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Kinetics and Mechanism of the Redox Reaction of Safranin - O with L - cysteine in Aqueous Acidic Medium

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ABSTRACT
Redox chemistry of L - cysteine is important in biological systems, pharmaceutical chemistry, environmental chemistry and chemical research. This importance arises mainly from the presence of thiol group in its molecular structure, which enables L - cysteine to participate in redox reactions. The kinetics and mechanism of the redox reaction of safranin - O with L – cysteine (LSH) have been investigated spectrophotometrically in aqueous acidic medium under the following conditions: $[H^+] = 2.0 \times 10^{-5}$ mol dm^{-3}, I = 0.3 C^2 mol dm^{-3} (NaCl), λ_{max} = 520 nm and T = 294 ± 1K. The aim of this study is to investigate the kinetics of the reaction of safranin - O with L - cysteine in aqueous acidic solution. The reaction was monitored at 520 nm under pseudo first order conditions by following the decrease in absorbance of the dye with time. The reaction was first order in both $[SO^+]$ and [LSH], with second order overall. Studies on the effect of hydrogen ion concentration revealed the participation of a deprotonated reactive species prior to electron transfer. Negative BrØnsted Debye salt effect was observed, suggesting participation of oppositely charged reacting species in the rate determining step. Spectroscopic investigation gave no evidence for the formation of an intermediate complex of significant stability in the reaction. Free radicals were not detected during the course of the reaction. The kinetics observations are consistent with an outer - sphere electron transfer pathway, hence proposed.

Keywords: Safranin - O, L - Cysteine, Kinetics, Redox Reaction, Acidic Medium, Mechanism

INTRODUCTION

Safranin - O (SO^+) is a cationic phenazine dye and it is among the oldest known synthetic dyes exhibiting strong basic property, due to the presence of four nitrogen atoms (Cooksey, 2019). It is soluble in water and shows yellow - red fluorescence in ethanol.

It has wide applications in multiple areas of human endeavor. In biology it is used as a histological stain (Vidovix *et al.,* 2021). It is also used for the detection of cartilage, mucin and mast cell granules (Wall and board, 2013). It serves as an indicator dye to determine membrane potentials and as an electron transfer mediator in potentiometric titrations (Wan *et al.,* 2019). It has wide applications in textile, which is mainly used to dye bast fibers, silk, wool, mordanted cotton, leather and paper (Kiruthika *et al*., 2022). Its conjugated structure gives rise to a strong visible absorption band, making spectrophotometry particularly suitable for monitoring its reactions in solution.

L - cysteine is a biologically important sulphur - containing amino acid possessing a reactive thiol (-SH) group that readily undergoes oxidation, making it an effective reducing agent in aqueous media. The redox chemistry of L - cysteine has attracted considerable attention because of its relevance in biological antioxidant systems (Dougherty *et al.,* 2017).

The kinetics of oxidation of L - cysteine by several oxidants have been reported (Abdulsalam *et al.,* 2020; Nkole and Idris, 2021, Ukoha *et al.,* 2023). The oxidation of cysteine by a trinuclear Mn (IV) species in aqueous acidic medium was reported to follow first order kinetics, with evidence for proton coupled electron transfer (Mukhopadhy *et al.,* 2013). Other kinetic investigations have shown that cysteine oxidation may involve the thiolate form of cysteine, whose concentration changes significantly with pH (Manago *et al.,* 2025).

Despite the extensive literature on cysteine oxidation and the known redox activity of phenazine dyes, to the best of our knowledge, kinetic information on the reaction of safranin - O with L - cysteine has not been documented. This makes the present investigation relevant to understanding the interaction between a phenazine dye and a biologically important thiol containing reductant. This study aims to investigate the kinetics and mechanism of the redox reaction of safranin - O with L - cysteine in aqueous acidic medium.

MATERIALS AND METHODS

The chemicals and reagents used were of analytical grade and were used without further purification. Safranin - O was obtained from Sigma - Aldrich, Germany. L- cysteine (CDH, 98%) Sodium chloride, (BDH, 99.8%) was used to keep the reaction's ionic strength constant. Magnesium chloride, $MgCl_2$. $6H_2O$ (BDH, 97%) and sodium nitrate, Na_2NO_3 (BDH, 99%), were used to ascertain the effect of added ions on the reaction rate. Hydrochloric acid, HCl (BDH), was used to study the effect of $[H^+]$ ion on the rate of reaction. Acetone (May and Baker, 99%) was used to investigate the solvent polarity (dielectric constant) on the reaction mixture. Acrylamide with methanol were used to check the participation of free radicals during the course of the reaction. Kinetic investigations were performed with JENWAY 7315 Spectrophotometer. Mass spectrophotometer was used for characterisation of the dye and reaction products.

Stock solution of safranin - O (7.5×10^{-4} mol dm^{-3}) was prepared by dissolving 6.6×10^{-3} g of the dye with distilled water and made up to the mark in 25 cm^3 volumetric flask.

Stock solution of 6 .0 × 10^{-2} mol dm^{-3} L - cysteine was prepared by dissolving 0.73 g of L -cysteine in distilled water and made up to the mark using 100 cm^3 standard flask.
Stock solution of 0.1 mol dm^{-3} hydrochloric acid was prepared by diluting 2.17 cm^3 of 36% HCl (specific gravity 1.18) in 250 cm^3 standard flask, then made up to the mark with distilled water. The solution was standardised titrimetrically with standard solution of sodium carbonate using methyl orange as indicator (Jeffery *et al.,* 1989).
Standard solutions, 1.0 mol dm^{-3} each of these compounds was prepared by dissolving 2.92g (NaCl), 10.17g ($MgCl_2.6H_2O$) and 4.25g (Na_2NO_3) in distilled water and made up to the mark in 50 cm^3 volumetric flask.

The Reaction Stoichiometry
The stoichiometry of the reaction was determined spectrophotometrically using the mole ratio method (Idris *et al.,* 2015). This was done by measuring the absorbance of solution containing various concentrations of [LSH] = (0.3 - 5.25) ×10^{-5} mol dm^{-3}, I = 0.1 mol dm^{-3} (NaCl), T = 302±1K and at constant concentration of $[SO^+]$ = 2.0 × 10^{-5} mol dm^{-3}. After the reaction had gone to completion, plot of absorbance against mole ratio was made and the point of inflection in the plot gave the number of moles of LSH consumed by 1 mole of the dye.

Kinetic Measurement
The order of reaction was determined by observing change in absorbance as the reaction proceeds at λ_{max} = 520 nm. The process was done using pseudo - first order conditions with the [LSH] 100-fold in excess over $[SO^+]$ at a constant ionic strength of 0.2 mol dm^{-3}(NaCl). Graphs of absorbance against time (t) were plotted for all the folds, and the observed rate constants, k_1 were obtained as the gradients of the graphs. The second order rate constants, k_2 were determined from equation 1

$$k_2 = k_1/[LSH] \quad (1)$$

The effect of hydrogen ion concentration was determined by change in $[H^+]$ in the range (0.4 - 2.8 ×10^{-5} mol dm^{-3}) of the stability of the dye. The salt effect of reaction medium was studied within the range of 0.1 - 0.8 mol dm^{-3} (NaCl), and the effect of dielectric constant (D) on the rate of reaction was investigated in the range determined. Solvent polarity was varied by the addition of acetone (2.0 cm^3) at constant concentrations of the reactants and temperature of 294±1K

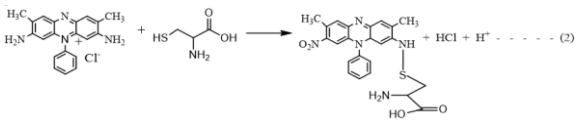
Effect of Added Ions on the Rate of Reaction
The effect of added ions on the rate of reaction was investigated under a constant concentration of oxidant, reductant, ionic strength, and at $[X^{2+}]$ = (2.0 − 12.0) ×10^{-3} mol dm^{-3}. (2)

Test for Free Radicals
A 2 cm^3 of acrylamide solution was added to a partially oxidized reaction mixture containing various concentrations of oxidant, reductant and hydrogen ion. This was followed by addition of an excess of methanol. The same treatment was applied to the solution of oxidant and reductant separately as a control.

Spectrophotometric Test for Intermediate
Test for the presence of stable intermediates formed during the course of the reaction was carried out spectrophotometrically. The intermediate complex was examined by scanning the partially reacted mixture at wavelength 400 - 600 nm and compared with the spectrum of the dye. Michaelis - Menten's plot of k^{-1} against $[LSH]^{-1}$was equally done.

Product Analysis
At the completion of the reaction, the product from the aqueous reaction mixture was extracted with diethyl ether in order to separate organic from the inorganic product. The organic product was analysed by Liquid Chromatography Mass Spectrum (LC - MS) method. The mass spectrum of the dye was obtained, and that of the reaction product was also obtained.

RESULTS AND DISCUSSION
Stoichiometry and Product Analysis
The result of stoichiometry study showed that one mole of SO^+ required one mole of L - cysteine in the course of the reaction and it is represented by equation 2 and Figure 1:
The result of the stoichiometry is similar with the previous report in the reaction of N, N^1 - phenylenebis(salicyalideneiminato) cobalt(III) with L - cysteine (Abdulsalam *et al.,* 2020). The stoichiometric equation of the reaction is as follows:



The stoichiometric result provides an important constraint on the proposed mechanism.

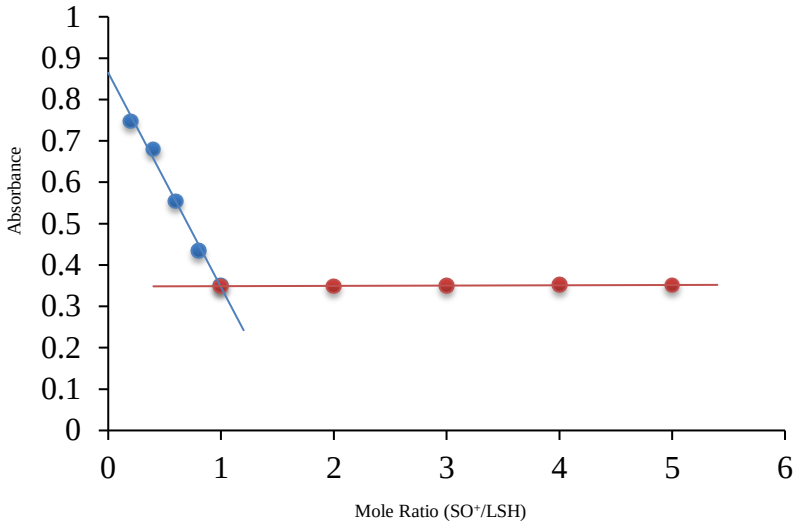


Figure 1: Plot Of Absorbance Versus Mole Ratio For The Determination Of Stoichiometry Of The Reaction Of SO^+ With LSH At $[SO^+] = 1.5 \times 10^{-5}$ Mol Dm^{-3}, [LSH] = $(0.3 - 5.25) \times 10^{-5}$ Mol Dm^{-3}, $[H^+] = 2.0 \times 10^{-5}$ Mol Dm^{-3}, I = 0.1 Mol Dm^{-3}, T = 302±1K And λ_{max} = 520 Nm

Mass spectrum of the dye had molecular ion peak at 315 (Figure II) which correspond to the molecular formula $C_{20}H_{19}N_4$. Also, mass spectrum of the reaction product had a molecular ion peak at 134.16 (Figure III), which corresponds to the cysteine fragment.

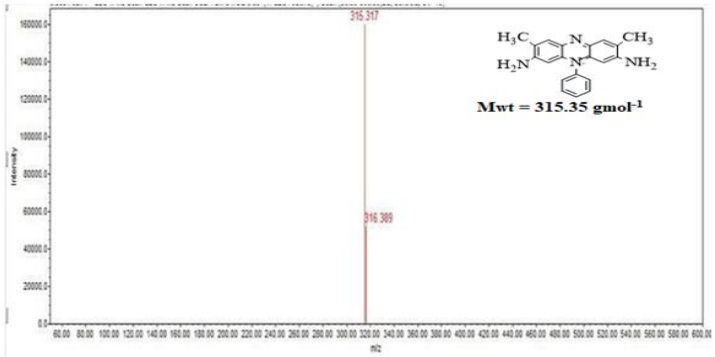


Figure 2: Mass Spectrum of Safranin - O Dye

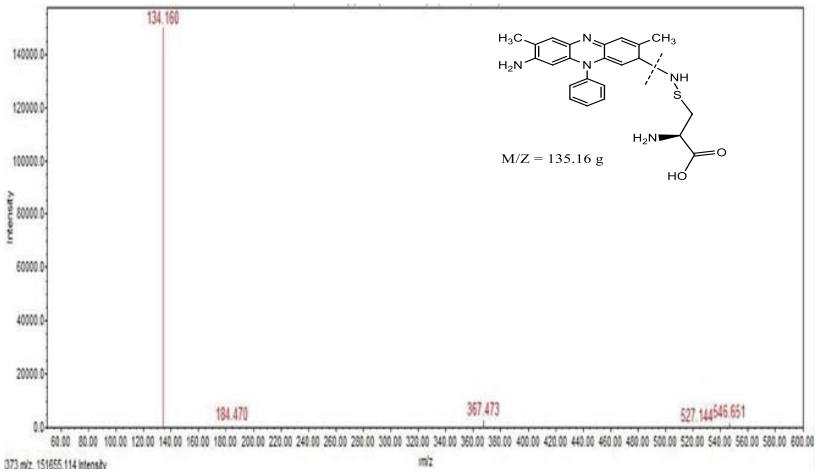


Figure 3: Mass Spectrum of the Reaction Product of SO^+ With L - Cysteine

Determination of Reaction Order

The reaction revealed first order with respect to the $[SO^+]$ as evidenced from the linearity of the graphs of log $(A_t - A_\infty)$ against time and a typical one is shown in Figure IV. Values of the observed rate constants are given in Table 1. A plot of log k_1 versus log [LSH] was linear with a slope of 0.95, which signifies first order in [LSH] as shown in Figure V. Similar result was obtained in the reaction of N, N^1 - phenylenebis(salicylideneiminato) cobalt (III) with L-cysteine (Abdulsalam *et al.*, 2020). First order reaction was

also observed in the catalysed oxidation of L - cysteine by salen schiff base complexes of Co (III), Fe (III) and Cr (III) (Abdel - Halim *et al.*, 2021).

The second order rate constants, k_2 was obtained from $k_2 = k_1/[LSH]$ was found to be fairly constant (Table 1). The rate law for the reaction is therefore given by equation (3):

$$\frac{-d[SO^+]}{dt} = k_2 [SO^+] [X] \quad (3)$$

X = [LSH], $k_2 = (36.72 \pm 0.11) \times 10^{-2} dm^3 mol^{-1} s^{-1}$,

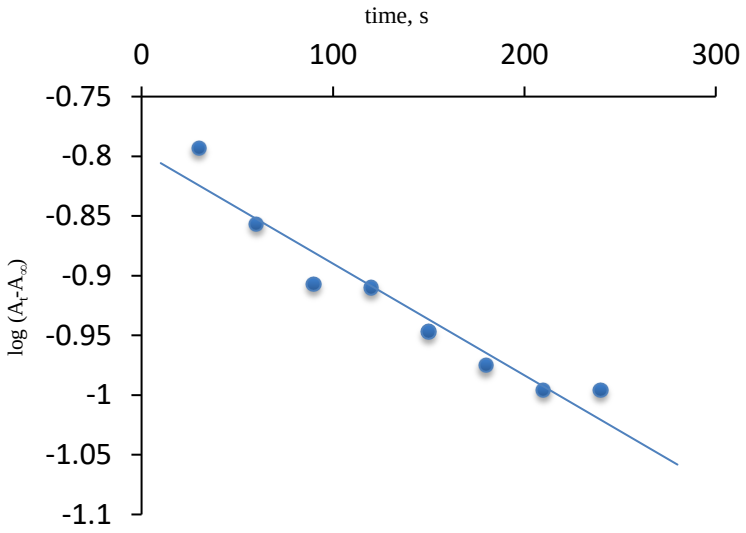


Figure 4: Typical Pseudo - First Order Plot for The Reaction of SO^+ With LSH At $[SO^+] = 1.5 \times 10^{-5}$ mol dm^{-3}, $[LSH] = 1.5 \times 10^{-2}$ mol dm^{-3}, $[H^+] = 2.0 \times 10^{-5}$ mol dm^{-3}, I = 0.2 C^2mol dm^{-3}, T = 294±1K and λ_{max} = 520 nm

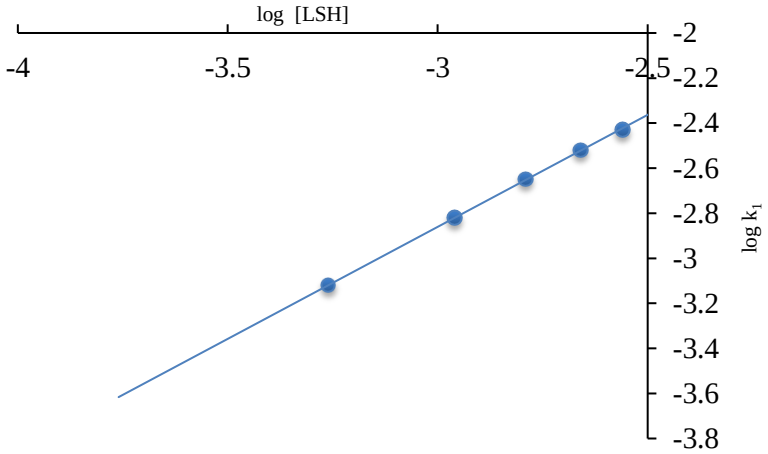


Figure 5: Plot of log k_1 versus log [LSH] for the reaction of SO^+ with LSH at $SO^+] = 1.5 \times 10^{-5}$ mol dm^{-3}, $[H^+] = 2.0 \times 10^{-5}$ mol dm^{-3} I = 0.2 mol dm^{-3}, T = 300±1K and λ_{max} = 520 nm

Table 1: Pseudo - First Order And Second Order Rate Constants For The Reaction Of SO^+ With LSH At $[SO^+] = 1.5 \times 10^{-5}$ Mol Dm^{-3}, T = 294 ± 1K And λ_{max} = 520 Nm

10^3 [LSH], mol dm^{-3}	10^5 $[H^+]$, mol dm^{-3}	I, mol dm^{-3}	10^2 k_1, s^{-1}	10^2 k_2, $dm^3 mol^{-1} s^{-1}$
7.5	2.0	0.2	27.40	36.53
15.0	2.0	0.2	54.80	36.53
22.5	2.0	0.2	82.25	36.56
30.0	2.0	0.2	110.23	36.74
37.5	2.0	0.2	137.70	36.72
45.0	2.0	0.2	164.46	36. 58
22.5	0.4	0.2	141.01	62.70
22.5	0.8	0.2	127.70	56.75
22.5	1.2	0.2	108.60	48.28
22.5	1.6	0.2	91.53	40 .68

10^3 [LSH], mol dm^{-3}	10^5 $[H^+]$, mol dm^{-3}	I, mol dm^{-3}	10^2 k_1, s^{-1}	10^2 k_2, dm^3mol^{-1} s^{-1}
22.5	2.0	0.2	82.24	36.55
22.5	2.4	0.2	71.40	31.73
22.5	2.0	0.1	111.80	49.69
22.5	2.0	0.2	104.00	46.23
22.5	2.0	0.3	93.83	41.70
22.5	2.0	0.4	98.18	43.64
22.5	2.0	0.5	82.25	36.56
22.5	2.0	0.6	71.55	31.80

Acid Dependence

The rate of reaction was observed to decrease in the presence of acid in the range investigated. The reaction showed a first order independence on $[H^+]$, (Table 1). A plot of k_2 versus $[H^+]^{-1}$ was found to be linear with a positive intercept (Figure VI) and the relationship is given by:

$k_2 = a + b\,[H^+]^{-1}$

where $a = 0.69\ dm^3\ mol^{-1}\ s^{-1}$; $b = 1.58 \times 10^{-6}\ s^{-1}$.

Substituting the expression for k_2 into equation 3 gives equation 4.

$$-d[SO^+]/dt = a+b\{[H^+]^{-1}\}\,[SO^+]\,[LSH] \qquad (4)$$

The equation suggests that the reaction occurs via acid independent pathway. This suggests H^+ deprotonation in a pre-equilibrium step prior to electron transfer. Given the nature of the reactant partners, the deprotonation is probably from the sulphydral group (SH). This indicates that the reactive species is likely the thiolate form of L - cysteine produced through a pre - equilibrium deprotonation (equation 5). The inverse hydrogen ion dependence therefore supports participation of a deprotonated cysteine species in the electron transfer step.

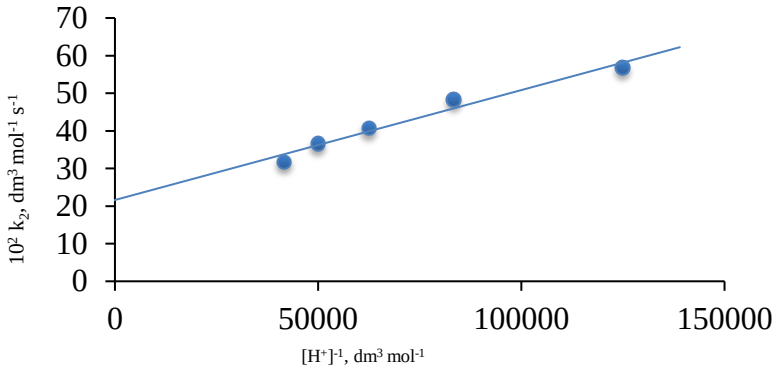


Figure 6: Plot of k_2 versus $[H^+]^{-1}$ for the reaction of SO^+ with LSH at $[SO^+] = 1.5 \times 10^{-5}$ mol dm^{-3}, $[LSH] = 2.25 \times 10^{-5}$ mol dm^{-3}, I = 0.2 C^2 mol dm^{-3}, T = 302± 1K and λ_{max} = 520 nm

Effect of Ionic Strength and Added Ions

Effect of changes in ionic strength of the reaction medium indicated that the rate constant decreases with increase in ionic strength (Table 1). Therefore, negative Bronsted - Debye salt effect was observed. This type of salt effect suggests an interaction of species carrying opposite charges in the activated complex. Plot of log k_2 against $I^{1/2}$ (Figure VI) gave a slope of - 0.60. Added cation and anion inhibited the rate of reaction (Table II). This effect exerted by the ions suggested that the reaction might be operating via the outer - sphere mechanism (Mayer and Taube, 1987).

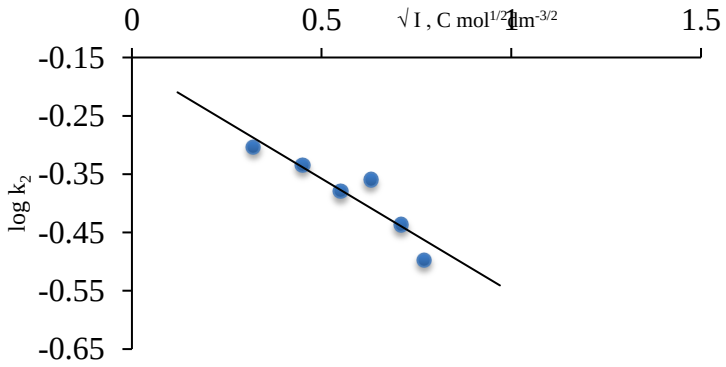


Figure 7: Plot of log k_2 versus √I for the reaction of SO^+ with cysteine at $[SO^+] = 1.5\times10^{-5}$ mol dm^{-3}, $[H^+] = 2.0 \times 10^{-5}$ mol dm^{-3}, T = 300±1K and λ_{max} = 520 nm

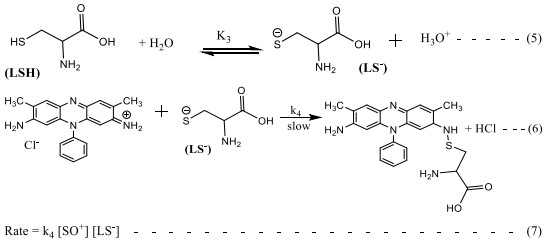
Table 2: Rate Data For The Effect Of Added Cations For The Reaction Of SO^+ With LSH At $[SO^+] = 1.5 \times 10^{-5}$ Mol Dm^{-3}, $[LSH] = 2.25 \times 10^{-2}$ Mol Dm^{-3}, $[H^+] = 2.0 \times 10^{-5}$ Mol Dm^{-3}, I = 0.2 C^2 Mol Dm^{-3}, T = 294 ± 1K And λ_{max} = 520 Nm

X	10^3 [X], mol dm^{-3}	10^4 k_1, s^{-1}	k_2, dm^3 mol^{-1} s^{-1}
Mg^{2+}	0.0	82.29	36.58
	2.0	72.93	32.41
	4.0	69.79	31.02
	6.0	60.96	27.09
	8.0	49.90	22.18
	10.0	36.85	16.38
$CH3COO^-$	0.0	81.89	36.40
	2.0	79.90	35.51
	4.0	78.20	34.76
	6.0	76.50	34.00
	8.0	75.00	33.33
	10.0	73.70	32.76

Other Determinations

Lack of spectrophotometric evidence for the formation of intermediate complex was inferred when no shift in λ_{max} (520 nm) was observed when the spectra of the reaction mixtures were compared with that of the SO^+ suggesting the absence of an intermediate and a probable outer - sphere mechanism. Also, the presence of intermediates in the reactions were ruled out from zero intercepts obtained from Michaelis-Menten plots of $\frac{1}{k_1}$ versus $\frac{1}{[LSH]}$ suggesting the absence of an intermediate and a probable outer - sphere mechanism. The negative result of polymerisation test suggests the absence of free radical intermediate in the reactions or the equilibrium constant for the formation of such radicals can be assumed to be negligible (Idris, 2005). This also favours the outer - sphere mechanism. Based on these experimental findings, an outer - sphere mechanistic pathway is proposed.

Reaction Mechanism



(5)

(6)

$$\text{Rate} = k_4 [SO^+] [LS^-] \quad (7)$$

$$\text{From equation 1, } [LS^-] = \frac{K_3[LSH]}{[H_3O^+]}$$

$$\text{Rate} = \frac{k_4 K_3 [SO^+] [LSH]}{[H_3O^+]} \quad (8)$$

Substituting $[LS^-]$ into equation 8

$$\text{Rate} = k' [SO^+] [LSH]$$

$$\text{where } k' = \frac{k_4 K_3}{[H_3O^+]}, [LSH] = [\text{L -cysteine}] \quad (9)$$

Equation 9 is analogous to equation 3

where $k_2 = k' = (36.72 \pm 0.11) \times 10^{-2}$ dm^3 $mol^{-1}s^{-1}$.

CONCLUSION

The redox reaction of safranin - O with L - cysteine in aqueous acidic medium was investigated kinetically using spectrophotometry at 520 nm. The reaction showed a first order behaviour under the experimental conditions. The rate exhibited an inverse dependence on hydrogen ion concentration, which is consistent with participation of a more reactive deprotonated cysteine species. A negative salt effect was observed with increasing ionic strength. The stoichiometric ratio was 1:1. Test for free radicals were negative, and kinetic observations gave no evidence for formation of a stable intermediate complex. Added ions inhibited the reaction, this effect suggested that the reaction is probably operating via the outer - sphere mechanism. In this study the product analysis is limited to LC - MS, therefore it is recommended that further product characterisation should be carried out in a similar study in order to establish what has actually been produced.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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