mean values of 5 experiments. C.V. of reaction rates <5 % of the means. [b]Mean values from two experiments. [c]Stock solution of $FeSO_4$ prepared in water. [d]At 1–10 μM Fe, no H_2O_2 could be determined.

alkaline solution Zwart *et al.*[14] identified parallel reactions that were first and second order with respect to Cu. (d) pH: at [Cu] around 1 μM the rate was shown to exhibit a maximum at pH 7.2–7.4,[7,8] in agreement with k_o' and k_o/K being lower at pH 8.1 than at pH 7.2 (Tables 4 and 5). The latter order is reversed, however, at higher [Cu].

The pH dependence indicates that a deprotonated state of the SH or NH_3^+ groups of cysteine[22,26] facilitates the formation of a reactive complex or its further reaction, and that the reaction may be counteracted by a competing ligand such as OH^-. Owing to the reactivity of Cu(II)–cysteine complexes, it has not been possible to determine their stability constants.

In their study of the Cu-catalysed autoxidation, Hanaki and Kamide[7] have assumed a ping-pong mechanism involving reduction of Cu(II) to Cu(I) within a cysteine complex and reoxidation of Cu(I) in Cu(I)SR by O_2. In the most penetrating kinetic study of the reaction [eqn. (1)],[14] carried out at pH 13.5, this operation of a Cu(II)/Cu(I) redox couple could be rejected, however, by the demonstration that the reduction of Cu(II) (determined under anaerobic conditions) is too slow to account for a major part of the reaction. A reaction mediated by a Cu(II) complex, presumably $Cu(SR)_2$ (cf. Refs. 17 and 34), would also be in line with EPR spectroscopic data showing 100 (±2) % of the Cu to be present as Cu(II). It is also consistent with the absence of catalytic action of Cu, shown in a separate study[11] to be mediated by Cu(I)SR, on reaction [eqn. (2)]. From their kinetic data Zwart *et al.*[14] propose two types of reaction pathway, first and second order with respect to copper, involving electron transfer via bonded free radicals (thiyl, RS·, and superoxide, $O_2^{\cdot-}$). The appearance of such radicals in the solution seems unlikely in view of the selectivity of the reaction. Jameson and Blackburn[35] have previously suggested a related mechanism.

The $[Cu]^2$-dependent mechanism led Zwart *et al.*[14] to suggest a binuclear complex with RSH and O_2, by analogy with Cu-containing enzymes [some of which contain Cu(II)]. EPR spectrometric studies have provided evidence for the existence of complexes with two or more Cu atoms.[36] See also the following Section.

The reaction conditions in the study of Zwart *et al.*[14] (pH ≈13.5, [RSH] = 0.15 M, [Cu] = 50–200 μM, 23 °C) are not directly translatable to the physiological conditions of the present study. A recalculation of the data obtained in the alkaline medium to pH 7.2, 1 μM Cu, equilibrium with air, and 37 °C, generates a rate that is some 20 times lower than that observed under these conditions. In this extrapolation, the $[Cu]^2$ term in the rate equation of Zwart *et al.* [eqn. (8)] loses practical importance below [Cu] = 10 μM, whereas in slightly alkaline solution (pH ≈8) the reaction order with respect to Cu was found to be decidedly > 1, probably around 2, at [Cu] = (0.3–5) μM (Fig. 3).

$$k_o \left(= -\frac{d[O_2]}{dt} \right) = k_I [O_2]^{0.5} \times [Cu] + k_{II} [O_2]^{0.5} \times [Cu]^2 \quad (8)$$

Therefore mechanisms other than those proposed for alkaline conditions cannot be excluded under physiological conditions.

For further elucidation of the mechanisms involved, the numerical solution of model equations, applied to data sets from experiments designed for such analysis, appears to be required.

Fe catalysis. One of the main differences between the results of this study and those of Taylor *et al.*[32] is due to the reaction being first and zeroth order, respectively, with respect to cysteine. One factor responsible for this difference could be identified, viz., the way in which the Fe salt is dissolved. Conditions favouring formation of colloidal Fe(III) oxyhydroxide were found to shift the kinetics towards zeroth order (Fig. 6), indicating that the Fe(III) sol acts as a surface catalyst, and the catalyst–substrate complexes then display greater stability than those in the reactions catalysed by monomeric Fe. The zeroth-order characteristics of mixed Fe–Cu catalysis (see Table 6 and following section) indicate further that contamination with other transition metals could be an additional factor contributing to the kinetics observed by Taylor *et al.*[32] Other findings in their study such as the rate maximum at pH 8.1 and the two-thirds order with respect to Fe could not be confirmed in the present study (cf. Figs. 5 and 7).

With monomeric Fe the observed dependence on the catalyst concentration is compatible with first order above 10 μM (Fig. 7), in agreement with, e.g., data for the Fe-catalysed oxidation of mercaptoacetate by O_2.[37] The intermediate complex is not known. The role of a binuclear complex has been discussed;[37,38] this would be in line with a contribution from a second-order dependence on Fe at low [Fe] (Fig. 7) – although the shape of this curve might have some trivial explanation such as relatively greater losses of the catalyst, e.g. through adsorption to vessel walls, at lower concentrations.

Fe(III)/Fe(II)[31,37,39] and Fe(III)/Fe(I)[32] redox couples have been proposed to operate in the transfer of electrons from RSH to O_2. The reaction follows stoichiometrically eqn. (3), formation of H_2O_2 as an intermediate being unlikely in alkaline solution. Formation of H_2O_2, in agreement with a two-electron transfer, has been reported, however.[38] Free-radical intermediates ($O_2^{\cdot-}$, ·OH) have also been proposed for the Fe-catalysed oxidation of mercaptoacetate.[37] Such a mechanism would explain the induced oxidation of unsaturated compounds[37] including lipid peroxidation by Fe(II) + cysteine, and would also account for the inhibition of the latter effect by hydroxyl-radical scavengers and antioxidants.[40] It is likely, however, that the release of free radicals into the medium is accidental rather than representative of a major reaction pathway. If the

latter had been the case, the Tris buffer (0.04 M), which is a good scavenger of hydroxyl radicals, would have caused partial prevention of RSH oxidation; however, within a few per cent the disappearance of 4 mol equivalents RSH was concomitant with the consumption of 1 mol of O_2 (footnote *d* to Table 6), and Tris was shown not to affect the reaction rate.

Mixed Cu–Fe catalysis. At concentrations 0.3–1 μM, Fe has hardly any catalytic effect on the oxidation of cysteine by O_2 (Table 6) whereas it strongly enhances the oxidation by H_2O_2 (Table 2). At these concentrations, Fe(II) added to the Cu–cysteine reaction mixture was therefore expected to enhance the overall reaction [(3) and (4)], concomitantly with a decrease in the $[H_2O_2]$ [reaction (2)]. The decrease or disappearance of H_2O_2 is, however, accompanied by a slowing down of the overall reaction, at the same time as the kinetics is shifted towards stricter zeroth order (Fig. 8, Table 7). This indicates that the catalytic effects of Cu and Fe are not independent, i.e. not additive, under these conditions.

With 1 μM Cu(II) + (0.3–1) μM Fe(II), $-d[RSH]/dt$ assumes a value which is about 40 % of that obtained with 1 μM Cu(II) alone. The stricter zeroth-order character of the reaction could be described in terms of a different, lower value of the Michaelis constant, K of eqn. (6c). In the reaction sequence (Cat = catalyst) in eqn. (9) K has the meaning given by eqn. (10).

$$\mathrm{RSH} + \mathrm{Cat}\ (+\ \mathrm{O_2}) \underset{k_b}{\overset{k_a}{\rightleftharpoons}} \mathrm{RSH\text{–}Cat(\text{–}O_2)} \xrightarrow{k_c} \mathrm{Cat} + \mathrm{Products} \tag{9}$$

$$K = \frac{k_b + k_c}{k_a} \tag{10}$$

Since the influence of Fe on k_c is but moderate, its main effect could be to stabilize the intermediate complex through a decrease in k_b/k_a (provided, of course, that the influence of O_2 – which is so far unknown – on the stability of the intermediate complex is the same in the absence and in the presence of Fe).

The characteristics of this mixed catalysis might be taken to indicate the role of a binuclear complex containing both metals, e.g. by Fe replacing one site in an intermediate dicopper complex. Such a replacement appears possible in view of similarity in ionic radius of Cu^{2+} and Fe^{2+}. An analogous binuclear cysteine complex, $Co_2(SR)_2$, of Co^{2+} which has approximately the same ionic radius as Fe^{2+} and Cu^{2+}, has been isolated.[41,42] Since the catalysed reaction involves reduction of oxygen to water [eqn. (3)] without the formation of reactive intermediates such as H_2O_2, it bears a resemblance to, or may be considered analogous to, four-electron transfer oxidases based on mixed Cu–Fe catalysis such as cytochrome oxidase and the oxidation of Fe(II) by ceruloplasmin.[43,44]

A certain structural specificity is indicated by the fact that cysteamine, which for the Cu-catalysed reaction has nearly the same values of k_o, K and k_2 as cysteine (to be published), does not exhibit the shift towards stricter zeroth-order kinetics, and shows a more moderate decrease of the H_2O_2 concentration during the course of the reaction, upon addition of Fe to the Cu-catalysed system. This may indicate a role of the carboxy group of cysteine in the formation of the postulated intermediate complex. It should be noted, however, that although presence of Fe lowers the level of H_2O_2 it has little effect on the rate of disappearance of cysteamine. Possibly, an Fe–Cu catalysed oxidation of H_2O_2 of the kind described by Uri[33] might play a role in this context.

2. Applications. A few applications of the results to radiobiological and other experiments with cultivated cells will be briefly summarized in this section.

Check for contamination of glassware and chemicals by Cu and/or Fe. It was suggested some 60 years ago that RSH autoxidation could be used as a monitor of contaminating trace metals.[45,12] This is now easily done using Ellman's reagent.[46] Information as to whether Cu or Fe predominates can be obtained by determining $d[RSH]/dt$ at pH 7.2 (where the rate is highest with Cu) and 8.1 (where it is highest with Fe), or by a simultaneous determination of RSH and H_2O_2, the latter being absent with Fe or mixed Fe–Cu catalysis.

H_2O_2 in cell-cultivation media. The appearance of H_2O_2 as an intermediate in the Cu-catalysed two-step oxidation of RSH by O_2 [eqns. (1) and (2)] explains[2,3] the enigmatic toxicity of radioprotective thiols sometimes observed at low but not at higher concentrations of a thiol such as cysteamine.[1] Owing to the reduction of H_2O_2 by RSH [eqn. (2)] $[H_2O_2]$ approaches steady-state concentrations which are lower at higher [RSH] [eqn. (11)].

$$[H_2O_2]_{SS} = \frac{k_o}{k_2'(K+[RSH])} \left(\rightarrow \frac{k_o}{k_2'[RSH]}\right) \tag{11}$$

The generation of H_2O_2 also contributes to physiological effects such as RNA-synthesis inhibition and enhancement of the radioprotective effect of the thiol.[4] The apparent growth stimulation of lectine-stimulated lymphocytes by a polypeptide (FV) from the red kidney bean[47] was found to be the result of effective chelation of trace metals;[48] FV is rich in cysteine,[49] as are certain protease inhibitors that have similar effects.[50]

The validity of the rate constants for cysteine oxidation was investigated in complete media which were 0.1 and 0.5 mM with respect to the nineteen other amino acids. At the lower concentration, k_o' and k_2' were practically unchanged, but at 0.5 mM a certain reduction appeared, evidently due to the formation of Cu(II)–cysteine–histidine complexes[51] competing with the formation of catalytically

active Cu(II)–cysteine complexes. (This competition gives the order of magnitude of the stability of the Cu–Cys complex.) In the presence of bacteria (*E. coli,* 10^7 cells ml^{-1}), slight changes in reaction rates were observed.

The disturbing H_2O_2 generation due to the Cu-catalysed autoxidation of RSH may be counteracted by the addition of Fe at a suitable concentration[4] (Fig. 8). On the other hand, within certain limits, a relatively constant level of pre-administered H_2O_2 may be maintained in the medium; at equal rates of formation and consumption, $[H_2O_2]$ is determined by k_o', k_2', and [RSH] according to eqn. (11).

Experimental

Materials. All chemicals were of analytical grade. Inorganic chemicals – NaH_2PO_4, Na_2HPO_4, $CuCl_2 \cdot 2H_2O$, $FeSO_4 \cdot 7H_2O$, $FeNH_4(SO_4)_2 \cdot 12H_2O$, H_2O_2, NaOH, H_2SO_4 and $TiOSO_4$ – were obtained from Merck, Darmstadt, and organic chemicals – L-cysteine HCl, cysteamine HCl, tris (hydroxymethyl)aminomethane hydrochloride (Tris HCl), ethylenediaminetetraacetic acid disodium salt (EDTA) and 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) – were obtained from Sigma Chemical Co., Saint Louis, MO.

Water free of Cu (and other trace metals) was prepared by distilling deionized water in a glass apparatus followed by two redistillations in a quartz apparatus. Since trace metals adsorbed from tap water onto glass walls cannot be removed by washing with deionized water, all glassware, after being washed according to standard laboratory procedures for laboratory-dish cleaning, was washed with 0.05 M EDTA, then rinsed in Cu-free distilled water, and finally dried at 200 °C.[48] The absence of autoxidation catalysts in chemicals and vessels was checked by the stability of 1 mM cysteine under efficient aeration, in the respective buffer solutions (cf. Warburg[45]). The criterion for stability was that the cysteine concentration should remain unchanged, within the accuracy of the analysis (± 2 %), over 40 min at 37 °C; usually, much greater stability was obtained.

Most reactions were studied at 37.0 °C (in certain cases 29.0 °C) in 40 mM Tris buffer pH 7.2 or 8.1, measured at the experimental temperature. Oxygen concentration in equilibrium with air or O_2, free of CO_2 and saturated with H_2O, was maintained by bubbling the gas through the reaction, with vigorous stirring. Reactions were started by adding a small volume of catalyst stock solution to the reaction mixture, usually 30 ml. Except in special studies of the consequences of hydrolysis, stock solutions of Fe(II) and Fe(III) were prepared by dissolving $FeSO_4 \cdot 7H_2O$ and $FeNH_4(SO_4)_2 \cdot 12H_2O$, respectively, in 0.05 M H_2SO_4, and equivalent amounts of NaOH were added to reaction mixtures before the start of reactions. The copper content of stock solutions was determined by atomic absorption, and iron, Fe(II) after oxidation by H_2O_2, was determined spectrophotometrically at 304 nm.

Reactions were followed by the determination, in aliquots, of RSH using Ellman's[46] reagent, and, when applicable, of H_2O_2 with Marklund's[52] modification of Bonet–Maury's[53] Ti(IV) reagent ($TiOSO_4$), concentrations being read from relevant calibration curves. In certain cases the disappearance of RSH and oxygen uptake were determined, the latter by the Warburg technique, in parallel experiments.

In separate studies of reaction [eqn. (2)] the second-order rate constants, k_2', of the oxidation of RSH by H_2O_2 were determined under anaerobic conditions obtained by bubbling through N_2 or Ar. The reaction was initiated by mixing solutions of the reactants, prewarmed to the reaction temperature.

Calculations. Standard methods[19] were used to determine reaction order, i.e. k_1' of the Fe-catalysed oxidation from semilogarithmic plots of [RSH] vs. time (cf. Fig. 4), and k_2' of reaction (2) *inter alia* in reactions of equivalent amounts of RSH and H_2O_2 by plotting $[RSH]^{-1}$ and $[H_2O_2]^{-1}$ versus time. k_o' of eqns. (6a) and (6b) was calculated from experimental values as given in eqn. (12).

$$k_o' = \frac{1}{2}\left(\frac{-\,\mathrm{d[RSH]}}{\mathrm{d}t} + \frac{2\mathrm{d[H_2O_2]}}{\mathrm{d}t}\right) \tag{12}$$

Usually, k_o' assumed the same value when determined from eqn. (6a) using the experimental value of k_2'. The Michaelis–Menten type kinetics of the reaction [eqn. (1)] was verified by a Lineweaver–Burke presentation (cf. Fig. 2). The parameters k_o and K (and, in some cases, k_2') from (6a) and (6b) were estimated by solving the equations and minimizing the sum of the squares (the subscripts o and e denote observed and expected values, respectively):

$$\Sigma\{[RSH]_o(t_i) - [RSH]_e(t_i, k_o, K)\}^2 + \Sigma\{[H_2O_2]_o(t_j) - [H_2O_2]_e(t_j, k_o, K)\}^2 \tag{13}$$

Since the differential equations have no explicit solution they were solved numerically with a search in the parameter space for the values of k_o and K that minimize expression (13). The program package MLAB[54] (*M*odelling *L*aboratory) handles easily this otherwise laborious problem, since MLAB allows the definition of the function required to fit to the data implicitly, through a system of differential equations.

In order to facilitate biological applications, the minute was used as the unit of time.

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