

ESR Studies of Free Radicals in Solution. IV. Kinetics of the Cysteinyl Free Radical Generated by Ceric Ion Oxidation*

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The rate of disappearance of the thiyl free radical generated upon oxidation of cysteine with ceric ion has been followed at pH 1.5 using an ESR stopped-flow technique. The kinetics of the reaction were studied over a range of concentration ratios in both aerated and de-aerated solutions. A dependence on RSH concentration was demonstrated, but a clear-cut reaction order could not be established. The rate data pointed out the actual marked complexity of an apparently simple reaction system. The rates of disappearance of the $RS\cdot$ radical were interpreted in terms of consecutive and concurrent reactions, taking into account the rates of formation, interaction of $RS\cdot$ with RSH, the possibilities of overoxidation by ceric ion, the possible effect of back reactions, and the influence of oxygen, as well as the classical dimerization mechanism. Various reaction mechanisms were postulated for the reaction of the thiyl free radical with RSH. The implications of these findings in relation to the mechanisms of radiobiological processes are discussed.

INTRODUCTION

Considerable interest in thiyl free radicals as intermediates in radiobiological processes has led to a number of studies on their generation, identification, and reactivity by fast reaction techniques. Thiyl free radicals have been identified by ESR continuous-flow methods in aqueous solution (1-9) and detected by absorption spectroscopy following pulse radiolysis generation (10-16). Various studies on the kinetics of disappearance of $RS\cdot$ radicals generated by the pulse radiolysis technique have been published (16-18). The results of this method suggest a short-lived species, whose dimerization rate constant is on the order of 10^9 - $10^{10} M^{-1} sec^{-1}$. While the exact mechanism of action of thiyl free radicals as radioprotective agents has not been clearly defined, it has been postulated that they are unreactive species which do not cause further radiolytic damage (19-24). The above reaction rate constants are somewhat inconsistent with such a mechanism, and in the present study we have attempted to investigate the rate of disappearance of thiyl free radicals by the direct observation afforded by ESR stopped-flow techniques.

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In preceding papers (1, 4, 5), we described the nature of the thiyl and related free radical signals generated upon oxidation of a variety of primary, secondary, and tertiary thiols with ceric ion solution. These signals were observed under steady-state flow conditions by ESR spectroscopy and were ascribable to at least six different species, designated by the letters "A", "B", "C", "D", "E", and "H" with g -values at 2.0106, 2.0186, 2.0032, 2.0151, 2.0097, and 2.0050, respectively. The "A" species was conclusively identified as the $RS\cdot$ (thiyl) free radical (2), and the "B" species was tentatively assigned to an $RSO_2\cdot$ structure (3). The "C" and "D" signals were only tentatively ascribed to carbon and peroxy radical species, respectively, while the nature of the "E" species remains uncertain, although various possible structures have been postulated (3). Subsequent studies on the oxidation of hydroxy thiols (6) led to the identification of the "H" species as the alkoxy free radical, $RO\cdot$, formed in an intramolecular hydrogen transfer reaction. In this paper, we examine the results of ESR stopped-flow measurements on the rate of disappearance of the thiyl free radical generated upon oxidation of cysteine with ceric ion and report on additional properties of the "C" species and its possible relationship to the $RS\cdot$ free radical.

EXPERIMENTAL

The ESR stopped-flow system used, which was specially designed for ESR kinetic studies performed in conjunction with signal-averaging techniques, has been described in detail elsewhere (25). A flow rate of 5.4 ml/sec was generally used with occasional tests at 7.5 ml/sec. ESR spectra were recorded with a Varian Model E-3 ESR spectrometer with associated 4-in. magnet, which operated at an X-band microwave frequency of 9.5 GHz and a modulation frequency of 100 kHz. Each sample was recorded at a modulation amplitude of 2.5 gauss and a microwave power of 200 mW. There was no evidence of power saturation of the two spectral subcomponents studied in this work. Qualitative spectra of the complete cysteinyl radical triplet signal were recorded on 50-gauss scans, whereas for quantification of spin concentration the central peak of the triplet and also the "C" signal were recorded individually on 20-gauss scans. A 1-sec filter was routinely used for scanning experiments.

In order to record kinetic decay curves, the ESR spectrometer was interfaced with a Hewlett Packard 5400A Multichannel Analyzer for signal-averaging by means of a Varian E-227 adaptor assembly and additional electronic circuitry designed for this purpose. These interfaces permitted stored information to be read out on the E-3 XY recorder. The E-3 spectrometer filter was turned off and a passband to optimize signal-to-noise (S/N) ratios was selected with an external Krohn-Hite model 3200 RC filter. A 40-Hz low-pass filter yielded optimum S/N improvement. To record decay curves, the spectrometer was set at the magnetic field value which represented the maximum of the first-derivative curve of the signal peak, and the stopped-flow system was activated. The output of the spectrometer, which gives a signal proportional to the concentration of the transient species being observed, was monitored by triggering the signal averager at the onset of each experiment, stopping the flow, and instantaneously recording the disappearance of the signal as a function of time. System triggering was accomplished by means of a line synchronous solenoid programmer (LSSP) which has been previously described (25). The LSSP permits a variable restore time for establishment of steady-state conditions while flowing, followed by a variable delay time in which the

initial intensity of the signal is defined. The beginning of the delay time period marks the triggering of the signal averager, while at the end of the delay period, closing of the solenoid valves is triggered. At the end of the selected sweep time, a preset repetition of the specified cycle occurs. The LSSP parameters normally used were sweep times of 512, 1024, or 2048 msec, delay times one-tenth of the corresponding sweep times, and restore times of 400 msec. A minimum of 128 repetitions of the sweep cycle was normally averaged to produce a single decay curve. In a typical kinetic run, a tenfold spectrometer gain increase over that used for continuous-flow studies was employed to yield the maximum S/N ratios.

Fit of the data to first- and second-order kinetic equations and other mathematical manipulations of kinetic parameters were calculated by means of a Mathatronics 480 desk model computer. Computer programs developed for ESR data treatment will be published in detail elsewhere.

All reagent solutions were prepared in distilled water. L-Cysteine · HCl was obtained from Fox Chemical Co., lot No. F-1011, and used without further purification. Cystine was from Eastman Organic Chemicals. The ceric salt used was ceric potassium nitrate, $\text{CeK}_2(\text{NO}_3)_6$, purchased from K & K Laboratories, while cerous chloride, CeCl_3 , 99.9% anhydrous, was obtained from Alfa Inorganics. A stock solution of 1 *N* HCl was prepared from 37% hydrochloric acid, Mallinckrodt analytical reagent, for subsequent dilutions. Concentrations of cysteine solutions utilized ranged from 0.02 to 0.32 *M*, while those of $\text{CeK}_2(\text{NO}_3)_6$ were 0.005–0.16 *M*. Concentrations of CeCl_3 greater than 0.02 *M* could not be used due to the limited solubility of this salt. The pH of the reagent solutions was adjusted so that the pH of the reaction mixture would be 1.4–1.5 by addition of the appropriate amount of HCl to each. The concentration of $\text{RS}\cdot$ radicals has been shown (26) to reach a maximum at this pH in this reaction system. All solutions were prepared just prior to use. It was necessary to record the ESR spectra as soon as possible after dissolution of reagents to avoid auto-oxidation (27, 28) of thiol and auto-reduction (29, 30) of the ceric solution. For experiments performed on de-oxygenated or O_2 -saturated solutions, solvent was placed in the flow system and bubbled with N_2 or O_2 gas for 40 min prior to addition of reagents. O_2 concentration was monitored (25) by an IBC thermocompensated probe and discrete O_2 analyzer.

All pH measurements were made with a Corning Model 10 pH meter, equipped with a semi-micro combination electrode. A flow method devised to determine the pH of the reaction mixtures exiting from the ESR flat cell has been described in related work (26). Spectra were recorded when reaction mixtures were at a temperature of $23.2 \pm 0.4^\circ\text{C}$. Temperatures were detected by a YSI 404 tubular-glass temperature flow probe in the sample stream (25).

$\text{VO}_2\text{SO}_4 \cdot 2\text{H}_2\text{O}$ crystals, obtained from Fisher Scientific Co., were purified (31) by repetitive washings with methanol and drying under vacuum. Standard VO_2SO_4 solutions (3.6974×10^{-4} *M*) were prepared for use as ESR spin concentration primary standards from the purified crystals dissolved in 1 *M* H_2SO_4 . The accurate determination of radical concentrations is a difficult problem in ESR spectroscopy since the best known primary solutions give a reproducibility of 10%, whereas the absolute accuracy may be in error by 50% (32, 33). The procedure for estimating radical concentrations, based on double integration of the ESR first-derivative signal (34), utilizing a computer analysis to facilitate baseline correction procedures, will be published elsewhere. The

VO^{2+} ion exhibits an 8-peak spectrum, where the area of each peak is approximately equal. An average of the second, third, and fourth peaks of this spectrum, chosen for double integration, was compared to the central peak of the cysteinyl triplet spectrum or to the "C" singlet, recorded at each concentration ratio used to generate the kinetic decay curves, in order to determine the $\text{RS}\cdot$ or "C" spin concentrations represented.

RESULTS AND DISCUSSION

When cysteine·HCl is oxidized by $\text{CeK}_2(\text{NO}_3)_6$ under continuous-flow conditions at pH 1.5 at various concentration ratios, the ESR spectra consist of a triplet at $g = 2.0106$ characteristic of the $\text{RS}\cdot$ free radical and a singlet at $g = 2.0032$ for the "C" species. These spectra are illustrated in Fig. 1, and the steady-state spin concentrations

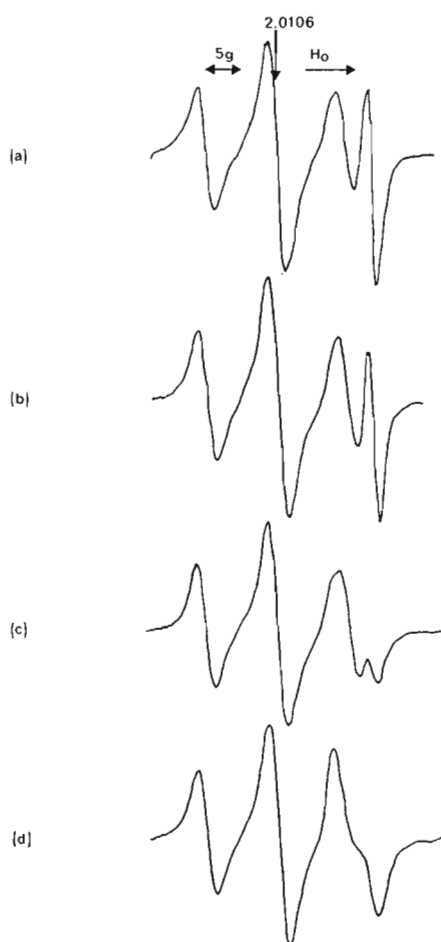


FIG. 1. First-derivative ESR spectra of solutions obtained by mixing (a) 0.32 *M* cysteine·HCl with 0.02 *M* $\text{CeK}_2(\text{NO}_3)_6$ in 0.2 *N* HCl; (b) 0.02 *M* cysteine·HCl in 0.225 *N* HCl with 0.02 *M* $\text{CeK}_2(\text{NO}_3)_6$ in 0.2 *N* HCl; (c) 0.2 *M* cysteine·HCl in 0.2 *M* HCl with 0.16 *M* $\text{CeK}_2(\text{NO}_3)_6$; and (d) 0.12 *M* cysteine·HCl in 0.15 *N* HCl with 0.12 *M* $\text{CeK}_2(\text{NO}_3)_6$ in 0.1 *N* HCl, all at flow rate of 5.4 ml/sec. Mod. amp. 2.5 gauss.

observed for the $RS\cdot$ radical under various experimental conditions are listed in Table 1. The spin concentration of "C" is always considerably less than that of the $RS\cdot$ radical. For example, at a concentration ratio of 2/2, the spin concentration of "C"

TABLE 1

PSEUDO-FIRST-ORDER RATE CONSTANTS AND RATE DATA OBTAINED FOR DECAY CURVES OF THE THIYL ($RS\cdot$) FREE RADICAL FORMED UPON REACTION OF CYSTEINE· HCl WITH $CeK_2(NO_3)_6$ AT pH 1.5 AT VARIOUS CONCENTRATION RATIOS

[RSH]/[Ce ⁴⁺] ratio <i>M</i> × 10 ²	Steady-state [RS·] <i>M</i> × 10 ⁶	Pseudo-first-order <i>k</i> ' _{obs} , sec ⁻¹	Tests for <i>k</i> ₂ , <i>M</i> ⁻¹ sec ⁻¹	Initial rates $\frac{dRS\cdot}{dt}$ <i>M</i> /sec × 10 ⁵
2/0.5	2.76	6.57 ± 1.51	—	2.05
2/2	6.01	6.84 ± 0.34	—	3.88
2/4	6.56	6.35 ± 1.36	157.8	4.94
2/6	10.73	8.73 ± 1.32	145.5	9.25
2/8	11.94	8.61 ± 0.81	107.7	8.99
2/16	15.13	9.63 ± 0.39	60.2	12.66
4/2	6.74	9.77 ± 0.65	—	5.34
6/2	7.08	11.20 ± 1.40	186.7	5.97
8/2	6.91	15.25 ± 1.12	190.6	6.52
16/2	5.89	22.06 ± 3.22	137.9	8.99
32/2	5.27	28.15 ± 7.50	88.0	9.36
4/4	6.46	10.06 ± 0.38	—	4.70
8/8	12.92	13.34 ± 1.37	—	14.32
12/12	14.37	16.63 ± 1.21	—	16.53
16/16	16.02	20.31 ± 0.91	—	21.29

is $3.06 \times 10^{-7} M$, while that of $RS\cdot$ is $6.01 \times 10^{-6} M$ (Fig. 1b). In Table 1 it is seen that when the cysteine concentration was held constant at 0.02 *M* and [Ce⁴⁺] was raised eight-fold, an approximately fivefold rise in $RS\cdot$ spin concentration was observed. The apparent "C"/ $RS\cdot$ ratio decreased with increasing [Ce⁴⁺], but the "C" singlet was seen to merge asymmetrically with and broaden the third peak of the $RS\cdot$ triplet (Fig. 1c). However, when [Ce⁴⁺] was held constant at 0.02 *M* and [RSH] was increased 16-fold, only a small increase in [RS·] was exhibited, which then began to decrease at highest [RSH], while the "C" singlet became progressively relatively larger (Fig. 1a). When both [RSH] and [Ce⁴⁺] were increased equally eight-fold from 2/2 and 16/16 ratios, [RS·] was found to rise to a value only slightly more (16.02×10^{-6}) than at a ratio of 2/16 (15.13×10^{-6}). At the same time, the "C" signal broadened the third peak of the $RS\cdot$ triplet and eventually merged with it, so that "C" was impossible to measure alone (Fig. 1d).

Typical decay curves for the $RS\cdot$ radical obtained by the ESR stopped-flow method shown in Fig. 2 correspond to the reaction conditions described for Fig. 1. Preliminary analysis of decay kinetics was performed by use of the integrated kinetic equations,

$$k = \frac{1}{t} \ln \left(\frac{I_0}{I_x} \right) \quad \text{and} \quad k = \left(\frac{I_0}{I_x} - 1 \right) \left(\frac{1}{I_0 t} \right),$$

where I_0 , the initial intensity, is proportional to $RS\cdot$ steady-state spin concentration, and I_x is the amount remaining at time t , for first- and simple second-order rate constants. These equations were programmed and rate constants were computed by means of the Mathatronics desk computer. Pseudo-first-order rate constants were found to describe

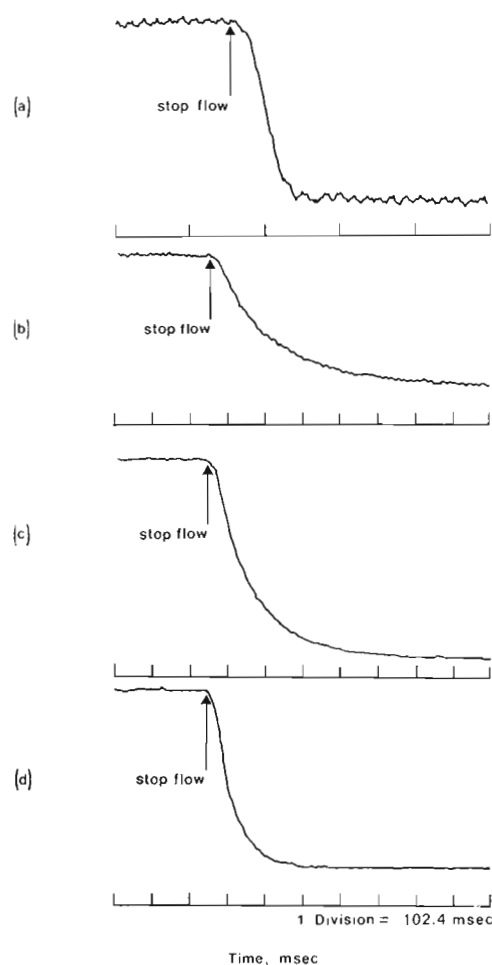


FIG. 2. Direct recording of thiol free radical signal amplitude decay as a function of time in msec at pH 1.5. Decay occurs upon stopping the mixing of the cysteine and $CeK_2(NO_3)_6$ concentrations indicated for the corresponding (a-d) ESR triplets shown in Fig. 1, when the magnetic field is positioned at maximum amplitude of the central peak, all at 5.4 ml/sec. Sweep times: (a) 512 msec, (b-d) 1024 msec. Each curve represents an averaging of 128 time scans.

the decay curves obtained for all reaction conditions examined, apparently since the concentrations of initial reagents are generally about four orders of magnitude greater than the steady-state level of the $RS\cdot$ radical produced. Those for the variation of $(RSH)(Ce^{4+})$ ratios are given in Table I, along with the initial rates of disappearance $-dRS\cdot/dt$, determined at a point 12.85 msec after flow has stopped.

In experiments performed to test the influence of molecular O_2 , the rates of disappearance of $RS\cdot$ were seen to be unaffected whether the reagent solutions had 0.2

ppm O_2 after bubbling with N_2 , 8.4 ppm in the presence of air, or greater than 40 ppm after bubbling with O_2 . O_2 apparently exerts some influence on the rate of formation, since $[RS\cdot]$ is generally slightly increased when generated in O_2 -saturated solutions. The "C" species, which was also analyzed for pseudo-first-order rate constants, generally disappeared or was reduced substantially in intensity in O_2 -saturated solutions. In order to test for the participation of possible back-reactions, the influence of excess $CeCl_3$ and/or cystine added to the initial reagent solutions was examined. Again no effect on $-dRS\cdot/dt$ was observed. However, in the presence of 0.02 M $CeCl_3$, the "C"/ $[RS\cdot]$ ratio was always noticeably reduced. The addition of excess KCl to solutions of low $[RSH]/[Ce^{4+}]$ ratios demonstrated that increased ionic strength was not responsible for the rate changes observed at higher RSH and Ce^{4+} concentrations.

Tests for the Determination of Reaction Order

Since the trend of pseudo-first-order rate constants was to increase as the initial concentration of reagents was raised, the data were treated with Ostwald's method of isolation (35-37). In the present system, $RS\cdot$ concentration is always small relative to that of the initial reagents, RSH and Ce^{4+} ion, which may thus be taken as effectively constant. The rate equation thus has the approximate form:

$$-dRS\cdot/dt = k_{obs}[RSH]^n [Ce^{4+}]^m [RS\cdot]^n$$

and since

$$[Ce^{4+}] \gg [RS\cdot] \quad \text{and} \quad [RSH] \gg [RS\cdot],$$

then

$$-dRS\cdot/dt = k'_{obs}[RS\cdot],$$

where

$$k'_{obs} = k_2[Ce^{4+}] \quad \text{or} \quad k'_{obs} = k_2[RSH].$$

When applied to the increasing $[Ce^{4+}]$ series, the results only indicate that the disappearance of $RS\cdot$ is not first order in ceric ion as the values of k_2 obtained (Table 1) were seen to decrease with increasing $[Ce^{4+}]$. If $-dRS\cdot/dt$ were independent of ceric ion, then a constant k_2 should have been found. In the case of the increasing cysteine series, a dependence on $[RSH]$ was demonstrated. Since the values for k_2 (Table 1) also decreased with increasing RSH, a clear-cut first-order dependence on RSH could not be demonstrated. Thus, the different conditions of isolation indicate that the reaction is complex, as its mechanism may be influenced by concentration changes, and suggest that an interaction of $RS\cdot$ with RSH appears to occur at high RSH concentrations.

In order to further examine the reaction with respect to $RS\cdot$ concentration, the differential method in which the order of a reaction, n , may be found as the slope of a plot of $\log(-dRS\cdot/dt)$ versus $\log[RS\cdot]$ was utilized. Out of the plots for the various conditions examined, only those for equal initial $[RSH]$ and $[Ce^{4+}]$ exhibited linearity, with slopes ranging from 0.63 to 1.35, indicating a fractional rate order. It is clear that the reaction has some complicating feature and the kinetics are not a simple second-order function of $RS\cdot$ concentration.

Since the data did thus not appear to obey any of the simple rate laws, the method of initial rates was applied (36). When the logs of initial rates are plotted against logs of initial concentrations for a series of initial concentrations, a straight line will be obtained if a reaction is not complex, and the slope will be the order. For the three series of initial

[RSH]/[Ce⁴⁺] ratios listed in Table 1, the results are illustrated in Fig. 3. In the increasing [Ce⁴⁺] series, the plot was fairly linear with a slope of 1.23, whereas for the increasing [RSH] series, the plot exhibited a wide curvature. In the plot representing the increase of both [Ce⁴⁺] and [RSH], the best agreement with linearity was attained, the line exhibiting a slope of 1.82, apparently an average reaction order. This graphical approach again demonstrates that the disappearance of the RS· radical does not fit a simple kinetic description, and an interaction of the RS· free radical with RSH is indicated.

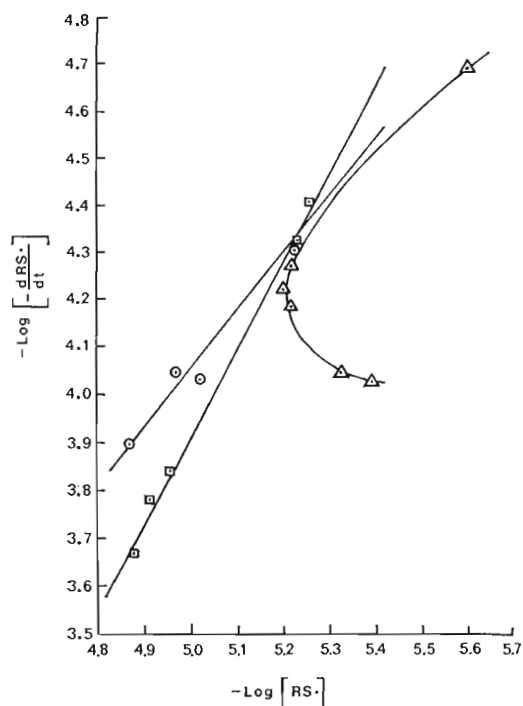
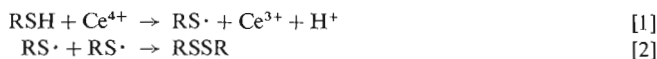


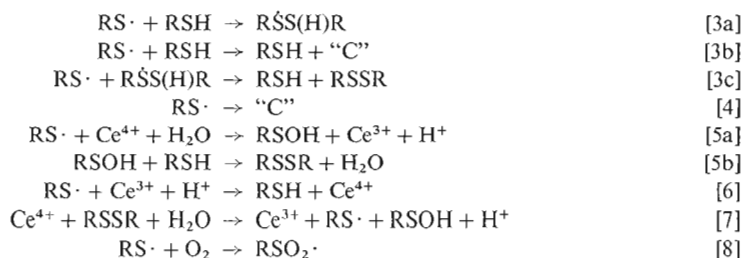
FIG. 3. Log-log plot of initial rates vs concentration of the thiyl free radical at various concentration ratios of cysteine/Ce⁴⁺ ion: (O) excess Ce⁴⁺, (Δ) excess cysteine, (□) equal concentrations of each.

The results of all of these kinetic treatments suggest that the apparent rate constants for the RS· disappearance reaction do not describe a single process, but rather are a perhaps complicated function of many rate constants. As there are many reactions which do not allow assignment of an order, the disparity of observed trends has provided a clue to the actual marked complexity of an apparently simple reaction system, and has suggested further analysis in terms of competitive and/or consecutive reactions.

Possible Reaction Mechanisms

Because of the complex nature of the kinetics of the cysteine thiyl free radical indicated by all of the standard tests for reaction order, further analysis was based upon consideration of the following series of reactions:





1. Formation

Reaction [1] represents the formation of $\text{RS}\cdot$ free radicals in a one-electron transfer reaction. The rate of disappearance of ceric ion, $-d\text{Ce}^{4+}/dt$, has been followed spectrophotometrically in the oxidation of α -mercaptocarboxylic acids (38) in H_2SO_4 . An intermediate complex involving metal-ion-sulfur coordination was discussed, but no significant concentration of complex was indicated.

In preliminary studies in this laboratory, the rate of appearance of the cysteinyl free radical had been examined by monitoring $-d\text{Ce}^{4+}/dt$ spectrophotometrically at 416 nm, using a manual stopped-flow system, and found to be first order in ceric ion. However, the second-order rate constants calculated from the pseudo-first-order plots varied with $[\text{RSH}]$, indicating a more complex dependence on cysteine. These rate "constants" ranged from 1500 to 195 $M^{-1} \text{sec}^{-1}$ as cysteine was increased from 0.0016 to 0.08 M at a constant $[\text{Ce}^{4+}]$ of 0.003 M .

In a study of $-d\text{Ce}^{4+}/dt$ presently in progress, which is being carried out spectrophotometrically by Samuni (39), the general validity of our previous very preliminary data has been confirmed. The reaction of cysteine, as well as that of glutathione and thioglycolic acid, is always first order in ceric ion, but varies in a very complex manner with the concentration of RSH. At high (RSH), a second-order dependence on $[\text{RSH}]$ was observed. At high cysteine (0.1–0.01 M), the second-order rate "constant" calculated was 600 $M^{-1} \text{sec}^{-1}$. It increased to 1200 $M^{-1} \text{sec}^{-1}$ with 0.0001 M cysteine and to 1500 $M^{-1} \text{sec}^{-1}$ at still lower $[\text{RSH}]$. For comparison, values of 260–650 $M^{-1} \text{sec}^{-1}$ were obtained for glutathione.

Czapski (40) has emphasized the necessity of considering the rate of appearance of free radicals generated by metal ion oxidations in ESR stopped-flow systems before the decay kinetics can be properly analyzed. He demonstrated that in many studies of organic free radicals produced by the $\text{Ti}^{3+}-\text{H}_2\text{O}_2$ system, it was incorrectly assumed that formation of the radical was complete prior to entry of the solutions into the cavity, and erroneous kinetic analyses resulted. For the present system, estimations of k_1 , the rate constant for formation of $\text{RS}\cdot$, were made from an expression relating the steady-state concentration, R_{ss} , of a free radical to its appearance and dimerization rate constants, derived by Czapski, for reactions which are slow and still proceeding in the cavity:

$$R_{ss} = \left(\frac{k_1[A_0][B_0]e^{-k_1A_0t}}{2k_2} \right)^{\frac{1}{2}}$$

A $2k_2$ of $1.0 \times 10^{10} M^{-1} \text{sec}^{-1}$, reported in pulse radiolysis work (18) for the $\text{RS}\cdot$ radical was assumed and k_1 was calculated using the $\text{RS}\cdot$ concentrations in Table I for several of the initial reagent concentrations where $A_0 \gg B_0$ at the observation time,

t , of 14.1 msec. The estimates yielded a k_1 on the order of 200–1000 $M^{-1} \text{ sec}^{-1}$ in agreement with the preliminary spectrophotometric data. All these lines of evidence thus indicate that the present system must be analyzed in terms of consecutive reactions, since the generation of $\text{RS}\cdot$ by ceric ion appears to be relatively slow in the context of radical reactions and it is likely that formation still is taking place in the cavity at the point of observation where decay kinetics are recorded.

2. Dimerization

Reaction [2] represents the generally accepted bimolecular dimerization mechanism for disappearance of the $\text{RS}\cdot$ radical. A transient absorption at 330 nm, detected by kinetic absorption spectrophotometry, has been assigned to $\text{RS}\cdot$ radicals produced from protonation of the RSSR^- radical anion and/or directly from reaction of H atoms with disulfides during pulse radiolysis. The decay kinetics (16–18) for a variety of $\text{RS}\cdot$ radicals indicated $2k_2$'s ranging from $3.4 \times 10^9 M^{-1} \text{ sec}^{-1}$ for glutathione to $1.0 \times 10^{10} M^{-1} \text{ sec}^{-1}$ for cysteine at pH 1.0. Second-order kinetics were also observed for $\text{RS}\cdot$ radicals generated from reaction of RSH with hydroxyl radicals (18) under conditions of “relatively high” $[\text{RS}\cdot]$ ($5 \times 10^{-5} M$) and “relatively low” ($5 \times 10^{-4} M$) $[\text{RSH}]$. (The highest $[\text{RS}\cdot]$ in the present study was $1.6 \times 10^{-5} M$.) The $2k_2$ found at pH 6.4 for cysteine ($3.4 \times 10^9 M^{-1} \text{ sec}^{-1}$) and at pH 4.3 for glutathione ($1.5 \times 10^9 M^{-1} \text{ sec}^{-1}$) was seen to be somewhat lower than those at pH 1.0, with an estimated error in the values of $\pm 15\%$.

The kinetics of oxidation of cysteine, glutathione, and other thiols by a variety of oxidizing agents (41–44) and by air (auto) oxidations (27, 45) have been studied, and although mechanisms were proposed involving rapid dimerization of intermediate thyl radicals to account for the overall kinetics, definitive second-order rate constants for this reaction were not reported.

3. Reaction of $\text{RS}\cdot$ with RSH

Reactions [3a–c] are proposed to account for the observed dependence of the rate of disappearance of the thyl free radical on RSH concentration. Reaction [3a] represents the formation of the hydrothiothyl free radical, $\text{R}\dot{\text{S}}\text{S}(\text{H})\text{R}$. This species has been postulated as an intermediate, produced either from protonation of RSSR^- radicals formed by direct attack of e_{aq}^- on RSSR , or from reaction of H atoms with disulfides (46, 47) in acidic solutions, and has a lifetime of $\ll 10^{-7} \text{ sec}$ (17). If $\text{R}\dot{\text{S}}\text{S}(\text{H})\text{R}$ is formed in the present system at pH 1.5, and if proton abstraction by $\text{RS}\cdot$ occurs faster than the dissociative rate of $\text{R}\dot{\text{S}}\text{S}(\text{H})\text{R}$, then reaction [3c] may provide an alternate pathway for disappearance of $\text{RS}\cdot$ and formation of the product cystine.

The postulation of reaction [3b] as a possible path for the disappearance of $\text{RS}\cdot$ is suggested by the characteristics of the “C” signal. While a definitive structural assignment for “C” has not yet been possible due to its lack of hyperfine splitting, and therefore it may be formed in a reaction independent of $\text{RS}\cdot$ (a parallel reaction), the following evidence all indicates that it is formed in a secondary reaction, i.e., is a daughter species of the $\text{RS}\cdot$ radical.

(a) The “C” singlet decreases in intensity with increasing flow rates, while the $\text{RS}\cdot$ triplet increases, in the range 1.0–7.5 ml/sec (corresponding to a mixing chamber

dead time of 27–3.6 msec), demonstrating that “C” is formed at a later point in time than the $RS\cdot$ radical.

(b) Temperature studies (unpublished results) over the range of -5 to $+68^\circ\text{C}$ have shown a marked increase in “C” singlet intensity, paralleled by a drop in $RS\cdot$ signal at the highest temperatures. At -5°C , an intense $RS\cdot$ triplet is recorded, while the “C” species is no longer detectable, suggesting that the longer lifetimes afforded by lower temperatures enhance the $RS\cdot$ signal by increasing the stability of the $RS\cdot$ radical so that “C” cannot form appreciably to the level of detection. Some preliminary results, illustrating these trends, are plotted in Fig. 4.

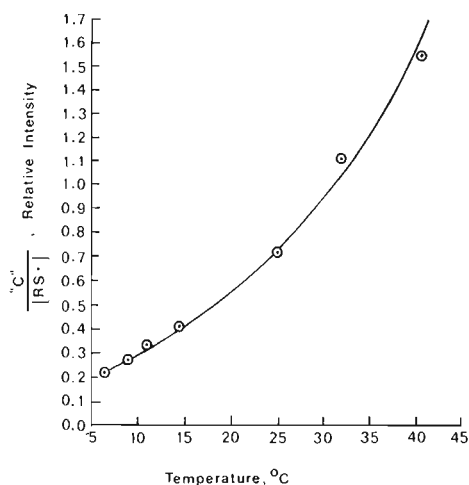


FIG. 4. Variation of relative intensities of the “C”/ $RS\cdot$ ESR signals with increasing temperature for a reaction mixture 0.02 M each in cysteine and $CeK_2(NO_3)_6$, comparing peak-to-peak height of the “C” singlet to that of the second peak of the $RS\cdot$ triplet.

(c) If flow rates can be accelerated sufficiently so that a reaction mixture is swept from the flow cell before the formation reaction can yield a detectable steady-state level of free radicals, then the rate of formation can be studied by stopped-flow ESR spectroscopy (48–50). This has not yet been feasible for observation of the rate of appearance of $RS\cdot$ since at the fastest flow rate available, 7.5 ml/sec, its signal is still observed in good intensity. Under these conditions, an “apparent” appearance curve may be recorded, which is a function of flow rate, rather than rate of formation alone, as seen in Fig 5a, which shows a sweep trace of $RS\cdot$ signal intensity as a function of time during a flow–stop–flow–stop sequence at 7.5 ml/sec. The steady-state concentration is first attained during continuous flow. Then flow is stopped and the decay curve is obtained. When the flow path is opened again, the “apparent” appearance is observed, which returns to the steady-state level. Figure 5b is an analogous scan for “C” when generated in good intensity during continuous flows at 5.4 ml/sec. When the flow rate is raised to 7.5 ml/sec, the steady-state concentration of “C” is decreased from 3.06×10^{-7} to $1.7 \times 10^{-7} M$. In Fig. 5c the influence of flow rates too fast for realization of the maximum steady-state radical concentration on stopped-flow kinetics can be seen.

Thus, although the real formation curves cannot yet be accurately measured, the continuing formation of "C" at a point in time later than that of the $RS\cdot$ radical and also the visible influence of incomplete appearance reactions on the drop-off point of the decay curve for disappearance have been clearly demonstrated.

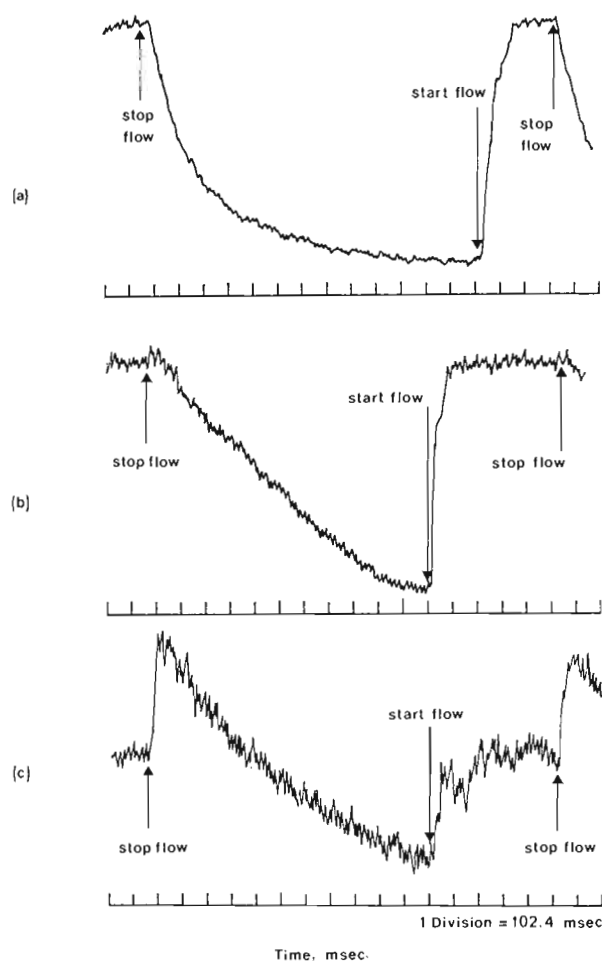


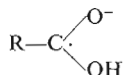
FIG. 5. Curves recorded for the decay and "apparent" formation of (a) the thiyl free radical, and (b, c) the "C" species, observed during start-stop-flow sequences, mixing 0.02 *M* cysteine·HCl in 0.225 *N* HCl with 0.02 *M* $CeK_2(NO_3)_6$ in 0.2 *N* HCl at flow rates of (a) 7.5, (b) 5.4, and (c) 7.5 ml/sec. All at sweep times of 2048 msec. Each curve was time averaged (a) 128, (b, c) 196 sweeps.

(d) When the reagent solutions are saturated with O_2 , the "C" signal is no longer detectable, whereas the $RS\cdot$ triplet appears in greater intensity. The "C" species may be formed and react rapidly with O_2 , generating a peroxy free radical, as suggested by previous studies of penicillamine. Since the penicillamine $RS\cdot$ ESR signal is a singlet, the "C" and "D" free radicals also generated (which absorb at nearby magnetic field) are more clearly recorded than in the presence of the cysteine triplet. As penicillamine solutions are saturated with O_2 , "C" disappears while "D", at $g = 2.0151$, characteristic

of peroxy radicals, grows in intensity (2), supporting the identity of "C" as a carbon free radical.

4. Intramolecular Reaction

In the presence of added excess CeCl_3 or of high $[\text{Ce}^{4+}]$, the "C" peak intensity relative to the $\text{RS}\cdot$ signal was diminished and the third peak of the $\text{RS}\cdot$ triplet was broadened (Fig. 1c, d). This kind of behavior, characteristic of radical ions whose ESR spectra weaken and broaden in the presence of metal ions with which they form ion pairs (51), suggests that "C" may represent a radical ion and "D" a metal ion pair, whose intensity is enhanced in the presence of O_2 due to formation of a higher concentration of cerous ions. Such a radical ion might have the carboxylate structure:



Carboxylate radical ions of this type have been described (52) at 77°K, with g -values of 2.0026–2.0032. If "C" is a species of this nature, it may be formed by reaction [4], which represents an intramolecular attack of the cysteinyl radical on its carboxyl group, in addition to intermolecular processes. The ability of $\text{RS}\cdot$ radicals to participate in sterically favorable intramolecular hydrogen transfer reactions has been previously demonstrated (6).

5. Overoxidation

Studies of reaction stoichiometry have been performed on the ceric ion oxidation of α -mercaptocarboxylic acids by thermometric titrimetry (53) and of various thiols by a spectrophotometric technique designed to follow RSH concentration (54). These studies showed that there is generally more than 1 mole of oxidant consumed per mole of thiol oxidized. It is thus likely that in the present system, overoxidation, as expressed by reaction [5a], may participate to a small extent in the mechanisms for disappearance of the $\text{RS}\cdot$ radical. Further reaction of an intermediate sulfenic acid, such as that in reaction [5a], according to a reaction such as [5b] to form the dimer, cystine, have been substantiated (27, 55, 56).

6. Back Reaction

The possibility of a contribution of a back reaction, according to reaction [6], to the overall kinetics of disappearance of the $\text{RS}\cdot$ radical was considered. To test this, an excess of CeCl_3 was added to the reagent solutions at several concentration ratios. No reproducible effect on either the rates of disappearance of the $\text{RS}\cdot$ radical or its steady-state spin concentration were observed, indicating that the participation of this reaction is unlikely. The influence of added cerous ion on the behavior of the "C" signal has been described above.

7. Back Fission

A possible reaction in which ceric ion may oxidize the cystine dimer to produce $\text{RS}\cdot$ radicals, as in the homolytic back fission of peroxides, is described by reaction [7]. This was tested by the addition of an excess of cystine to the reaction mixtures at several

initial concentration ratios. Neither the rates of disappearance of $RS\cdot$ nor its steady-state concentration were changed, suggesting that this reaction is not important in the present system.

8. Combination with Oxygen

Reaction of $RS\cdot$ with O_2 to produce a sulfonyl free radical, $RSO_2\cdot$ (species "B"), has been previously discussed (2). The reaction conditions chosen in the present studies were not favorable for the observation of the $RSO_2\cdot$ singlet. As the rates of disappearance of the $RS\cdot$ radical from both de-aerated and air-exposed solutions were identical, it was concluded that decay of $RS\cdot$ by this pathway is not important at pH 1.5.

System Rate Law and Further Analysis

Integrated equations for consecutive and parallel reactions are obviously of very complex forms and do not permit simple experimental verifications, without some direct knowledge of the ratios of the several rate constants. Experimentally, both consecutive and concurrent reactions are characterized, although not distinguished, by the fact that the kinetic decay curves will not fit any of the simple rate laws, as has been demonstrated in the present system. In practice, there are no general methods for such a distinction (35), as it is an individual problem for each reaction studied and must be made on an experimental basis.

Since the preliminary spectrophotometric data on the rate of formation of the $RS\cdot$ free radical and approximations from Czapski's equation (40), relating the steady-state spin concentration of a free radical to its appearance and disappearance dimerization rate constants, described above under reaction [1], appear to describe a k_1 on the order of 200–1000 $M^{-1} \text{ sec}^{-1}$, then reaction [1] must be considered as the rate-limiting step, and this $RS\cdot$ radical reaction system must be analyzed in terms of consecutive reactions.

There are, in general, no exact solutions to any sequences of consecutive reactions higher than first order, since the differential equations are no longer linear, and non-linear equations only have exact solutions in a few very specific cases (35). A couple of exact methods are available for studying only some aspects of these systems.

On the basis of all the preceding considerations, it appears that the rate law which represents the most likely possible reactions, based on reactions [1], [2], [3], and [5], may be expressed as:

$$-d[RS\cdot]/dt = 2k_2[RS\cdot]^2 + k_3[RSH][RS\cdot] + k_5[Ce^{4+}][RS\cdot] - k_1[Ce^{4+}][RSH].$$

Here, k_1 is the rate constant for formation as in reaction [1], $2k_2$ is for disappearance by dimerization as in reaction [2], k_3 is for disappearance by means of reaction [3], and k_5 for disappearance by overoxidation as in reaction [5]. In the present system, the steady-state concentration of the $RS\cdot$ free radical has been measured and found to be very small compared to the concentrations of the initial reactants, and the rates of disappearance of this intermediate have been determined. The use of the ESR stopped-flow technique alone precludes specific determination of absolute rate constants in a system as complex as that described by the rate equation given above. For a more definitive analysis of the kinetics of formation and disappearance of the $RS\cdot$ radical, even by a simplified treatment of the kinetic equations for consecutive reactions, a

knowledge of the rate of disappearance of at least another reactant, $-d[\text{RSH}]/dt$, must also be established. Investigations are under way to define these additional quantities.

CONCLUSIONS

It has been demonstrated by ESR stopped-flow spectroscopy that the disappearance of the thiyl free radical in solution can proceed through pathways other than the concurrent bimolecular classical dimerization reaction in a significant manner. The predominance of these simultaneous reactions has been seen to be dependent on the concentrations of the $\text{RS}\cdot$ radical and RSH.

Although at this stage of the analysis ESR techniques have not yet yielded concrete values for absolute rate constants, substantial evidence has been gained for the relative participation of the various possible mechanisms, which describe the reactivity of the thiyl free radical. Thus, when reactive molecules other than free radicals interact to a significant extent with those free radicals in a complex reaction scheme, that system cannot be described by ESR techniques alone. But rather, evidence from a variety of precise methods for the study of fast reactions used to determine the reactivity of free radicals with these other reactive species must be unified and correlated in order to piece together the complete mechanistic picture. Further studies are required to accurately delineate the rate of appearance of the $\text{RS}\cdot$ free radical, the rate of disappearance of RSH, the influence of O_2 and possible back reactions, and the effects of increased (physiological) pH.

These studies thus represent a first step in the correlation of data derived from ESR stopped-flow techniques, kinetic absorption spectrophotometry-rapid-mixing devices and -pulse radiolysis methods (17, 18, 47) necessary to clearly define the nature of the reactivity of the thiyl free radical. The delineation of reactions, other than interactions solely with other radicals as in dimerization or free radical scavenging (57), will be of great importance in the consideration of some of the hypotheses describing the mode of action of the $\text{RS}\cdot$ intermediate in radioprotective processes that have been proposed (19-24). This work has provided support for the high rate constants of 10^9 - $10^{10} \text{ M}^{-1} \text{ sec}^{-1}$ for $\text{RS}\cdot$ radical dimerization measured in pulse radiolysis studies (16-18), and evidence for various modes of reaction of the $\text{RS}\cdot$ radical with RSH, demonstrating that the thiyl free radical is not a stable, unreactive species capable of disappearing by dimerization alone.

Note added in proof. Full details of the computer analyses used are currently in press in "Computer Programs in Biomedicine" (1974).

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