Anaerobic Oxidation of Cysteine to Cystine by Iron(III). Part 2.† The Reaction in Basic Solution

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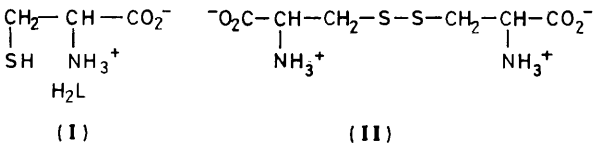
The anaerobic oxidation of cysteine (H_2L) to cystine by iron(III) has been followed over the pH range 8.2—11.6 by use of a stopped-flow high-speed spectrophotometric method (the results obtained below pH 8.6, however, were not reproducible and no attempt was made to establish a rate law over this range). Between pH 8.6 and 11.2 the experimental data were accurately reproduced by assuming that the bis-cysteine complex, $[Fe(OH)L_2]^{2-}$, reacts with the mono-cysteine complex, [Fe(OH)L], to yield two iron(II) ions, one cystine, and an unoxidised cysteine with a second-order rate constant of 8.36×10^3 dm^3 mol^{-1} s^{-1}. The predominant complex species present in solution is the purple $[Fe(OH)L_2]^{2-}$ which exhibits an absorption maximum at 545 nm (shoulder at 445 nm) and has a molar absorption coefficient of 3 175 dm^3 mol^{-1} cm^{-1}. The other complex present is also purple and is [Fe(OH)L]. The absorption spectrum of this species (obtained over the pH range 5.5—8.0) exhibits a maximum at 503 nm (shoulder at 575 nm) and has a molar absorption coefficient of 1 640 dm^3 mol^{-1} cm^{-1}. The kinetic results were also used to calculate values of the first and second protonation constants for cysteine ($\log K_1^H = 10.34$, $\log K_2^H = 8.32$) which compare very favourably with previously published values (obtained by potentiometric titration). Finally, a value of $\log K_2^M = 4.76$ for the reaction $[Fe(OH)L] + L^{2-} \rightleftharpoons [Fe(OH)L_2]^{2-}$ was also extracted from the kinetic data. All measurements were carried out at 25 °C in solution of ionic strength 0.10 mol dm^{-3} (KCl).

In strongly alkaline solutions cysteine (H_2L) reacts with iron(III) to form the deep purple complex, $[Fe(OH)L_2]^{2-}$, which rapidly disappears with the formation of iron(II) and cystine [1,2] (**II**). The



(I) (II)

colour is regenerated by shaking in air (oxygen) and in fact the colour can be used for the estimation of iron(III) if the estimation is carried out with an excess of oxygen present.

Following our study [3] of the reaction between iron(III) and cysteine in acid solution in which the predominant cysteine-containing species is the bright blue $[FeL]^+$ we report here an investigation of the reaction in alkaline solution. Once again we have made use of the high-speed spectrophotometric stopped-flow technique which is capable of much higher precision than the classical kinetic methods employed in earlier studies of this system.[1,2]

Fortunately, the spectra of the mono-cysteine complex, [Fe(OH)L], and the bis-cysteine complex, $[Fe(OH)L_2]^{2-}$, are sufficiently different (see Figure 1) to enable the reaction to be followed by monitoring the spectrum of the latter over the whole of the pH range where it predominates, namely pH 8.8—11.2. The previous classical studies had not been able to establish this very important point. {The spectrum of the mono-cysteine complex, [Fe(OH)L], was obtained over the pH range 5.5—8 and a molar absorption coefficient of 1 640 dm^3 mol^{-1} cm^{-1} obtained.[4]}

† Part 1 is ref. 3.

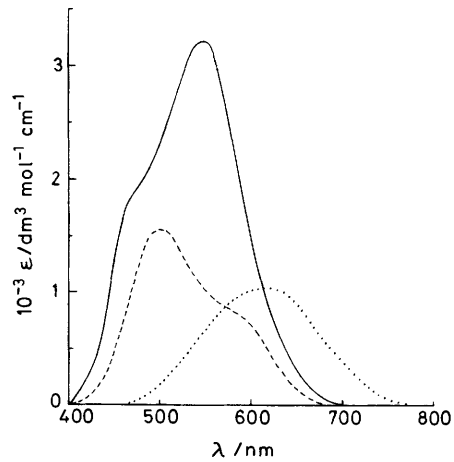


Figure 1. Electronic spectra of cysteine complexes of iron(III): (· · · ·) $[FeL]^+$, (— — —) [Fe(OH)L], (———) $[Fe(OH)L_2]^{2-}$

Results

In order to work at a reduced pseudo-order, cysteine was always in large excess, and the shape of the kinetic curves and constancy of the component spectra (Figure 2) showed that we were following the formation and then decay of a single species. However, another species did make its appearance at pH < *ca.* 8.8, and a single run at pH 11.6 strongly suggested that a third species was then present. The former species was found [4] to be the mono-cysteine complex, [Fe(OH)L], whereas the latter could be [1] the tris-cysteine complex, $[FeL_3]^{3-}$, or a polymeric species but this was not further investigated.

The first part of the kinetic curves represents the formation of a coloured species which is identified with the bis-cysteine

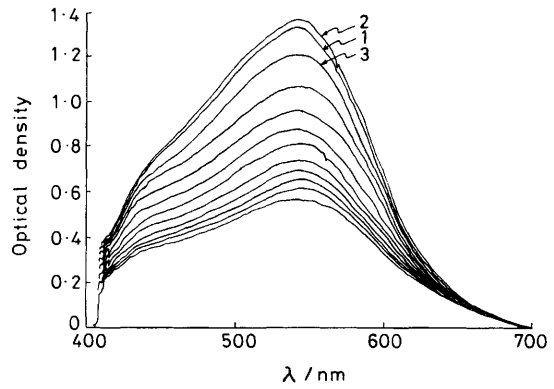


Figure 2. Constancy of electronic spectrum during kinetic run. $[Cys]_0$ = 0.025 mol dm^{-3}, $[Fe]_0$ = 0.0005 mol dm^{-3}, pH 9.58; time interval 9 999 ms between spectra

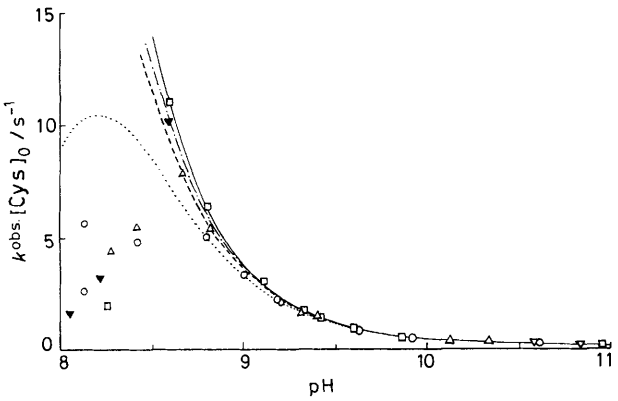


Figure 3. Dependence of $k^{obs.}[Cys]_0$ on pH: $[Cys]_0$ = 0.005 (○) (· · · ·), 0.010 (△) (— — —), 0.015 (▼) (— · —), and 0.025 mol dm^{-3} (□) (———). Theoretical lines (see text) and experimental points: $[Fe]_0$ = (5—15) × 10^{-4} mol dm^{-3}

complex, $[Fe(OH)L_2]^{2-}$, but it was decided that the data were not complete enough to enable a study of the formation kinetics to be made.

The second, decay, part of the curves represented a much slower reaction and was amenable to standard methods of examination. In all cases a plot of the reciprocal of the optical density *versus* time gave straight lines indicating accurate second-order kinetics over more than 85% of the reaction being followed, independent of the amount of iron(III) originally present. The basic experimental rate law could thus be written as in equation (1) in which $k^{obs.}$ is calculated from the slopes of

$$-d[Fe(OH)L_2{}^{2-}]/dt = k^{obs.}[Fe]_T{}^2 \quad (1)$$

the (optical density)$^{-1}$ *versus* time plots by the relationship $k^{obs.} = \varepsilon l$(slope) where ε is the molar absorption coefficient of the complex and l is the pathlength of the optical cell (here 2 cm). The molar absorption coefficient of the absorbing species was calculated from the extrapolation of these lines (plotted for the absorption maximum at 545 nm) to zero time, yielding a value of ε = 3 175 dm^3 mol^{-1} cm^{-1} {based on total iron(III) concentrations, $[Fe]_T$} for all solutions and this compares very favourably with the previously reported[1,2] value of 3 050 dm^3 mol^{-1} cm^{-1}.

Variation of the Observed Second-order Rate Constants, $k^{obs.}$ *with* $[Cys]_T$ *and* $[Fe]_T$.—Some typical results are given in the Table and the values of $k^{obs.}[Cys]_0$ obtained for all the runs carried out are plotted against pH in Figure 3. Above a pH of *ca.* 9.5 all the results lie on a single line and thus above this pH the rate of disappearance of the bis-cysteine complex, $[Fe(OH)L_2]^{2-}$, depends on $[Cys]_T{}^{-1}$ and is second-order with respect to $[Fe]_T$ ($[Cys]_0$ and $[Cys]_T$ are initial and total cysteine concentrations). This implies that the basic experimental rate law at constant $[H^+]$ takes the form of equation (2) over the pH range 9.5—11.0.

Table. Observed second-order rate constants

pH	$10^2[Cys]_0$/mol dm^{-3}	$10^4[Fe]_0$/mol dm^{-3}	$k^{obs.}[Cys]_0/s^{-1}$
11.58	2.50	2.50	0.11
10.84	1.50	2.50	0.16
10.57	1.50	1.50	0.22
10.55	1.50	5.00	0.22
10.33	1.00	2.50	0.28
9.91	0.50	2.50	0.49
9.58	2.50	5.00	0.97
9.41	1.00	2.50	1.45
9.32	1.00	5.00	1.72
9.33	2.50	5.00	1.75
9.23	0.50	2.50	2.17
9.18	0.50	2.50	2.25
9.08	2.50	2.50	3.99
8.82	5.00	2.50	5.01
8.82	2.50	5.00	6.31
8.84	1.00	2.50	5.37
8.62	1.50	5.00	10.11

$$-d[Fe(OH)L_2{}^{2-}]/dt = (k^{obs.}[Cys]_0)[Fe]_T{}^2[Cys]_T{}^{-1} \quad (2)$$

Dependence of $k^{obs.}[Cys]_0$ *on* $[H^+]$.—The shape of the $k^{obs.}[Cys]_0$ *vs.* $[H^+]$ curve strongly suggested that the true dependence was on one of the deprotonated forms of cysteine, rather than $[Cys]_T$. Furthermore the most likely species is the deprotonated ion since this is co-ordinated to the iron in the complexes.

Now the two equilibria of concern are (3), for which equation (4) applies, and (5), for which equation (6) applies. Over the pH

$$L^{2-} + H^+ \rightleftharpoons HL^- \quad (3)$$

$$K_1^H = [HL^-]/[H^+][L^{2-}] \quad (4)$$

$$HL^- + H^+ \rightleftharpoons H_2L \quad (5)$$

$$K_2^H = [H_2L]/[H^+][HL^-] \quad (6)$$

range concerned here, namely 9.5—11.0, the total cysteine concentration is given by equation (7) (the presence of metal

$$[Cys]_T = [L^{2-}] + [HL^-] + [H_2L] \quad (7)$$

complexes can be ignored as ligand is always in large excess).

Thus from (4), (6), and (7) we obtain equation (8) (where β_2^H =

$$[Cys]_T = [L^{2-}](1 + K_1^H[H^+] + \beta_2^H[H^+]^2) \quad (8)$$

$K_1^H K_2^H$). If, therefore, the rate of reaction is actually inversely proportional to $[L^{2-}]$ rather than to $[Cys]_T$ then (2) and (8) lead to equation (9), where the rate constant k' is defined by

$$k^{obs.}[Cys]_0/(1 + K_1^H[H^+] + \beta_2^H[H^+]^2) = k' \quad (9)$$

$$-d[Fe(OH)L_2{}^{2-}]/dt = k'[Fe]_T{}^2/[L^{2-}] \quad (10)$$

equation (10). A simple curve-fitting routine gave the following values of the three constants, and the relevant line in Figure 3 was calculated using them: log $K_1^H = 10.34$, log $\beta_2^H = 18.66$, $k' = 0.145$ s^{-1}. {Note that, as in Part 1,[3] the empirical relationship: $[H^+] = 10^{-[(pH-0.131)/0.982]}$, obtained[5] by titrating HCl with KOH was used to convert pH values to hydrogen ion concentrations.}

These protonation constants agree well with the published values (for example Lenz and Martell[6] report 10.11 and 18.24 respectively for them) but the variation in the literature is quite high and so the kinetically determined values above were considered the best for use in all these kinetic studies. The apparent rate constant k' was also determined by Leussing *et al.*[2] who obtained 0.105 s^{-1} (but see Discussion section).

Finally, as will be justified below, the separation of the single line in Figure 3 into four separate lines depending on the value of $[Cys]_0$ was ascribed to the fact that the bis-cysteine complex no longer predominates below pH 9.5 and that the presence of the mono-cysteine complex must be taken into account. (The appearance of the spectrum of the mono-cysteine complex towards the very end of some of these relatively low pH runs confirmed that this was so.) The equilibrium concerned is as in equation (11), for which equation (12) applies. The value of

$$[Fe(OH)L] + L^{2-} \rightleftharpoons [Fe(OH)L_2]^{2-} \quad (11)$$

$$K_2^M = [Fe(OH)L_2{}^{2-}]/[Fe(OH)L][L^{2-}] \quad (12)$$

5.79×10^4 dm^3 mol^{-1} for this constant (log $K_2^M = 4.76$) was obtained by calculating the best fit to the data obtained between pH 8.6 and 9.5 [the method employed is derived in section (*b*) below]; the lines drawn in Figure 3 were calculated on this basis. The only previously reported[2] value for this constant is 2.44×10^4 dm^3 mol^{-1} (log $K_2^M = 4.39$).

Interpretation of the Rate Law.—(*a*) *Results above* pH 9.5. The simplest explanation for the second-order dependence on total iron(III) concentration is that two iron-containing species are reacting in the rate-determining step. Similarly, the inverse dependence on total cysteine concentration at constant pH [equation (2)] or, more generally, on $[L^{2-}]$[equation (10)] suggests that one species contains one fewer co-ordinated cysteine than the other. Thus, in agreement with Leussing *et al.*,[2] we propose that the initial rate-determining step is that in equation (13), whose rate is given by equations (14) and (15),

$$[Fe(OH)L] + [Fe(OH)L_2]^{2-} \longrightarrow \text{products} \quad (13)$$

$$\text{Rate} = k[Fe(OH)L][Fe(OH)L_2{}^{2-}] \quad (14)$$

$$\text{Rate} = kK_2^M[Fe(OH)L]^2[L^{2-}] \quad (15)$$

and which must now be expressed in terms of total iron(III), $[Fe]_T$, and total cysteine, $[Cys]_T$.

At the pH values concerned in the present studies all of the iron will be complexed by cysteine, and hence $[Fe]_T$ is given by equations (16) and (17), so that equation (14) becomes equation

$$[Fe]_T = [Fe(OH)L] + [Fe(OH)L_2{}^{2-}] \quad (16)$$

$$[Fe]_T = [Fe(OH)L](1 + K_2^M[L^{2-}]) \quad (17)$$

$$\text{Rate} = kK_2^M[L^{2-}][Fe]_T{}^2/(1 + K_2^M[L^{2-}])^2 \quad (18)$$

(18). Above pH 9.5 a further simplification can be made to equation (18) since we may make the assumption (19), *i.e.* we

$$[Fe(OH)L_2{}^{2-}] \gg [Fe(OH)L] \quad (19)$$

now have equation (20), or using equation (8) this gives equation (21). Comparison of equations (20) and (10) then gives

$$\text{Rate} = k[Fe]_T{}^2/K_2^M[L^{2-}] \quad (20)$$

$$\text{Rate} = k(K_2^M)^{-1}(1 + K_2^M[H^+] + \beta_2^H[H^+]^2)[Fe]_T{}^2[Cys]_T{}^{-1} \quad (21)$$

the required result, namely equation (9), where equation (22) applies.

$$k' = k/K_2^M \quad (22)$$

(*b*) *Results below* pH 9.5. Below pH 9.5 the plots of $k^{obs.}[Cys]_0$ *vs.* pH (or $[H^+]$) no longer form a coincident set, but lie on curves determined by the original cysteine concentration, $[Cys]_0$. This can be explained by the fact that the assumption (19) now no longer holds, although because of the relative absorptions of the two species (see Figure 1), the kinetic data are still valid. Thus it is only necessary to interpret these data using the full expression (18). Combining (18) with (22) and comparing the result with the basic rate expression (1) leads to equation (23) in which only K_2^M is unknown for each kinetic

$$k^{obs.} = k'(K_2^M)^2[L^{2-}]/(1 + K_2^M[L^{2-}])^2 \quad (23)$$

run: a mean value of $(5.79 \pm 0.08) \times 10^4$ dm^3 mol^{-1} (log $K_2^M = 4.76$) was obtained for this constant and used to calculate the lines drawn in Figure 3. Finally, from the values of k' and K_2^M a value of 8.36×10^3 dm^3 mol^{-1} s^{-1} is obtained for the rate constant k [reaction (13), rate expression (14)].

Discussion

One of the advantages of the use of a high-speed spectrophotometric method in the study of this system is that we are able to report accurate spectra of the complexes formed. In the earlier studies[1,2] it was assumed that the spectrum obtained from air (oxygen) saturated solutions was identical to that obtained under anaerobic conditions. This does not appear to be the case, however, and we find a distinct shift in the maximum of the absorption spectrum of the bis-cysteine complex, $[Fe(OH)L_2]^{2-}$, from 545 to 575 nm when oxygen is present. A similar small, but distinct, shift from 503 to 490 nm is observed when oxygen is present in solutions containing the mono-cysteine complex, [Fe(OH)L].

The main disagreement, however, between our results and those of Leussing *et al.*[2] is that we find no evidence for a reaction between two molecules of the bis-cysteine complex (or the formation of a dimer molecule). They base their evidence, however, on the existence of an intercept in the $[L^{2-}]$ *vs.* $k^{obs.}$ graph. If their results are recalculated using the present values of the protonation constants of cysteine in order to obtain $[L^{2-}]$ values and the $k^{obs.}$ values corrected to make use of our value for the absorption coefficient then it is highly significant that the straight line then passes through the origin. Furthermore, the slope now has the value 0.133 s^{-1} (instead of 0.105 s^{-1}) which is in very good agreement with our value of 0.145 s^{-1}.

The reaction between the two species exhibits several points of interest. First, the most likely mode of interaction is *via* one, or less likely, two hydroxyl bridges linking the two iron(III) centres. Two electrons could then be transferred into this bridge system from the two cysteine ligands bound to the same iron atom (particularly favoured if the co-ordinated sulphur donor atoms are adjacent in the co-ordination sphere) yielding two iron(II) species, one molecule of cystine (**II**), and one unoxidised cysteine.

This mechanism is closely allied to that proposed[7] for the iron(III)-catalysed oxidation of cysteine by molecular oxygen in which strong evidence is put forward for the involvement (as rate-determing step) of a reaction between molecular oxygen and the bis-cysteine complex. In this case the oxidation again involves the transfer of two electrons from the co-ordinated cysteine ligands through the iron atom, but this time to the oxygen molecule (yielding peroxide). However, in order to explain the initial co-ordination of the O_2 to the iron it must be assumed that a large degree of charge transfer is present in the bis-cysteine complex enabling the iron atom at the centre to function in many ways as iron(II). Similarly, in the case of the copper(II)-catalysed oxidation of ascorbic acid by molecular oxygen where it was found necessary[8] to postulate the interaction of the O_2 with a dimer of the copper(II) complex the copper centres are behaving as copper(I) towards dioxygen rather than as copper(II). In fact this non-innocent behaviour of the complexes of these readily oxidised ligands such as ascorbic acid, adrenaline, dopa [3-(3,4-dihydroxyphenyl)alanine], and cysteine seems not only to enable them to co-ordinate oxygen to the central metal ion by the latter functioning as if it were in a lower oxidation state, but also to give them some considerable stability. Thus the bis-cysteine complex of iron(III) only undergoes an internal redox reaction when either a molecule of the mono-cysteine complex or a molecule of dioxygen is co-ordinated to the central iron and whereupon *both* electrons

$$2\,(\mathbf{I}) \longrightarrow (\mathbf{II}) + 2e^- \qquad (24)$$

required for reaction (24) can be transferred through the metal ion bound in the bis-cysteine complex.

Despite the fact that at pH < 7 the redox reaction would seem to involve[4] the interaction of two mono-cysteine complexes, the kinetic results over the pH range studied here, namely 8.6—11.2, do not allow any significant involvement of this reaction under these conditions. In the pH range 6.9—8.6 precipitation of iron-containing species prevented any kinetic studies being carried out.

Experimental

Experimental conditions were as described in Part 1;[3] tris-(hydroxymethyl)aminoethane ('Tris') and borax were used to buffer the reaction mixtures.

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