The Reaction of Cysteine with the Pentacyanonitrosylferrate(2−) Ion

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The title reaction has been studied and it has been found that in the presence of oxygen a catalytic path for the oxidation of cysteine to cystine is established. The rate law is presented, and the mechanism is discussed on the basis of observed effects of the reagent concentrations on the extrapolated initial absorbance of the solution at 522 nm, and on the experimental pseudo-first-order rate of disappearance of colour. Cyanide ion has a remarkable influence on the course of the reaction.

THE ion pentacyanonitrosylferrate(2−) (nitroprusside) is known to react with a wide variety of bases.[1-4] Several types of interaction have been described, involving either direct reaction of the NO group with the incoming base or previous reaction with hydroxide ion to generate a reactive intermediate. In a number of cases, pentacyanonitritoferrate(3−) is first generated by reaction with hydroxide and subsequently aquated.[4]

The reaction of cysteine with pentacyanonitrosylferrate(2−) was first described over 40 years ago,[5] and has been proposed as a test for this amino-acid. Reactions with other sulphur-containing bases have also been described, namely HS^-,[6] thioglycolic acid,[7] other thiols,[7] and the reactions of ' photolyzed ' pentacyanonitrosylferrate(2−) with thiourea and thiocyanate.[8,9]

The reports of the reactions with thiourea and thiols are to a certain extent contradictory. We have pursued analysis of the reaction products and kinetics of the reaction of cysteine in an effort to elucidate the mechanism of generation of different coloured species. The reaction itself has relevance in the field of iron–sulphur proteins, since it provides a model for iron redox reactions with disulphide formation.[10]

EXPERIMENTAL

All reagents were analytical grade. Water was doubly distilled before use. The oxygen content was controlled by boiling and/or bubbling purified nitrogen through the solutions.

Visible spectra were obtained on a Cary 14 spectrophotometer. Infrared spectra were measured as KBr discs, using a Perkin-Elmer 427 spectrometer. The pH was measured with a Radiometer M52 pH meter. Potentiometric titrations were performed with a Metrohm piston burette. Electron spin resonance measurements were obtained on a Varian EM 500 spectrometer.

Kinetic experiments were performed spectrophotometrically at 522 nm, by following changes in absorbance with the disappearance of the intermediate, on a Hitachi-Perkin-Elmer 139 spectrophotometer. The working temperature was 25.0 ± 0.1 °C, the ionic strength was kept at 1.5 mol dm^{-3} (NaCl), and the pH was varied between 7.5 and 11.0 by using suitable buffers, which were first shown not to react with nitroprusside (except for the formation of the nitrito-complex at higher pH values).[4] Cysteine in solution is very prone to oxidation, especially in alkaline medium, even when deaerated solutions are stored in well stoppered flasks; therefore, fresh solutions were used in each run. Experiments were performed with various ratios of nitroprusside to cysteine, at various pH values and cyanide-ion concentrations. The resulting data were treated as usual to find the kinetic order. In every case pseudo-first-order behaviour was shown by the rate of disappearance of colour, *i.e.* $d(\ln A)/dt$ was constant during at least 70% reaction (and usually up to 90% bleaching). Experiments were done in duplicate or triplicate, and reported values are the average of at least two measurements which did not differ by more than 10%, usually less than 5%. The error bracket for individual k values was always found to be less than 3% for 95% confidence limits, so it is estimated that reported values are correct to within 5%.

The characterization of the main species participating in the reaction was achieved by observing the spectral changes on changing pH, i.r. characterization of the solid separating from solutions containing large amounts of reacted cysteine, and other experiments to be described in the Results section.

RESULTS

The reaction of $[Fe(CN)_5(NO)]^{2-}$ with cysteine in the pH range 7.5—11.0 gives rise to an intense red colour which develops ' instantaneously ' and then fades gradually. The spectrum of the coloured intermediate shows a maximum at 522 nm (see Figure 1).

The red colour is due to the formation of the adduct $[Fe(CN)_5(NO)(SR)]^{3-}$ [equation (1)].[5-7] The formation reaction (1) is fast, and no kinetic information can be

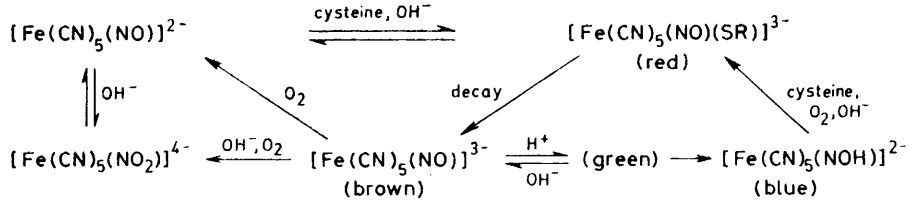
$$[Fe(CN)_5(NO)]^{2-} + SR^- \rightleftharpoons [Fe(CN)_5(NO)(SR)]^{3-} \quad (1)$$

obtained by our techniques. On the other hand, reaction (1) behaves as a fast pre-equilibrium and the equilibrium constant can be evaluated.

The primary products of the fading reaction are $[Fe(CN)_5(NO)]^{3-}$ and cysteine, as evidenced from the following observations. (*a*) Acid addition to the faded reaction mixture gives rise to changes in the colour, following the sequence brown ⟶ green ⟶ blue. The spectral changes are shown in Figure 2. These changes correspond to the reported behaviour of $[Fe(CN)_5(NO)]^{3-}$ and its protonated form $[Fe(CN)_5(NOH)]^{2-}$ [11,12] and are also consistent with the observations described below. (*b*) Several cycles of the form red $\xrightarrow{H^+}$ blue $\xrightarrow{OH^-}$ red can be achieved. (*c*) By repeatedly adding cysteine to slightly alkaline $[Fe(CN)_5(NO)]^{2-}$ solution, repeated development of colour is observed, as shown in Figure 3. In this way, taking due precaution to maintain adequate aeration, up to 10 mol

of cysteine can be made to react per mol of nitroprusside. These colour changes are not observed in the reaction of $[Fe(CN)_5(OH_2)]^{3-}$ with cysteine.[13] Thus, Scheme 1 can be postulated for the reaction. (*d*) ' Brown ' $\{[Fe(CN)_5(NO)]^{3-}\}$

On the other hand, the rate of disappearance of colour is only slightly sensitive to pH; see Table 1. There seems to be a decrease in the rate at pH values between 8.6 and 8.9, the origin of which is not clear.



SCHEME 1

species suffer an ageing process causing changes in the time scale of formation of the blue species. Further experimental work on this process is in progress, and will be reported independently. (*e*) The i.r. spectrum of the solid phase appearing when large amounts of cysteine have reacted was identical to the spectrum of pure cystine.

pH Dependence.—Below pH 7.5 the colour is barely observable. In the range 7.5—11.0, the extrapolated initial

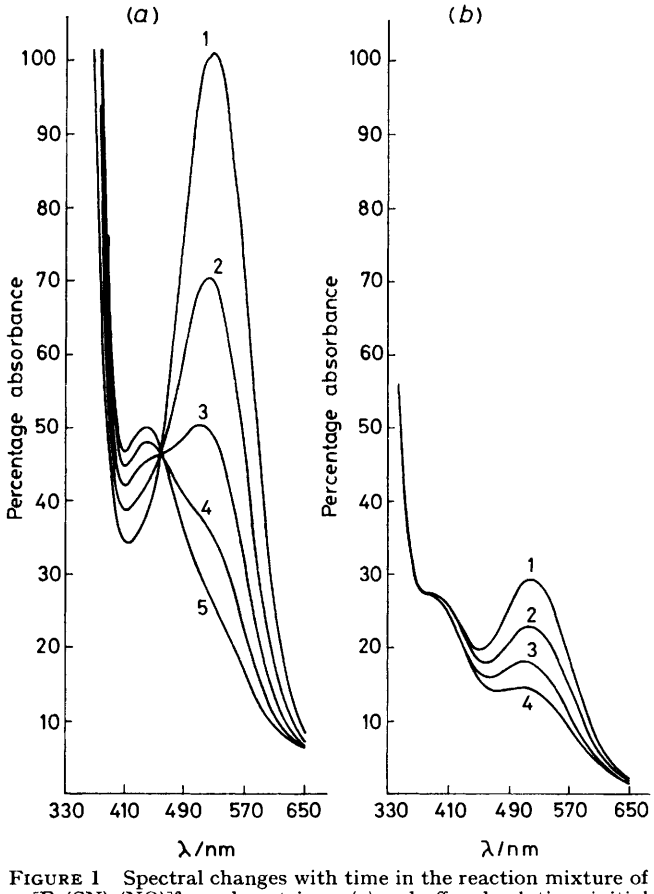


FIGURE 1 Spectral changes with time in the reaction mixture of $[Fe(CN)_5(NO)]^{2-}$ and cysteine: (*a*) unbuffered solution, initial pH *ca.* 8.9; (*b*) buffered solution, pH 8.9. Spectra are numbered in sequential order; time interval between consecutive spectra was of the order of some minutes

value of the absorbance of identical solutions varies as shown in Figure 4. The sigmoid curve shows its inflection point at pH *ca.* 9.1.

In Table 1, first-order experimental rate constants $k_{exp.} = -d\{\ln[A - A_\infty)/(A_0 - A_\infty)]\}/dt$ are presented.

Pentacyanonitrosylferrate(2—) *Ion Dependence.*—When

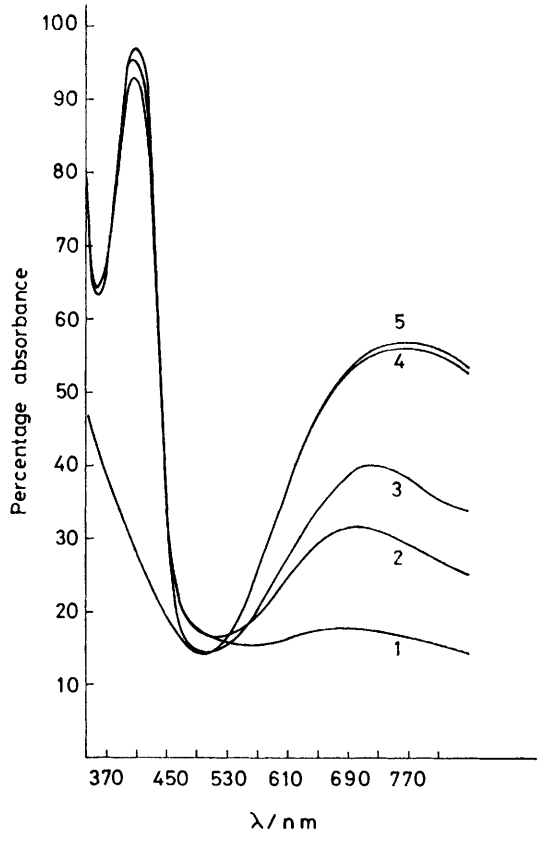


FIGURE 2 Spectral changes with addition of acid to reaction mixtures of $[Fe(CN)_5(NO)]^{2-}$ and cysteine. Curves 1 to 5 represent increasing amounts of acid added to the mixture

the concentration of $[Fe(CN)_5(NO)]^{2-}$ is increased at constant cysteine concentration, the extrapolated initial absorbance value increases, until a ' saturation ' value is obtained when the ratio of complex ion to cysteine reaches a value of *ca.* 300 (at pH 8.9). This is shown in Figure 5. On the other hand, the first-order $k_{exp.}$ for the fading reaction does not show any dependence on the complex-ion concentration, as shown in Table 2. No second-order path is substantiated by our data (*cf.* ref. 7).

Cysteine Dependence.—The cysteine concentration affects

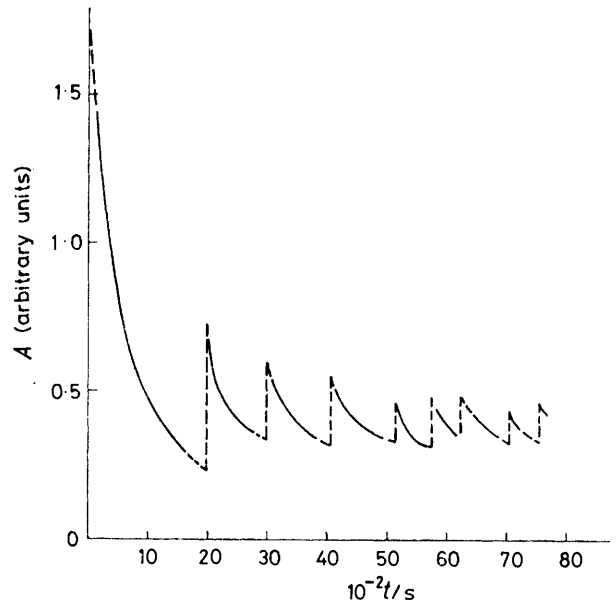


FIGURE 3 Absorbance changes with time at 522 nm and pH 8.9; $[Fe(CN)_5(NO)^{2-}] = 2.5 \times 10^{-3}$ mol dm^{-3}; 5 mg cysteine was added at the times with sharp absorbance changes

the formation of $[Fe(CN)_5(NO)(SR)]^{3-}$ in a similar fashion. However, it effects the fading reaction in a more complex way. At low cysteine to complex-ion concentration ratios the pseudo-first-order rate constant obtained is independent of cysteine concentration, but at high values (10 : 1—50 : 1) the pseudo-first-order rate constant increases linearly with cysteine concentration, as shown in Table 2. Consequently, the fading is very fast and the colour barely detectable.

Cyanide Ion Dependence.—The influence of cyanide-ion concentration on $k_{exp.}$ is shown in Figure 6. The changes

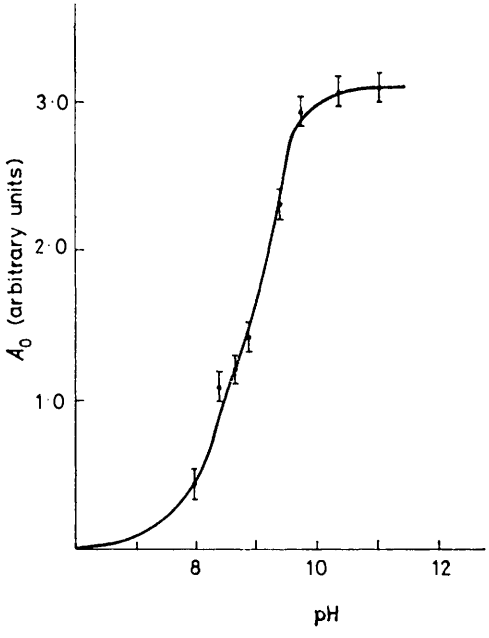


FIGURE 4 pH dependence of the extrapolated initial absorbance at 522 nm; $[Fe(CN)_5(NO)^{2-}] = 2.5 \times 10^{-2}$ mol dm^{-3}; original [cysteine] = 5.0×10^{-4} mol dm^{-3}

TABLE 1

Rate constants ($k_{exp.}$) at various pH values. [HSR] = 5×10^{-4} mol dm^{-3}, $[Fe(CN)_5(NO)^{2-}] = 2.5 \times 10^{-2}$ mol dm^{-3}, I = 1.5 mol dm^{-3} (NaCl), T = 298 K

pH	$10^3 k_{exp.}$/s^{-1}
8.00	3.61
8.39	4.07
8.64	4.17
8.89	2.50
9.39	2.22
9.67	2.05
10.32	2.85
11.02	2.55

brought about by cyanide are not purely kinetic; the spectrum of the reaction mixtures shows, upon cyanide addition, increased absorbance in the short-wavelength region [probably due to hexacyanoferrate(4—)]. Also the

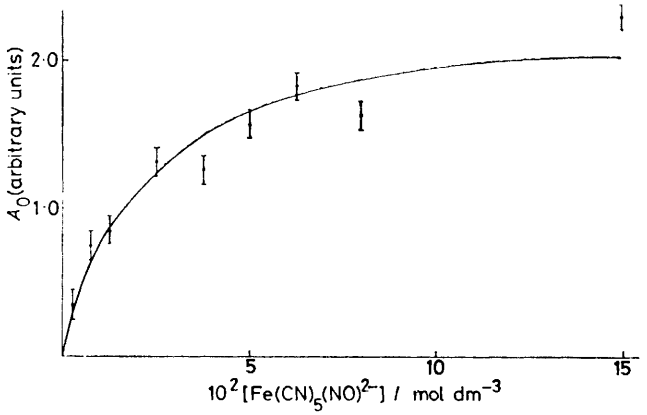


FIGURE 5 Change of extrapolated initial absorbance at 522 nm with $[Fe(CN)_5(NO)^{2-}]$; original [cysteine] = 5.0×10^{-4} mol dm^{-3}; pH = 8.9

capacity to process a large excess of cysteine is largely diminished, pointing to the formation of different iron-containing products.

The Role of Oxygen.—When the ratio of cysteine to nitroprusside is sufficiently low (<0.01 : 1), the influence of

TABLE 2

Rate constants ($k_{exp.}$) at various cysteine and complex-ion concentrations; I = 1.5 mol dm^{-3} (NaCl), pH = 8.9, T = 298 K

[HSR]/ mol dm^{-3}	$[Fe(CN)_5(NO)^{2-}]$/ mol dm^{-3}	$10^3 k_{exp.}$/ s^{-1}
5×10^{-4}	2.5×10^{-3}	4.22
5×10^{-4}	7.5×10^{-3}	2.64
5×10^{-4}	1.3×10^{-2}	2.80
5×10^{-4}	2.5×10^{-2}	2.62
5×10^{-4}	3.8×10^{-2}	3.05
5×10^{-4}	5.0×10^{-2}	3.47
5×10^{-4}	6.3×10^{-2}	3.05
5×10^{-4}	7.5×10^{-2}	3.34
2×10^{-3}	2.5×10^{-3}	3.92
2.5×10^{-3}	2.5×10^{-3}	4.06
3×10^{-3}	2.5×10^{-3}	4.50
3×10^{-3}	2.5×10^{-3}	3.30 *
3.5×10^{-3}	2.5×10^{-3}	3.26
3.75×10^{-3}	2.5×10^{-3}	3.78
5×10^{-3}	2.5×10^{-3}	10.0
1×10^{-2}	2.5×10^{-3}	19.6
1.5×10^{-2}	2.5×10^{-3}	26.7
2.5×10^{-2}	2.5×10^{-3}	50.0

* Air was not excluded.

TABLE 3

Rate constants ($k_{exp.}$) in aerated and anaerobic conditions at various cysteine to nitroprusside ratios; I = 1.5 mol dm^{-3} (NaCl), pH = 8.9, T = 298 K

[HSR]/[Fe(CN)$_5$(NO)$^{2-}$]	$10^3 k_{exp.}$/s^{-1} Aerated solutions	$10^3 k_{exp.}$/s^{-1} Anaerobic conditions
8×10^{-3}	3.32	3.34
2×10^{-2}	3.47	4.22
1.2	3.30	4.50
10	48.3	50.0

oxygen is kinetically not noticeable; the same situation arises when the ratio is sufficiently high (>10 : 1). In the intermediate range, however, oxygen quenches the rate of colour disappearance, as shown in the comparative studies included in Table 3.

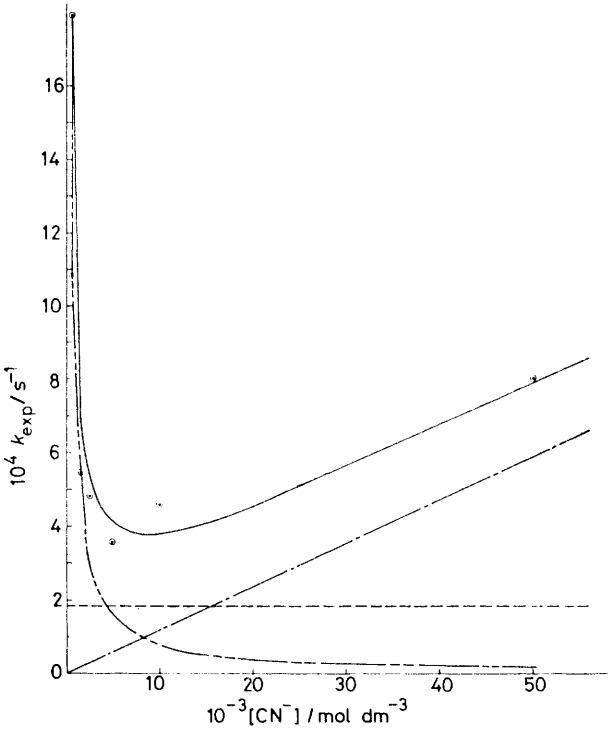


FIGURE 6 Changes of the first-order rate constant $k_{exp.}$ with cyanide-ion concentration; [Fe(CN)$_5$(NO)$^{2-}$] = 1.5×10^{-2} mol dm^{-3}; [cysteine] = 5.0×10^{-4} mol dm^{-3}; pH = 8.9. Broken curves represent the different terms in the rate law, the solid curve the total calculated change (see text), and individual points are experimental values

DISCUSSION

Formation of the Red Species.—Several experiments indicate that the red species is formed through an equilibrium reaction between pentacyanonitrosylferrate(2—) and cysteinate anion (see refs. 5—7). Thus, maximum values for the extrapolated initial absorbance are attained only at pH values higher than 10.5, and with a large excess of one of the reagents. Due to the reaction of cysteine with the red species, this is best seen at high iron concentrations, a 300-fold excess over cysteine being required to yield values which are independent of the concentration of reagents. The typical sigmoid curve (Figure 4) reflects the competition for cysteinate between the metallic moiety and H^+; the equilibrium characteristic of reaction (1) is depicted in Figure 5. From the slope of the plot of the inverse iron concentration against the inverse absorbance (A^{-1}) at constant cysteine concentration (Figure 7), the equilibrium constant K =

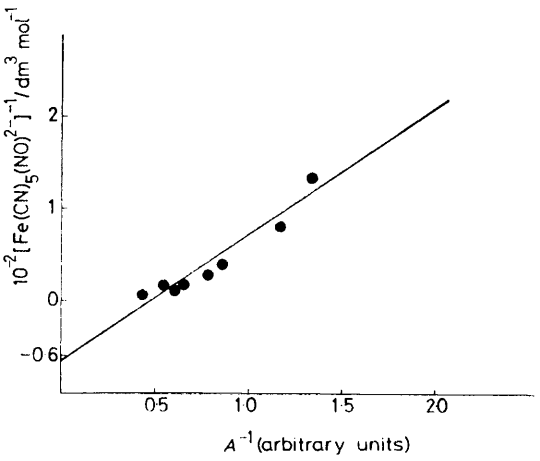


FIGURE 7 Relationship between inverse iron concentration and inverse absorbance. For further data, see Figure 5

[Fe(CN)$_5$(NO)(SR)$^{3-}$]/[Fe(CN)$_5$(NO)$^{2-}$][HSR] can be calculated to be 56 dm^3 mol^{-1}. Combining this with data from Figure 4, it is estimated that K' = [Fe(CN)$_5$(NO)(SR)$^{3-}$]/[Fe(CN)$_5$(NO)$^{2-}$][SR$^-$] = 145 dm^3 mol^{-1}. In order to obtain this figure, an apparent pK_a value of 9.1 was used for cysteine (see Figure 4); the literature value for the second pK_a is 8.33;[14] our attempts to demonstrate through potentiometric titrations that in our media this value was shifted to 9.1 did not succeed, but it was still felt that 9.1 was the best value to use in our (approximate) calculations.

In a similar way, the molar absorption coefficient of the complex can be calculated to be *ca.* 1×10^4 dm^3 mol^{-1} cm^{-1}.

The bond between cysteine and the NO ligand probably occurs *via* nitrogen, in view of previous data for the analogous reaction with SH$^-$.[6]

Fading of the Red Species.—In the reaction of SH$^-$ with the NO group,[6] it has been postulated that hydrogen-atom migration in the intermediate gives rise to the Fe–N(S)O arrangement, which in turn may give rise to Fe–SNO. The absence of a hydrogen atom on the sulphur of cysteine precludes the occurrence of this pathway, and in its place a simple electron-transfer reaction seems to be operative, giving rise to [Fe(CN)$_5$(NO)]$^{3-}$ and cystine.

The reactions of photolyzed nitroprusside with thiocyanate and thiourea give rise to analogous colours,[8,9] and a different reaction scheme (Scheme 2) has been postulated to explain these, involving equations (2)—(5).

The experiments performed by adding successive small amounts of cysteine show that pentacyanonitrosylferrate(2—) is consumed only slowly, and therefore, in our medium, Scheme 1 is a better representation of the course of the reaction.

In particular, the gross stoicheiometry of the fading reaction seems to be as in reaction (6). In oxygen-free

Alkaline medium

$$[Fe(CN)_5(NO)]^{2-} + 2OH^- \longrightarrow [Fe(CN)_5(NO_2)]^{4-} + H_2O \quad (2)$$

$$[Fe(CN)_5(NO_2)]^{4-} + H_2O \longrightarrow [Fe(CN)_5(OH_2)]^{3-} + NO_2^- \quad (3)$$

Acidification

$$[Fe(CN)_5(OH_2)]^{3-} \xrightarrow{O_2} [Fe(CN)_5(OH_2)]^{2-}$$

$$[Fe(CN)_5(OH_2)]^{2-} + SC(NH_2)_2 \longrightarrow [Fe(CN)_5\{SC(NH_2)_2\}]^{2-} + H_2O \quad (4)$$

$$[Fe(CN)_5\{SC(NH_2)_2\}]^{2-} \longrightarrow \text{Iron(II) products + sulphur oxidation products} \quad (5)$$

SCHEME 2

solutions, the kinetic behaviour indicates that many competing pathways are operative in the mechanism of this reaction. When the concentration of iron is in

$$2\,[Fe(CN)_5(NO)(SR)]^{3-} \longrightarrow 2\,[Fe(CN)_5(NO)]^{3-} + RSSR \quad (6)$$

excess, pseudo-first-order behaviour is observed (see Table 1), but cyanide ion produces a remarkable and noticeable effect. The data in Figure 6 can be represented by equation (7) with $k_1 = 8.0 \times 10^{-7}$ mol dm^{-3}

$$k_{exp.} = k_1[CN^-]^{-1} + k_2 + k_3[CN^-] \quad (7)$$

s^{-1}, $k_2 = 1.8 \times 10^{-4}$ s^{-1}, and $k_3 = 1.2 \times 10^{-2}$ dm^3 mol^{-1} s^{-1}. In this rate law, k_2 represents a simple intramolecular electron-transfer reaction, by which an electron mainly located on cysteine is transferred to a metal-centred orbital. On the other hand, the path involving the inverse cyanide concentration probably represents a route by which two metal complexes are brought together in an activated complex through the prior loss of cyanide ion; its occurrence is rather unexpected under our conditions of a large excess of $[Fe(CN)_5(NO)]^{2-}$. The dimeric species must be formed from one intermediate and one unreacted $[Fe(CN)_5(NO)]^{2-}$ molecule; this is in agreement with our observed first-order kinetic behaviour. Our data also imply that the dimer decomposes by a simple intramolecular electron-transfer reaction, but with a much higher rate constant than that of the monomer. The influence of cyanide ion had been previously described,[7] but in our study no indication of second-order kinetics was found, ruling out the possibility of two RS^- groups interacting in the activated complex.

The occurrence of a cyanide-enhanced path is novel; for this term we postulate a secondary reaction involving the aquation of co-ordinated NO(SR), assisted by cyanide through the well known dependence of the rate of release of ligands from $[Fe(CN)_5L]^{n-}$ on the concentration of incoming ligand.[15] This path, if the hypothesis is correct, should give rise to $[Fe(CN)_6]^{3-}$ as the main reaction product, and no catalytic oxidation of cysteine should be observed. In good agreement, in high cyanide concentrations, the phenomenon described in Figure 3 is quenched to a high extent. No direct unambiguous observation of hexacyanoferrate(4−) could be made, however.

In excess of cysteine, direct attack [reaction (8)] is observed. Electron spin resonance spectra of the

$$[Fe(CN)_5(NO)(SR)]^{3-} + SR^- \longrightarrow [Fe(CN)_5(NO)]^{3-} + \text{other products} \quad (8)$$

aerated solutions, in accord with previous evidence,[7] did not show any signal.

Collecting all the evidence described, the following reaction pathways (Scheme 3) seem feasible for the fading reaction (C represents the coloured species).

$$C \xrightarrow{k_2} [Fe(CN)_5(NO)]^{3-} + \tfrac{1}{2}RSSR$$

$$C \xrightarrow{K_1} [Fe(CN)_4(NO)(SR)]^{3-} + CN^-$$

$$\downarrow k_1'$$

$$[Fe(CN)_5(NO)]^{3-} + \tfrac{1}{2}RSSR \quad (k_1 = k_1'K_1)$$

$$C + CN^- \xrightarrow{k_3} [Fe(CN)_6]^{3-} + \text{products}$$

$$C + SR^- \xrightarrow{k_4} [Fe(CN)_5(NO)]^{3-} + \text{products}$$

SCHEME 3

In the presence of sufficient oxygen, nitroprusside is regenerated in a fast step, and the gross stoicheiometry of the overall process is shifted towards a catalyzed cysteine oxidation. When [cysteine]/[nitroprusside] is low, the rate constant is not noticeably affected (see Table 3) but when larger amounts of cysteine are present the rate is lowered, until even at higher cysteine concentrations both the rate constants become equal again.

In the low cysteine concentration range, effectively 100% of the ligand is complexed, and the rapid re-oxidation of the metallic complex does not alter the kinetic behaviour; at higher cysteine concentrations, two opposing factors influence the value of $k_{exp.}$; the direct attack by cysteine becomes increasingly important, and the coloured species is regenerated through equilibrium (1) as the fading reaction proceeds. Experimentally, the rate constant remains roughly constant, until direct attack by cysteine predominates. In this intermediate range, regeneration of $[Fe(CN)_5(NO)]^{2-}$ by oxygen will add to this complex picture unless rigorous air-free conditions are ensured; therefore, the values for $k_{exp.}$ should not be expected to give much information in this range. At very large cysteine concentrations, traces of oxygen which might have remained in solution are rapidly consumed by the system; and during actual kinetic measurements rigorous anaerobic conditions are established.

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