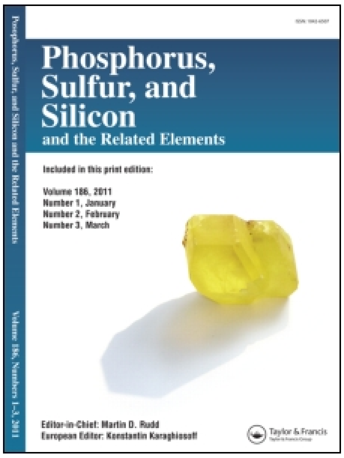

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KINETICS AND MECHANISM OF THE OXIDATION OF O-MERCAPTOBENZOIC ACID (A MODEL FOR PEPSIN) BY METHYLENE BLUE IN METHANOL-WATER MEDIUM

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Key words: o-Mercaptobenzoic acid; oxidation by methylene blue

The oxidation of o-mercaptobenzoic acid (TSA: a model for pepsin) by methylene blue (MB) has been investigated kinetically in aqueous methanol and in presence of sulphuric acid. The principal reactants interact in a molar ratio of 2:1 forming diphenyldisulfide-2,2′-dicarboxylic acid as the oxidation product. The order in the oxidant is unity while the order in the substrate varies from zero to half at its larger concentrations. The rate depends on pH showing an optimum. The order in hydrogen ion increases from 0.04 to 0.1 when the runs are made at low concentrations of TSA (ca. 1.25×10^{-3} M). The variation in ionic strength does not influence the rate but an increase in dielectric constant increases the rate. Here again, the rate attains a limiting value after a particular concentration of methanol (50% v/v). The addition of reaction products retards the rate showing a variation in order of reaction in methylene blue from unity to zero on adding the leuco base. The possibility of the participation of half reduced MB was checked by making the run in presence of acrylonitrile and it was overruled. Activation parameters were evaluated and a reaction scheme presuming the participation of triplet methylene blue has been proposed.

In recent years the sulphur chemistry has attracted a considerable attention owing to multifacet involvement of sulphur compounds especially the sulphydryl compounds in metabolic pathways. Largely due to this, the oxidation kinetics of a variety of structurally related sulphydryl substrates employing 2,6-dichlorophenolindophenol (DCIP, a model for coenzyme Q) and hexacyanoferrate (III) ion have been investigated by Mishra and coworkers.[1-11] These studies have highlighted the steric and environmental effects on the kinetic features of these reactions and thus, the investigations have been extended to enzyme model sulphydryl substrates. The applied oxidant (methylene blue) is known to participate in the electron transfer reactions used as model systems for elucidating the mechanism of the oxidation of flavin-bound enzymes in mitochondria. Keeping this in view, in the present work the kinetics of the oxidation of o-mercaptobenzoic acid (a model for pepsin) has been described. It may be mentioned here that the kinetics of the oxidation of this substrate by DCIP in presence of hydroxyl ions has been reported previously[3] but the results obtained now show a complete divergence and highlight the specific nature of these reaction systems.

EXPERIMENTAL

The solution of o-mercaptobenzoic acid (TSA or RSH) was prepared by dissolving an exactly weighed quantity of a sample supplied by M/s Evans chemetics Inc., U.S.A. (assay 99%) in methanol (E. Merck, G. R. grade). The solution of methylene blue (MB) was prepared by dissolving an exactly weighed quantity in double distilled water. The solutions of the oxidant and the substrate were stored in a dark place in order to preclude the possibility of a photochemical interference. Other chemicals such as potassium chloride, sulphuric acid etc. were E. Merck's G.R. grade samples. The disulfide of TSA was prepared by oxidising o-mercaptobenzoic acid with ferric alum,[13] while the leuco methylene blue was obtained by bleaching a known solution of the oxidant by passing SO_2 gas liberated from potassium metabisulphite.[1,12] The solutions were always stored in a nitrogen atmosphere.

The kinetics of the reaction was followed colorimetrically by measuring the decrease in [MB] (ε = 4.5×10^4 lit mole^{-1} cm^{-1} at 660 nm) by a klett-Summerson photoelectric colorimeter fitted with an orange light filter. The use of this filter is justified because the leuco base of methylene blue, TSA and its oxidation product absorb strongly in the UV part of the spectrum.

Reaction vessels made of pyrex glass and coated black from outside with Black Japan were used. These were thermostated for a sufficient length of time (variation ± 0.1°C) and the aliquots were analysed at different time intervals with the help of Beer's law plots. The law is found to be obeyed by methylene blue in the concentration range employed presently.

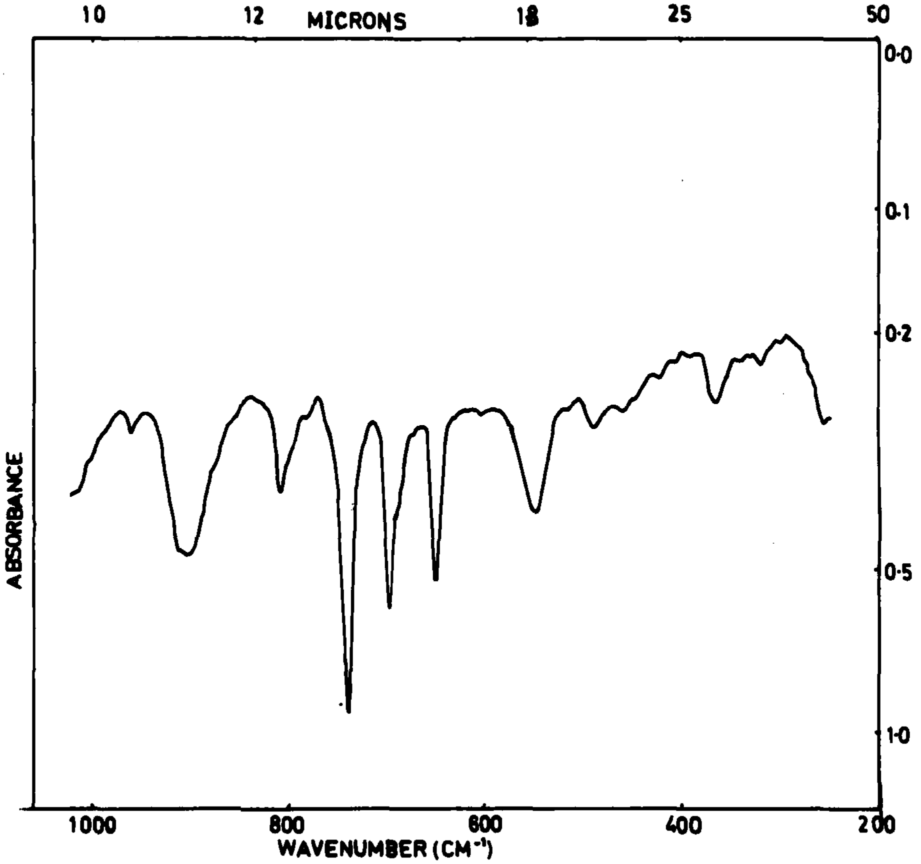


FIGURE 1 IR-Spectrum of diphenyldisulfide-2,2′-dicarboxylic acid.

RESULTS

The principal reactants are found to interact in a molar ratio of 2:1 forming the corresponding disulfide and the leuco base. The formation of the disulfide was confirmed by recording the IR spectrum of the product isolated from the reaction mixture (Run 5, Table I) after completion of the reaction (Figure 1). It is similar to the spectrum obtained for the disulfide prepared by oxidising TSA with ferric alum and gives a characeristic stretching mode at 550 cm^{-1} which is in agreement with the value reported for aryl disulfides.[14]

The reaction follows a pseudo first order kinetics with respect to methylene blue when 40 to 100 fold excess concentration of TSA over the oxidant is employed. The pseudo first order rate constants obtained for different [TSA] are given in Table I. The order in TSA was determined by making use of the runs given in Table I. The order in the substrate varies from zero to 0.5 on increasing [TSA] as revealed by the plot of log k_1 against log [TSA] which gives a straight line with a slope of 0.56.

The pseudo first order rate constant decreases on increasing [MB] as shown in Table II. The double log plot between the rate constant and [MB] gives a straight line with a negative slope of 0.4. The half order dependence of rate on [MB] was further verified in these variations by the initial rate measurement method. The formation of some inhibiting species during the course of reaction is indicated by such a diversity.

TABLE I

Rate constants at different [TSA]

Run	[TSA] × 10^3 M	k_1 × 10^4 sec^{-1}
1.	1.00	1.7
2.	1.25	1.7
3.	1.50	1.5
4.	1.75	2.5
5.	2.00	2.8
6.	2.50	2.9

[MB] = 2.5×10^{-5} M | $\mu = 2.3 \times 10^{-2}$ M
$[H_2SO_4] = 6.0 \times 10^{-3}$ M | CH_3OH = 30% (v/v)
[KCl] = 5.0×10^{-3} M | Temp = 30°C

TABLE II

Rate constant at different [MB]

[MB] × 10^5 M	k_1 × 10^4 sec^{-1}
3.5	2.5
3.0	2.5
2.5	2.8
2.0	3.0
1.5	3.8
1.0	4.1

[TSA] = 2.0×10^{-3} M | $\mu = 2.3 \times 10^{-2}$ M
$[H_2SO_4] = 6.0 \times 10^{-3}$ M | CH_3OH = 30% (v/v)
[KCl] = 5.0×10^{-3} M | Temp = 30°C

The rate increases on increasing [H^+] but after attaining a limiting value, the rate gradually decreases. A further increase in [H^+], however, again increases the rate (Table III). In these variations, the ionic strength of the reaction system was maintained constant (42×10^{-3} M) by adding the requisite amount of potassium chloride in the reaction mixture.

The rate constant remains unaffected on varying the ionic strength of the reaction system. The dielectric constant of the medium was varied by adding different volumes of methanol to the reaction mixtures. The rate constant decreases on increasing methanol content up to 50% (v/v) but beyond this composition, the rate attains a limiting value (Table IV).

TABLE III

Rate constant at different [H^+]

[H_2SO_4] $\times 10^3$ M	$k_1 \times 10^4$ sec^{-1}
1.0	2.3
2.0	2.3
4.0	2.5
6.0	2.8
10.0	3.1
12.0	2.6
13.0	2.9

[MB] = 2.5×10^{-5} M
[TSA] = 2.0×10^{-3} M
$\mu = 42.0 \times 10^{-3}$ M
CH_3OH = 30% (v/v)
Temp = 30°C

TABLE IV

Rate constants at different dielectric constant

CH_3OH (% v/v)	D	$k_1 \times 10^4$ sec^{-1}
20	70.0	3.2
30	65.2	2.8
40	60.5	1.9
50	55.7	1.7
60	51.0	1.6
70	46.2	1.7

[MB] = 2.5×10^{-5} M
[TSA] = 2.0×10^{-3} M
[H_2SO_4] = 6.0×10^{-3} M
[KCl] = 5.0×10^{-3} M
$\mu = 2.3 \times 10^{-2}$ M
Temp = 30°C

TABLE V

Rate constants in presence of reaction products

[RSSR] $\times 10^5$ M	$k_1 \times 10^4$, sec^{-1}	[H_2MB] $\times 10^6$ M	$k_1 \times 10^4$, sec^{-1}	$k_0 \times 10^4$ mol · lit^{-1} sec^{-1}
2.0	2.5	0.0	2.5	—
4.0	2.1	6.66	—	4.4
6.0	2.0	13.3	—	2.7
8.0	1.9	20.0	—	2.3
10.0	1.7	—	—	—

[MB] = 2.5×10^{-5} M
[TSA] = 2.0×10^{-3} M
[H_2SO_4] = 6.0×10^{-3} M
[KCl] = 5.0×10^{-3} M
$\mu = 2.3 \times 10^{-2}$ M
Temp = 30°C

The reaction products viz. TSA disulfide and the leuco base decrease the rate of reaction when added externally in a series of runs. Addition of leuco base, however, produces a change in order in MB from unity to zero (Table V).

The Arrhenius equation was found to be applicable and was used for the calculation of activation parameters. ΔH*, ΔS* and ΔF* are found to be 41.9 kJ mole^{-1}, −42.8 J K^{-1} mole^{-1} and 58.4 KJ mole^{-1}.

DISCUSSION

The present investigations were made in aqueous methanol (30% (V/V)) and in presence of sulphuric acid. Methylene blue is reported to be present in dimeric form in aqueous medium, while the monomeric species exists in alcohols.[15] It is further reported that methylene blue cation absorbs energy from visible radiation and is excited to a triplet state. Our kinetic results are best explained by presuming the triplet methylene blue cation as the principal reactant. For the sake of brevity, this configuration has been denoted as M′ in the foregoing discussion. The protonation of methylene blue has already been reported by Mukherjee and Ghosh[16–20] and here too, the protonated species (M′H$^+$) is presumed to react with nucleophile RS$^-$ to produce the leucobase (L) and the reactive sulphenyl cation (RS$^+$). It would be pertinent to mention here that the formation of species RS$^+$ has been reported earlier by authors such as Kolthoff, Kapoor and Sinha specifically using hexacyanoferrate (III) ion as an oxidant.[21] Thus,

$$M' + H^+ \underset{k_1}{\overset{k_1}{\rightleftharpoons}} M'H^+ \tag{1}$$

$$M'H^+ + RS^- \underset{k_2}{\overset{k_2}{\rightleftharpoons}} L + RS^+ \tag{2}$$

$$RS^+ + RSH \underset{k_3}{\overset{k_3}{\rightleftharpoons}} RSSR + H^+ \tag{3}$$

The participation of RS$^-$ seems justified because even in acidic medium RSH will partly dissociate as given by the equilibrium:

$$RSH \overset{K}{\rightleftharpoons} RS^- + H^+ \tag{4}$$

The participation of RS$^-$ seems even more logical because the thiolate anion of o-mercaptobenzoic acid is reported to be stabilized due to intramolecular hydrogen bonding[22] and secondly, the possibility of the participation of thiyl radical appears unlikely since the addition of acrylonitrile to the reaction system does not produce any change in the rate of reaction.

The rate of reaction for the above scheme would be given by:

$$-\frac{d[M']}{dt} = k_1 [M'][H^+] - k_1[M'H] \tag{5}$$

On presuming steady state for $M'H^+$ and RS^+:

$$[RS^+] = \frac{k_2[M'H^+][RS^-] + k_3[RSSR][H^+]}{k_2[L] + k_3[RSH]} \tag{6}$$

$$[M'H^+] = \frac{k_1[M'][H^+](k_2[L] + k_3[RSH]) + k_2[L]k_3[RSSR][H^+]}{k_1(k_2[L] + k_3[RSH]) + k_2[RS^-](k_2[L] + k_3[RSH]) - k_2[L]k_2[RS^-]} \tag{7}$$

On substituting these values in Equation (5)

$$-\frac{d[M']}{dt} = k_1[M'][H^+] \times \frac{k_2[RS^-](k_2)[L] + k_3[RSH]) - k_2k_2[L][RS^-] - k_1k_2k_3[L][RSSR][H^+]}{k_1(k_2[L] + k_3[RSH]) + k_2[RS^-](k_2[L] + k_3[RSH]) - k_2k_2[L][RS^-]} \tag{8}$$

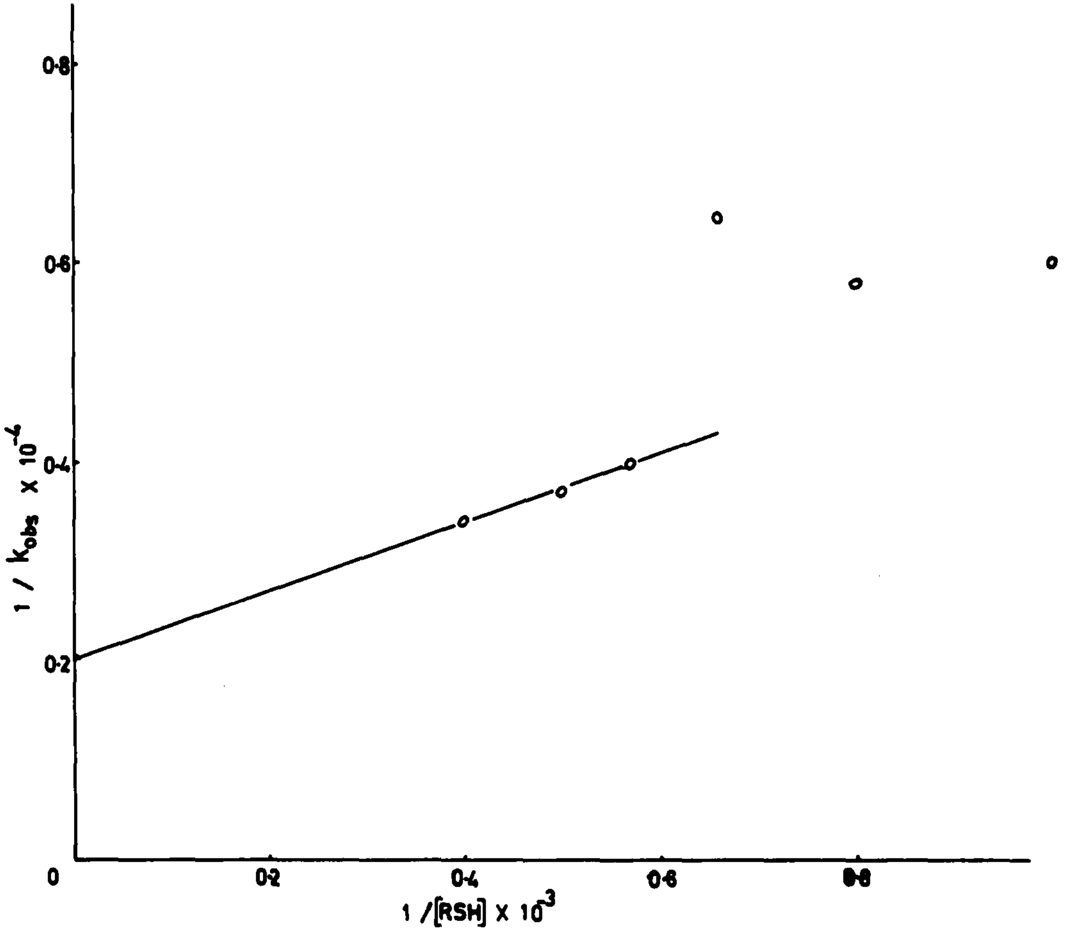


FIGURE 2 Rate constants at different [TSA]. $[MB] = 2.5 \times 10^{-5}$ M; $[H_2SO_4] = 6.0 \times 10^{-3}$ M; $[KCl] = 5.0 \times 10^{-3}$ M; CH_3OH = 30% (v/v); $\mu = 2.3 \times 10^{-2}$ M; Temp = 30°C.

Equation (8) explains a first order kinetics in methylene blue and also a complex dependence of rate on $[H^+]$. It also explains the retarding influence of the reaction products viz. the disulphide and the leucobase.

The concentrations of the leucobase and disulphide are taken as negligibly small and if $k_1 << k_2 [RS^-]$, then Equation (8) is reduced to:

$$-\frac{d[M']}{dt} = k_1[M'][H^+]\frac{k_2[RS^-](k_2[L] + k_3[RSH])}{k_1 + k_2[RS^-](k_2[L] + k_3[RSH])} \tag{9}$$

or

$$-\frac{d[M']}{dt} = \frac{k_1k_2[M'][H^+][RS^-]}{k_1 + k_2[RS^-]} \tag{10}$$

or

$$-\frac{d[M']}{dt} = \frac{k_1k_2K[M'][RSH][H^+]}{k_1[H^+] + k_2K[RSH]} \tag{11}$$

from Equation (11), the pseudo first order rate constant (k_{obs}) will be given by the expression:

$$k_{obs} = \frac{k_1k_2K[RSH][H^+]}{k_1[H^+] + k_2K[RSH]} \tag{12}$$

or

$$\frac{1}{k_{obs}} = \frac{k_1}{k_1k_2K[RSH]} + \frac{1}{k_1[H^+]} \tag{13}$$

Equation (13) was verified by plotting $1/k_{obs}$ against $1/[RSH]$ for the runs described in Table I to give a straight line with slope and intercept equal to 3.4 and 2.01×10^{-3} respectively (Fig 2).

From Equation (13),

$$\text{slope} = \frac{k_1}{k_1k_2K}$$

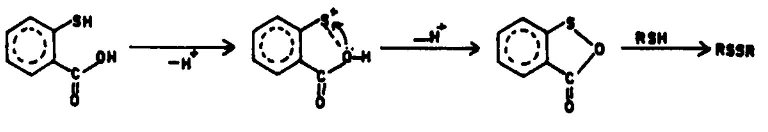
and

$$\text{intercept} = \frac{1}{k_1[H^+]}$$

With the help of these relations, the ratio $k_1[H^+]:k_2K[RSH]$ was calculated and it is found to be $10^{-6}: 1$ ($K = 10^{-5}$).[23] Under these conditions, $k_1[H^+]$ is negligibly small in the denominator in Equation (11) and thus, the simplified rate expression explains a zero order dependence on TSA as shown below:

$$-\frac{d[M']}{dt} = k_1[M'][H^+] \tag{14}$$

It has been reported by Danehy[24] that thiols having β-carboxylic group produce the cyclic sulfenic anhydride (II) and it seems that the cyclization through an electron deficient sulfur atom ($R\overset{\oplus}{S}H$) is favoured when [TSA] is sufficiently increased.



(15)

(I) (III)

Obviously the formation of RSSR inter alia ($R\overset{\oplus}{S}H$) will have a composite influence on the rate because of its influence on various specific velocity constants and k_2 may tend to decrease under these conditions so that $k_1[H^+]$ becomes comparable to $k_2K[RSH]$ in Equation (11). This explains a fractional order in TSA at higher concentrations.

This postulate may appear, at first sight, to be speculative but seems justified in the light of the variation in $[H^+]$ while employing low [TSA] (ca. 1.25×10^{-3} M). The rate constants obtained for such variations are given in Table VI. A plot of log k_1 against log $[H^+]$ gives a straight line with a slope of 0.1 which indicates that the order in H^+ ion is comparatively larger than the order observed at large concentrations of TSA.

TABLE VI
Rate constants at different $[H^+]$ at low [TSA]

$[H_2SO_4] \times 10^3$ M	$k_1 \times 10^4$ sec^{-1}
1.0	1.4
2.0	1.5
4.0	1.7
6.0	1.7
10.0	1.4
12.0	1.5

$[MB] = 2.5 \times 10^{-5}$ M $CH_3OH = 30\%$ (v/v)
$[TSA] = 1.25 \times 10^{-3}$ M $\mu = 42.0 \times 10^{-3}$ M

The proposed mechanism thus explains the principal kinetic features of the reaction excepting the transition in order of reaction in methylene blue from one to zero on adding externally the leuco base in the reaction mixture. It seems that in presence of leuco base, the protonated triplet state of MB tends to attain a limiting concentration as shown by step (2) in the reaction scheme and thus, causes a change in order of reaction in the oxidant. A further probe is expected to throw more light on this aspect of the reaction.

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