The Autoxidation of 5-Mercaptouracil and 5-Mercaptodeoxyuridine[1]

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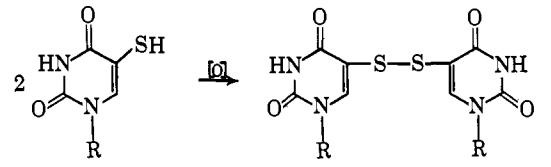
Abstract: 5-Mercaptouracil (I) and its N_1 deoxyribosides (III) undergo rapid autoxidation in aqueous solutions to the corresponding disulfides. This reaction was found to be dependent on the presence of trace amount of Fe^{3+} in the buffer solution. Addition of EDTA inhibited the oxidation and changed its over-all kinetic order. Addition of Fe salts at low substrate concentrations resulted in an initial rate increase, up to a certain "saturation" concentration of iron which was dependent upon, but much smaller than, the substrate concentration. The pH dependence of the oxidation rate closely paralleled that of the ionization of the SH group; ionization of the second proton of I (N_1–H) further increased, while that of III (N_3–H) decreased the rate. The kinetic data are consistent with the assumption that the autoxidation reactions depend on the formation of a 1:3 chelate between the Fe^{3+} and the thiolate ions and on its subsequent homolytic breakdown, to a Fe^{II} complex and a thiyl radical, the latter process being significantly promoted by the ionization of the N_1–H proton. Based on the proposed mechanistic scheme, it was possible to derive for the autoxidation reactions a general rate equation which satisfies all kinetic results.

An antimetabolite of thymine,[3] 5-mercaptouracil (I), undergoes extremely rapid autoxidation in aqueous solutions to the corresponding disulfide II. This reaction is so fast even in stoppered cuvettes that the correct ultraviolet spectra and pK_a constants of I could be determined only in the presence of a reducing agent, dithiothreitol.[4] The recently synthesized anomeric 5-mercaptodeoxyuridines[5] (III, α and β) are oxidized under the same conditions at appreciably slower rate.



I, R = H II, R = H
III, R = deoxyribosyl (α or β) IV, R = deoxyribosyl (α or β)

This paper presents a kinetic study of the autoxidation reactions of these compounds and of the effects of several variables upon the autoxidation rates. Because of certain structural properties of these compounds, the results obtained in this study seem to lend themselves to a more detailed mechanistic interpretation than those of previous studies reported in the literature concerning the autoxidation reactions of various other thiols.

Experimental Section

Materials. 5-Mercaptouracil (I) and 5-mercaptodeoxyuridine (III) were prepared by previously described methods.[4,5] Stock solutions of I and III were prepared in 5×10^{-4} *N* hydrochloric acid, under nitrogen atmosphere, and the dilutions were made with the appropriate buffer directly in the microcuvette, at the beginning of each experiment.

The following buffers were used for the rate studies at constant pH: (1) Tris-HCl, 0.25 *M*, pH 7.4 [tris(hydroxymethyl)aminomethane, purchased from Nutritional Biochemical Corp., was "enzyme grade," three times recrystallized, and HCl was Fisher certified reagent (Fe = 0.00001%, heavy metals as Pb = 0.00001%)]; (2) phosphate buffer, 0.05 *M*, pH 7.4 [prepared from KH_2PO_4 and K_2HPO_4, Baker's analyzed reagents (Fe = 0.0005%, heavy metals as Pb = 0.0002%)]. Both buffer solutions were prepared with deionized and distilled water; at the dilutions used, their Fe concentrations were approximately the same, *i.e.*, $1\text{–}2 \times 10^{-6}$ *M*. Parallel experiments, using the two different buffers, under otherwise identical conditions gave essentially the same results in the kinetic studies.

For the pH-dependence studies, the following buffers were used: between pH 3 and 5, 0.2 *M* acetate buffers; between pH 5 and 8, 0.1 *M* phosphate buffers; between pH 8 and 12, Sorensen's 0.1 *M* glycine buffers.

EDTA (ethylenediaminetetraacetic acid disodium salt) and $FeNH_4(SO_4)_2 \cdot 12H_2O$ were Fisher certified reagents.

Kinetic Measurements. The autoxidation rate studies were performed at 22° with a Beckman DU monochromator equipped with a Gilford multiple absorbance recorder, Model 2000, using 1.0 ml solutions in open microcuvettes (10-mm light path), and the change of absorbance with time was recorded at a given wavelength, usually at 290 mμ. The pH-dependent ultraviolet absorption spectra of I, II, III, and IV were previously reported.[4] For the purpose of the present study, the molar absorptivity differences between I and II and between III and IV (*i.e.*, equal to the change in absorbance that would result from the oxidation of 1 mole of thiol to 0.5 mole of disulfide) were determined for each pH and wavelength at which the experiments were conducted. These values were used as the basis of conversion of the ΔA/sec data, measured directly to the corresponding M sec^{-1}.

Figure 1 shows absorbance changes with and without EDTA for the "inhibited" reactions over a 3-hr time period. In all other experiments the reaction was followed only through the first 10-min period, and the "initial oxidation rates" were calculated from the slopes of the initial linear portions, or of the zero-time tangents, of the recorded curves. The estimated O_2 concentration at the beginning of the experiments was 2×10^{-4} *M* and did not appear to be limiting during the initial reaction period. In most experiments, the rates did not change during the first 5 min, and only at the highest oxidation rates ($>3 \times 10^{-7}$ *M* sec^{-1}) was a decline of the rate of oxidation observed 1–2 min after the beginning of the reaction.

The pH of the buffered solutions remained stable during the kinetic experiments within ±0.05 unit. Parallel experiments with either continuous or interrupted exposure of the solutions to the ultraviolet light showed no measurable difference in autoxidation rates.

All experimental values represent averages of duplicate runs with a reproducibility of ±5%.

(1) This investigation was supported by Grant CA-06695 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.
(2) To whom inquiries should be addressed.
(3) T. J. Bardos, R. R. Herr, and T. Enkoji, *J. Am. Chem. Soc.*, 77, 960 (1955).
(4) T. J. Bardos and T. I. Kalman, *J. Pharm. Sci.*, 55, 606 (1966).
(5) T. J. Bardos, M. P. Kotick, and C. Szantay, *Tetrahedron Letters*, 1759 (1966).

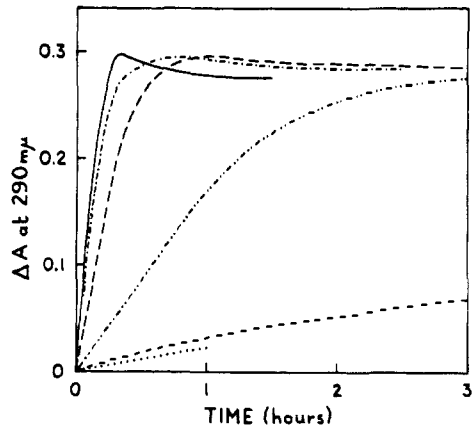


Figure 1. Change in absorbance of I (6.68×10^{-5} M, pH 7.4) at 290 mμ in the presence and absence of EDTA: ———, no EDTA; - - -, 1.0×10^{-5} M EDTA; — — —, 1.5×10^{-5} M EDTA; — · —, 2.0×10^{-5} M EDTA; - - - -, 1.0×10^{-4} M EDTA; · · ·, 1.0×10^{-3} M EDTA.

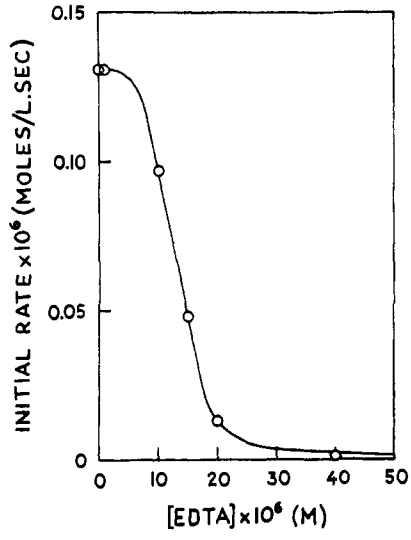


Figure 2. The initial rate of autoxidation of I (8.68×10^{-5} M, pH 7.4) as a function of the EDTA concentration.

Results and Discussion

Addition of EDTA to a buffered neutral or basic (pH 6–10) solution of I lowered the rate of autoxidation. Figure 1 shows the effect of various concentrations of EDTA on the autoxidation rate of I, as followed by the increase of absorbance at 290 mμ *vs.* time. In this experiment, the concentration of I was maintained constant, at 6.68×10^{-5} M, and the pH at 7.4. It can be seen that EDTA concentrations up to 1.0×10^{-5} M had only small effect, but with further increase of the concentration of the chelating agent, the autoxidation rate of I was drastically reduced, until it approached a limiting level at 1.0×10^{-4} M EDTA concentration. When the initial rates were plotted against the EDTA concentrations, an S-shaped "titration curve" was obtained (Figure 2).

Increasing the concentration of I reversed the rate-lowering effect of EDTA. Figure 3 shows the dependence of the initial rates of autoxidation upon the concentration of I. These experimental data satisfy the following equation

$$\text{initial rate} = k_{app}[\text{I}]^n$$

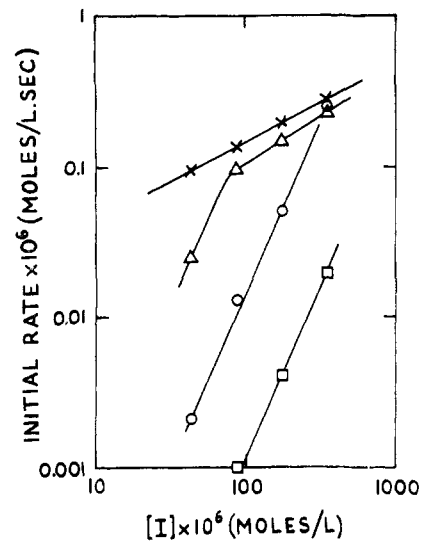


Figure 3. Dependence of the initial rate of autoxidation on the concentration of I, in the presence of various concentrations of EDTA: ×, no EDTA; Δ, 1.0×10^{-5} M EDTA; ○, 2.0×10^{-5} M EDTA; □, 4.0×10^{-5} M EDTA.

where k_{app} is the apparent rate constant of the over-all reaction, and the exponent n, given by the slopes of the linear log-log plots of Figure 3, expresses the "order" of the rate-determining reaction step(s) with respect to the substrate. It is of interest that n has a value of 0.5 for the "uninhibited" autoxidation (*i.e.*, in the absence of an *effective* concentration of EDTA), and a value of 2.3 for the "inhibited" process regardless of the EDTA concentration, provided that the latter is sufficient for producing a significant rate-lowering effect. Thus, the linear plots (Figure 3) corresponding to 2×10^{-5} and 4×10^{-5} M concentrations of EDTA are parallel to each other throughout the range of observation, while the plot corresponding to 1×10^{-5} M EDTA is parallel to the former only at low concentrations of I (when 1×10^{-5} M EDTA is still inhibitory), then shows a sharp break and approximates the slope of the "uninhibited" reaction when the substrate concentration becomes sufficiently high to reverse the inhibitory effect of this EDTA level. This change in slope (*i.e.*, in reaction order) indicates that the rate-determining step of the EDTA-inhibited oxidation is different from that of the "uninhibited" process.

The autoxidation of cysteine, glutathione, and other thiols was studied by numerous investigators, and a variety of mechanistic interpretations have been advanced.[6,7] However, considerable uncertainty still exists regarding the nature and mechanism of these reactions. It appears that the formation of a complex between the thiol and a metal ion is indispensible for the oxidation process. The rates of the autoxidation reactions were found, in most cases, to be proportional to the concentration of the complexing metal ions. With respect to the thiol, the rate of oxidation was usually reported to be zero order,[7] but first-order and intermediate (*e.g.*, "average 0.3-order"[8]) over-all kinetics

(6) D. S. Tarbell in "Organic Sulfur Compounds," Vol. 1, N. Kharasch, Ed., Pergamon Press, New York, N. Y., 1961, p 97.
(7) J. E. Taylor, J. F. Yan, and J. Wang, *J. Am. Chem. Soc.*, **88**, 1663 (1966).
(8) C. G. Overberger, K. H. Burg, and W. H. Daly, *ibid.*, **87**, 4125 (1965).

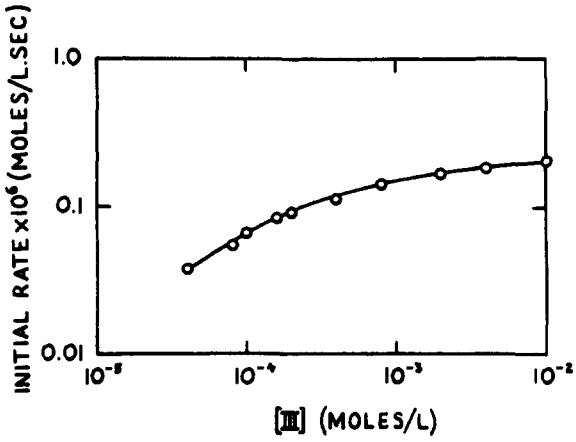


Figure 4. The initial rate of autoxidation of III as a function of the substrate concentration.

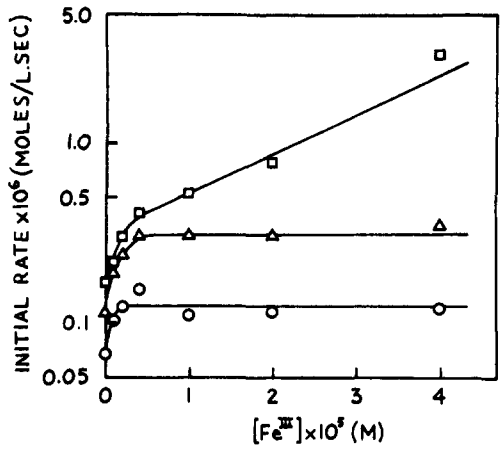


Figure 5. Effect of added Fe^{III} salt on the initial rate of autoxidation of III, at various substrate concentrations: ○, 1 × 10^{-4} *M* III; △, 4 × 10^{-4} *M* III; □, 2 × 10^{-3} *M* III.

were also observed. These experiments were usually conducted with introduction of molecular oxygen and addition of metal salts to the thiol solutions. Mechanisms involving thiyl radicals have been proposed by several authors.[6,8]

In our studies in which relatively low concentrations of I were used, the air and the trace metals (~10^{-6} *M*) present in the buffer solutions were apparently sufficient to permit the oxidation reaction to proceed at the observed high rate. The essential role of metal ions in the autoxidation of I was indicated by the inhibitory effect of EDTA, a powerful metal chelating agent. Moreover, the quantitative relationship seen in Figure 3 suggested that I reversed this inhibitory effect by being able to compete with EDTA and forming metal complexes that would catalyze the oxidation of 100 times larger concentrations (~10^{-4} *M*) of I. The change of reaction order from 0.5 to 2.3 in the presence of EDTA seemed to indicate that the formation of the "catalytic" metal complex (competitively inhibited by EDTA) requires two molecules of I, and that a third molecule of I may participate in the rate-determinating step without interference by this agent.

Since the low solubility of I did not permit the extension of this study to more concentrated solutions, and since, in the meantime, the N_1-2′-deoxyribosyl derivatives of I (III, α and β) have also become available[5] and showed much higher solubility, we continued our autoxidation studies with the latter compounds.[9] The initial rate of the autoxidation of III (Figure 4) in the 4 × 10^{-5} to 2 × 10^{-4} *M* concentration range followed the same, approximately 0.5-order, dependence on the thiol concentration as that of I (in the absence of EDTA), but the reaction approached zero order as the concentration of III was further increased. Figure 5 shows that at 1–4 × 10^{-4} *M* concentrations of III, addition of 1–4 × 10^{-5} *M* concentrations of Fe^{III} salt caused an up to two- to threefold increase of the initial oxidation rate, but addition of more Fe^{III} was without any further effect. However, at higher concentrations of III (2 × 10^{-3} *M*), the oxidation rate continued to show dependence upon the Fe^{III} salt concentration.

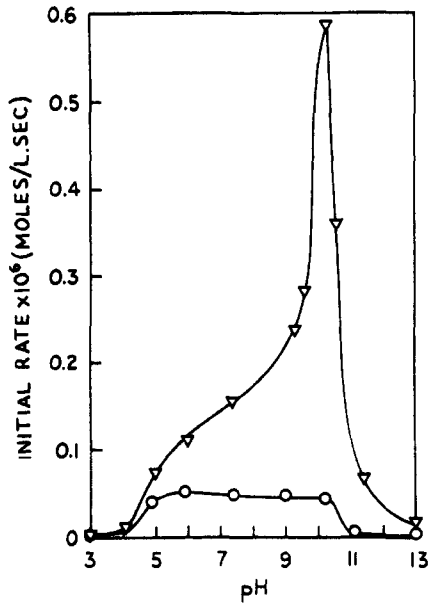


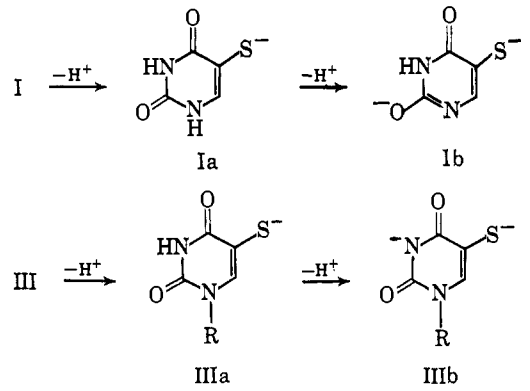
Figure 6. Effect of pH on the initial rates of autoxidation of I and III: ▽, I; ○, III.

The pH dependence of the autoxidation rates of I and III is shown in Figure 6. Both compounds are essentially stable to oxidation at pH <3.5, *i.e.*, in their nonionized forms.[10] The oxidation rates of both compounds rapidly increased between pH 4 and 6, *i.e.*, through the pH range corresponding to the first dissociation step which involves ionization of the SH group.[4] For III, the oxidation rate showed very slow decrease between pH 6 and 10.3 and then rapidly dropped, while in the case of I, the oxidation rate continued to increase, showed a steep rise between pH 9.5 and 10.4 (*i.e.*, through part of the second ionization step),[4] and then suddenly dropped. The second ionization step involves predominantly the $H_{(1)}$ proton for I and (of necessity) the $H_{(3)}$ proton for III;[4] consequently, the dianions of I and III may be represented by Ib and IIIb, respectively (each being one of the possible resonance structures)

The experimental results (Figure 6) thus indicate that (1) ionization of the thiol group is required for the rapid autoxidation of either I or III, and (2) that the

(9) The autoxidation rates of the two anomers were identical and, at pH 7.4, about one-third of that of I (see Figure 6). In subsequent studies, only the α anomer was used.

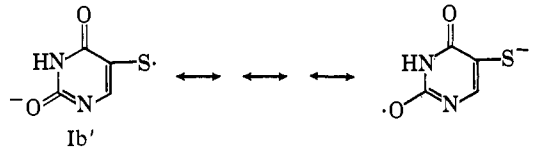
(10) The ionization constants for I and III are: pK_{a1} 5.3 and 5.0, pK_{a2} 10.6 and 10.5, respectively.[4]



Ia Ib

IIIa IIIb

dianion of I (Ib) is by far the most rapidly oxidized species, while the dianion of III (IIIb) appears to be slightly less reactive than the corresponding monoanion (IIIa). These facts are consistent with the assumption that the autoxidation of these compounds depends on the formation of intermediate Fe^{III}–thiolate complexes and on the subsequent breakdown of the latter with the transfer of one electron from the sulfur to the metal. The thiolate ions, Ia, Ib, IIIa, or IIIb, would react with the ferric ion to form five-membered chelates in which the metal is linked to the sulfur by ionic (or covalent) bond, and to the *o*-oxygen (4-O of the uracil nucleus) by coordination bond. A 1:2 Fe^{III}–thiolate complex, in which the two pyrimidine ligands can be coplanar, would be expected to form very rapidly and to be quite stable, while a 1:3 complex could be somewhat more labile and could either dissociate to the former (reversible equilibrium) or decompose homolytically, yielding a Fe^{II}–thiolate (1:2) complex and a thiyl radical. The latter process should be energetically most favored in the case of a Fe^{III}–Ib complex which on decomposition would yield a conjugated, resonance-stabilized radical ion (Ib′).



Ib′

In the case of the thiyl radicals which would arise upon decomposition of the Fe^{III} complexes of Ia, IIIa, and IIIb, respectively, there is no possibility of resonance between the sulfur and the 2-oxygen position of the uracil nucleus.

The participation of a Fe^{III}–thiolate complex in the oxidation reaction is further indicated by the observation that in relatively concentrated solutions of III, immediately upon the addition of a Fe^{III} (or Fe^{II}) salt, a strong blue color appears (λ_{max} 678 mμ) which fades on exclusion of air, but instantly reappears on aeration and finally disappears when the oxidation is completed to the disulfide (*cf.* ref 8). The blue color is visible only in the pH 4–11 range, *i.e.*, when the autoxidation rate of III is significant. There is no difference in the rapidity of appearance or in the intensity of the blue color whether Fe^{III} or Fe^{II} salt is added to the solution.

The following reaction scheme seems to be consistent with the above experimental results.[11]

$$USH \rightleftharpoons US^- + H^+ \quad (1)$$

$$Fe^{3+} + 2US^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [Fe^{III}(US)_2]^+ \quad (2)$$

$$[Fe^{III}(US)_2]^+ + US^- \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} [Fe^{III}(US)_3] \xrightarrow{k_3} [Fe^{II}(US)_2] + US\cdot \quad (3)$$

$$[Fe^{II}(US)_2] + 0.5O_2 + 2H^+ \xrightarrow{k_4} [Fe^{III}(US)_2]^+ + H_2O \quad (4)$$

$$2US\cdot \xrightarrow{k_5} USSU \quad (5)$$

Steps 1, 2, and 5 are very fast, and at pH 7.4 and in the absence of EDTA proceed virtually to completion. The oxidation step, 4, is also very fast as indicated by the rapid appearance of the blue color of the Fe^{III} complex upon the addition of a Fe^{II} salt (see above), and the concentration of oxygen does not seem to limit the *initial* autoxidation rates (see Experimental Section). Thus, step 3 appears to be rate determining. This reaction is first order with respect to the thiolate ion (US^-) but approaches zero-order kinetics in the presence of excess thiolate.

In the presence of EDTA or at relatively high OH^- concentration, respectively, the following reactions limit the availability of Fe^{3+} ions for step 2.

$$Fe^{3+} + EDTA^{4-} \rightleftharpoons [Fe^{III}\cdot EDTA]^- \quad (6)$$

$$Fe^{3+} + 3OH^- \rightleftharpoons Fe(OH)_3(aq) \rightleftharpoons Fe(OH)_3(ppt) \quad (7)$$

If we take into consideration these competing reactions, the general equation for the initial rate of autoxidation may be expressed in the following form[13]

$$\frac{d[0.5USSU]}{dt} = \frac{k_3[Fe_s^{III}]}{1 + \frac{K_1}{[US^-]}\left[1 + \frac{K_2}{[US^-]^2}\left(1 + \frac{[OH^-]^3}{K_{OH}} + \frac{[EDTA^{4-}]}{K_{EDTA}}\right)\right]} \quad (8)$$

where $[Fe_s^{III}]$ is the total concentration of iron in solution; $K_1 = (k_{-2} + k_3)/k_2$; $K_2 = k_{-1}/k_1$, *i.e.*, the over-all dissociation constant (actually, the product of the two-step dissociation constants) of the $[Fe^{III}(US)_2]^+$ complex; K_{OH} is the product of the three ionization constants of $Fe(OH)_3$ from eq 7; and K_{EDTA} is the dissociation constant of the $[Fe^{III}\cdot EDTA]^-$ complex from eq 6.

(11) USH = either I or III; USSU = II or IV; US^- = "free" monoanion Ia or IIIa; (US) = "metal-bound" monoanion Ia or IIIa; US· = thiyl radical. This reaction scheme, and the rate equations that follow, were written for the monoanionic species; however, the same equations with different constants and with appropriate modification of the ionic charges will apply also to the autoxidation of the dianions Ib or IIIb. The relative concentrations of the latter[12] are negligible at pH 7.4 where most of the studies were conducted, but at pH >9, the contribution of Ib to the over-all oxidation rate of I becomes very significant (see Figure 6).

(12) The fractional concentrations of the mono- and dianionic forms of I and III at any pH can be readily calculated from the pK_a data[10]

$$\frac{[US^-]}{[US]_{total}} = \frac{1}{1 + \frac{[H^+]}{K_{a_1}} + \frac{K_{a_2}}{[H^+]}}$$

$$\frac{[US^{2-}]}{[US]_{total}} = \frac{1}{1 + \frac{[H^+]}{K_{a_2}}\left(1 + \frac{[H^+]}{K_{a_1}}\right)}$$

where $[US]_{total}$ = all forms (ionized and nonionized) of I or III, respectively.

(13) Equation 8 is readily derived for conditions where k_4 and $k_5 >> k_3$, and there is still an excess of oxygen present (virtually all of the iron is in the Fe^{III} form), by assuming that a "steady state" exists for the concentration of the 1:3 complex, *i.e.*, $d[Fe(US)_3]/dt = 0$.

Since K_2 is very small, the initial rate in the absence of EDTA and at low OH^- concentration will become

$$\frac{d[0.5USSU]}{dt} = \frac{k_3[Fe_s^{III}]}{1 + (K_1/[US^-])} \quad (9)$$

and thus will approach zero order at high, and first order at very low US^- concentration ($0 < n < 1$) with respect to the substrate. The rate will increase proportionally with the amount of iron *in solution* (Fe_s^{III}) which is limited by the extremely low solubility of Fe^{III} at neutral or alkaline pH. The experimental data in Figure 5 are consistent with the assumption that the solubility of Fe^{III} is also dependent on the concentration of "chelating" thiolate ions. Figure 7, with the linearity of the reciprocal plots and the common intercept on the abscissa, supports the validity of eq 9.

In the presence of EDTA, owing to the very low value of K_{EDTA} ($\sim 10^{-25}$),[14] the $[EDTA^{4-}]/K_{EDTA}$ term will dominate the parenthesized expression in eq 8 even at a very low concentration of the chelating agent, and the initial rate will become

$$\frac{d[0.5USSU]}{dt} = \frac{k_3[Fe_s]}{1 + \frac{K_1}{[US^-]}\left[1 + \left(\frac{K_2}{[US^-]^2}\right)\left(\frac{[EDTA^{4-}]}{K_{EDTA}}\right)\right]} \quad (10)$$

which will approach third order with respect to $[US^-]$ if the $[EDTA^{4-}]/[US^-]^2$ ratio reaches a sufficiently high value. On increasing the concentration of the substrate at constant EDTA concentration, this ratio will drop sharply (quadratic dependence on $[US^-]$) and eq 10 will become similar to eq 9, *i.e.*, between zero and first order with respect to the substrate (see Figure 3).

A similar situation should exist if the OH^- concentration becomes sufficiently high (pH >10.2, see Figure 6). The initial rate will then suffer a sudden drop due to its third-power dependence on the OH^- concentration (see eq 8). Before reaching this critical OH^- concentration, *i.e.*, as long as the initial rate can be approximated by eq 9 which does not contain an $[OH^-]$ term, the pH dependence of the autoxidation rates would follow the changes in the relative contributions of the mono- and dianionic forms of the substrate.[12]

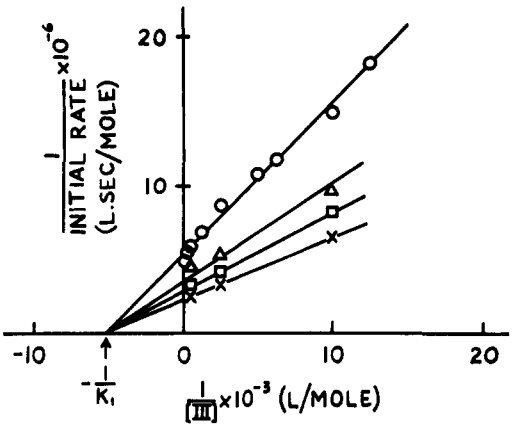


Figure 7. Double reciprocal plot of the initial rate of the autoxidation of III as a function of the substrate concentration, in the presence and absence of added Fe^{III}: ○, no Fe^{III} added; Δ, 1×10^{-6} *M* Fe^{III}; □, 2×10^{-6} *M* Fe^{III}; ×, 4×10^{-6} *M* Fe^{III} added. Intercepts on the ordinate give values of $1/k_3(Fe_s^{III})$ and the common intercept on the abscissa gives $-1/K_1$, as indicated. From these values, $k_1 = 1.9 \times 10^{-4}$ *M*, and $k_3 = 0.89$ sec^{-1}.

(14) A. I. Vogel, "Textbook of Quantitative Inorganic Analysis," John Wiley and Sons, Inc., New York, N. Y., 1961, p 418.