their singly protonated counterparts. In *n*-hexane, however, the neutral molecules and the respective singly charged cations were observed to fluoresce with about the same intensities. This suggests that the diminished quantum yields of fluorescence of the neutral molecules in water are not due to the inherent molecular structures of these species but rather to some interaction with the excited-state solvent cage, presumably hydrogen bonding at the quinuclidine nitrogen atoms. Excited-state hydrogen bonding is known to quench the fluorescences of a wide variety of molecules, presumably by coupling the molecule to the solvent quasilattice. The vibrational modes of the solvent quasilattice provide an efficient means of radiationless deactivation of the lowest excited singlet state. That the dissociations and solvent interactions of the quinuclidine nitrogen atoms have such a dramatic effect upon the fluorescent properties of quinine and quinidine indicates substantial electronic coupling in the lowest excited singlet state between the aromatic and aliphatic portions of the alkaloids. Normally, the solvent interactions and prototropic exchanges involving functional groups not directly bonded to the aromatic fluorophore or sterically free to interact directly with the fluorophore (12, 13) do not have much effect upon the fluorescence arising from the fluorophore.

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Electron Spin Resonance Studies of Free Radicals in Solution III: pH Dependence of Thiyl Free Radical of Cysteine

JEAN C. KERTESZ, MICHAEL B. WOLF, WALTER WOLF^x, and LI-YA A. CHEN

Abstract □ An electron spin resonance flow technique was utilized to follow the concentration of thiyl free radicals (RS·) generated upon the oxidation of cysteine with ceric ion over the 0.25–7.5 pH range. The thiyl free radical concentration was seen to decrease with increasing pH in a manner dependent on the extent of ionization of cysteine. The formation of thiyl free radicals was favored by the proportion of thiol present in the positively charged form. The diminution of RS· spin concentration at physiological pH was correlated with the increasing prevalence of singly and doubly negative thiolate ions. At acidic pH's below 1.5, thiyl free radical formation was depressed by solutions of high ionic strength. The utility of this technique in studying the potential role of thiyl free radicals in radioprotective processes is indicated.

Keyphrases □ Free radicals in solution—pH dependence of thiyl free radical formation from oxidation of cysteine, electron spin resonance □ Cysteine oxidation—pH dependence of thiyl free radical formation □ Electron spin resonance—monitoring, pH dependence of thiyl free radical formation from oxidation of cysteine

Recent studies in this laboratory identified and characterized thiyl (RS·) and related free radicals in aqueous solution by electron spin resonance (ESR) spectroscopy when they are generated by ceric-ion oxidation of various thiols at low pH (1–6). Since sulfhydryl compounds and thiyl free radicals exhibit roles of considerable importance in the mech-

anisms of radiation damage and radioprotective processes (7–12), it was of special interest to determine whether the free radicals observed at acidic pH's may also be detected and similarly characterized at physiological pH's (6.8–7.6). Since thiyl free radical signals are observed under steady-state flow conditions, it was necessary to modify the flow method so that as the ESR spectra are recorded the concomitant pH of the reaction mixture can be measured. This paper presents the results of a systematic investigation of the variation of the thiyl free radical spin concentration observed upon the oxidation of cysteine with increasing pH.

EXPERIMENTAL

The ESR continuous-flow system used was described in detail elsewhere (13). A flow rate of 1.0 ml/sec was used uniformly throughout the experiments. ESR spectra were recorded with a ESR spectrometer[1] 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 1.6 gauss and a microwave power of 200 mw on 50-gauss field scans. There was no evidence of power saturation of the spectra studied in this work. A 1-sec filter was routinely used

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[1] Varian model E-3.

for scanning experiments. All spectra were recorded when the reaction mixtures were at 23.5 ± 0.3°.

All reagent solutions were prepared in distilled water. *l*-Cysteine hydrochloride[2] and the ceric salt, ceric potassium nitrate[3] [CeK2(NO3)6], were used without further purification. A stock solution of 1 N HCl was prepared from 37% hydrochloric acid[4] for subsequent dilutions. Analytical reagent grade sodium hydroxide[4] was used.

The ceric potassium nitrate was readily water soluble, yielding a pH of 2.0 at a concentration of 0.005 M, the acidity due to hydrolysis of the salt. The pH of ceric-ion solutions could not be raised above 4 upon addition of sodium hydroxide prior to placement in the flow system, since a flocculent precipitate of cerium hydroxides resulted. Thus, the pH of the resultant reaction mixture could be adjusted only by addition of the appropriate concentrations of sodium hydroxide or hydrochloric acid to the initial cysteine solutions. Since, in neutral or basic aqueous solutions, thiols are oxidized by the air quite readily to the disulfides (14) or higher oxidation products (15), it was important that the ESR spectra be recorded as soon as possible after dissolution of the reagents. For this reason, the hydrochloride salt of cysteine was used routinely throughout the experiments, rather than the free base, because the latter dissolves much more slowly in neutral solutions. The concentration of sodium hydroxide required for the basic pH's studied was adjusted to neutralize the additional acid introduced by the hydrochloride salt form of cysteine. Thus, upon dissolution of reagents the solutions were rapidly removed to the flow system to avoid autoxidation of thiol and autoreduction of ceric ion which is catalyzed by glass surfaces upon standing (16, 17).

In a few experiments, a ceric–citrate complex was used which prevented the ceric ion from precipitating at pH 5. The intensity of the RS· triplet signals observed at this pH was equivalent to those obtained in the absence of the citrate anions. This confirmed that the strength of the ceric-ion solutions at the time of reaction in the flat cell at the higher pH's was not diminished by the subsequent formation of hydroxide precipitates as the reaction mixture flowed from the cavity.

Vanadyl sulfate double hydrate[5] crystals were purified (18) by repetitive washings with methanol and drying under vacuum. Standard vanadyl sulfate solutions were prepared from the purified crystals dissolved in 1 M H2SO4, which prevents oxidation of the VO+[2] ion, and were used as ESR spin concentration primary standards. The procedure for estimating free radical concentrations based on double integration of the ESR first derivative signal, adapted from the numerical method of Poole (19), will be published in detail elsewhere. At high pH values, the ESR spectra were somewhat noisy, and integration of the signals led to more ambiguous results. However, simultaneous recording of the signals with a multichannel analyzer[6] allowed suitable intensity enhancement.

All pH measurements were made with a pH meter[7], calibrated with pH 4 and 7 buffer solutions[8]. A flow method was devised to determine the pH of the reaction mixtures exiting from the ESR flat cell. Initially, a method utilizing a pH microblood assembly[8] was tested. This equipment bypassed fluids from the flat cell exit through a narrow polyethylene tube leading to a stream directly across the electrodes. A narrow diameter tubing was used to reduce the dead volume, so that pH readings as close as possible to those at the actual time of reaction in the flow cell could be recorded. Unfortunately, this arrangement obviated consistent determinations at the higher pH values, since cerium oxide precipitates obstructed the path of flow. This reduced the flow rate, affected the stability of the electrodes, and altered the true proportions of the reaction mixtures. An alternative method in which the flow path was unrestricted was then used. The pH was determined by flowing the reaction mixture exiting from a 0.63-cm (0.25-in.) i.d. tube over a semimicrocombination electrode with triple-purpose glass membrane immersed in a filter flask. This electrode reaches a stable reading within 30 sec, and the pH is

Table I—Dependence of RS· Spin Concentration on pH and Ionic Strength

pH	Concentration of Acid or Base in Reaction Mixture	RS· Spins/mole $\times 10^7$ M	Ionic Strength, M
0.25	3.00000 N HCl	10.92	3.040
0.60	1.50000 N HCl	13.42	1.540
0.80	1.00000 N HCl	15.24	1.040
1.50	0.25000 N HCl	17.86	0.290
2.10	0.00000 N HCl	14.62	0.0375
3.00	0.01000 N NaOH	11.43	0.0425
3.60	0.01500 N NaOH	9.95	0.0525
4.60	0.01625 N NaOH	9.04	0.05375
5.00	0.01650 N NaOH	8.13	0.0540
6.10	0.01875 N NaOH	6.81	0.0563
6.70	0.02500 N NaOH	4.52	0.063
7.50	0.03000 N NaOH	0.17	0.0675

monitored while the ESR readings are recorded over the entire range of pH studied, albeit at a dead time of 154 sec after reaction has occurred in the ESR flat cell.

RESULTS AND DISCUSSION

The thiyl free radical ESR signal observed upon the oxidation of cysteine with ceric ion is a 1:2:1 triplet at[9] g = 2.0106 ± 0.0002. The minimum concentrations of thiol and ceric ion necessary to produce a reasonable RS· signal had previously (2) been determined to be 0.02 M thiol and 0.005 M Ce+[4] ion. Since higher concentrations of ceric ion produce more acidic solutions, the minimum concentrations proved to offer the greatest flexibility for variation of pH over a wide range without excessive precipitation of ceric ion at the higher pH's. The pH was adjusted by addition of the appropriate concentration of hydrochloric acid or sodium hydroxide to the cysteine solution before mixing. The resultant concentrations of acid or base in the flow cell reaction mixture at the time of ESR observation and the concomitant spin concentrations are given in Table I. The relationship between the total intensity of the RS· signal and increasing pH is depicted in Fig. 1. This plot, which appears to have the shape of a "titration" curve in the 1.5–7.5 pH range, represents the steady-state concentration of RS· free radicals generated in the one-electron transfer (20) reaction of Scheme I.
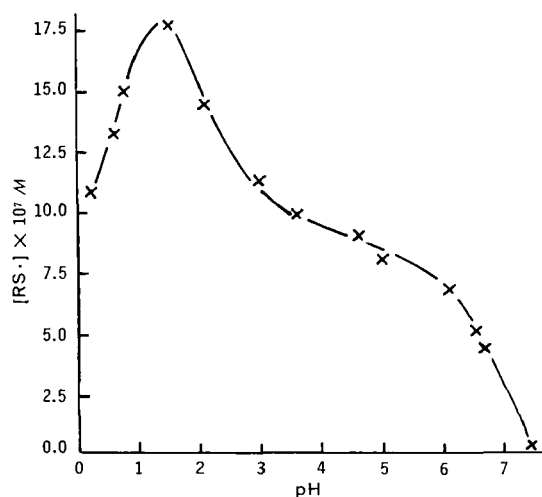
$$Ce^{+4} + RSH \longrightarrow Ce^{+3} + RS\cdot + H^+$$
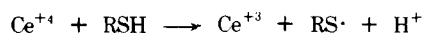
Scheme I

Figure 1—*pH dependency of thiyl free radicals generated upon oxidation of 0.02* M *cysteine hydrochloride with 0.005* M $CeK_2(NO_3)_6$.

[2] Fox Chemical Co., lot F-1011.
[3] K & K Laboratories.
[4] Mallinckrodt analytical reagents.
[5] Fischer Scientific Co.
[6] Hewlett Packard 5400 A.
[7] Corning model 10.
[8] Beckman.

[9] The g-value for a free radical is a dimensionless constant; it is also called the spectroscopic splitting factor.

The disappearance of the thiyl free radical may then occur either by dimerization:

$$RS\cdot + RS\cdot \longrightarrow RSSR$$
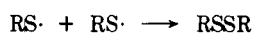
Scheme II

or interaction with other species:

$$RS\cdot + X \longrightarrow Y$$
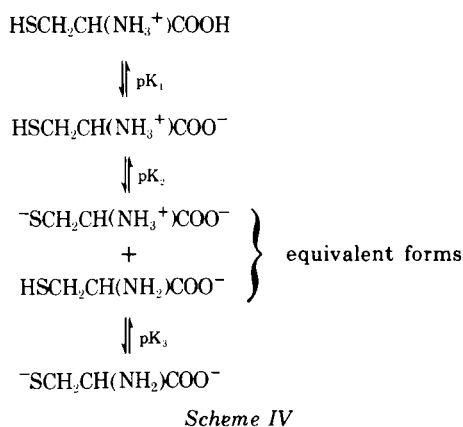
Scheme III

where the possible identity of X includes molecular oxygen or RSH. The nature of Schemes II and III, determined in kinetic studies, will be discussed elsewhere.

Amino acids are present in various ionic states depending on the pH of a solution. The form of the variation of RS· concentration with pH illustrated in Fig. 1 suggests that the rate of Scheme I is markedly dependent upon the state of protonation of the various functional groups of cysteine. Cysteine has three dissociable protons. The ionization of these protons may be represented by the steps shown in Scheme IV.

$$HSCH_2CH(NH_3{}^+)COOH$$

$$\Updownarrow pK_1$$

$$HSCH_2CH(NH_3{}^+)COO^-$$

$$\Updownarrow pK_2$$

$$\left.\begin{array}{c} {}^-SCH_2CH(NH_3{}^+)COO^- \\ + \\ HSCH_2CH(NH_2)COO^- \end{array}\right\}\text{ equivalent forms}$$

$$\Updownarrow pK_3$$

$$^-SCH_2CH(NH_2)COO^-$$

Scheme IV

The concentration of RS· radicals is seen to reach a maximum at pH 1.5. At lower pH's, RS· concentration is depressed, apparently due to ionic strength factors which will be discussed later. As the pH rises above 1.5, the RS· concentration decrease parallels the first dissociation constant of cysteine. The rate of decrease in the pH 3–6 region is slower; beyond pH 7.5, there is no longer a steady-state concentration of RS· radicals sufficient for ESR detection.

Correlation with Ionic Species—The ability to generate the thiyl free radical is apparently strongly influenced by the ionic form of the various substituents of cysteine. To determine whether the ease of formation of the thiyl free radical could be correlated with the presence of a specific charged parent molecule, the concentrations of ionic species as a function of pH were calculated (21, 22) utilizing the reported (23) pKa values of $pK_1 = 1.71$, $pK_2 = 8.33$, and $pK_3 = 10.78$ for cysteine. Due to the alternative ways of ionization of the —SH and —NH$_3{}^+$ groups, there is considerable divergence in the literature as to the exact values of these pKa's. Various pKa values, as reported for cysteine by different workers (23–27), range from 1.71 to 1.96 for pK_1, from 8.15 to 8.7 for pK_2, and from 10.28 to 10.78 for pK_3. These differences may be responsible for a slight shift in the concentration of ionic species in relation to that of the thiyl free radical. The log of each ionic species concentration as a function of pH is plotted in Fig. 2, along with the log of the observed number of spins of the thiyl free radical for comparison. These curves indicate that formation of the RS· radical is greatly favored by the presence of the positively charged HSCH$_2$CH(NH$_3{}^+$)COOH species, which is predominant at pH's below 1.5. The RS· radical concentration begins to fall as the zwitterion, HSCH$_2$CH(NH$_3{}^+$)COO$^-$, becomes predominant and nearly parallels the concentration of this species past the isoionic point (pI 5.02), falling gradually as the negatively charged $^-$SCH$_2$CH(NH$_3{}^+$)COO$^-$ species increases with pH. As the pH rises beyond 6.7, the thiyl free radical concentration begins to fall rapidly. At this range, the zwitterion is still prevalent, while the concentrations of both the singly and doubly negative charged thiolate ions are both rapidly increasing; beyond pH 7.5 the RS· signal is no longer detectable at the initial concentra-
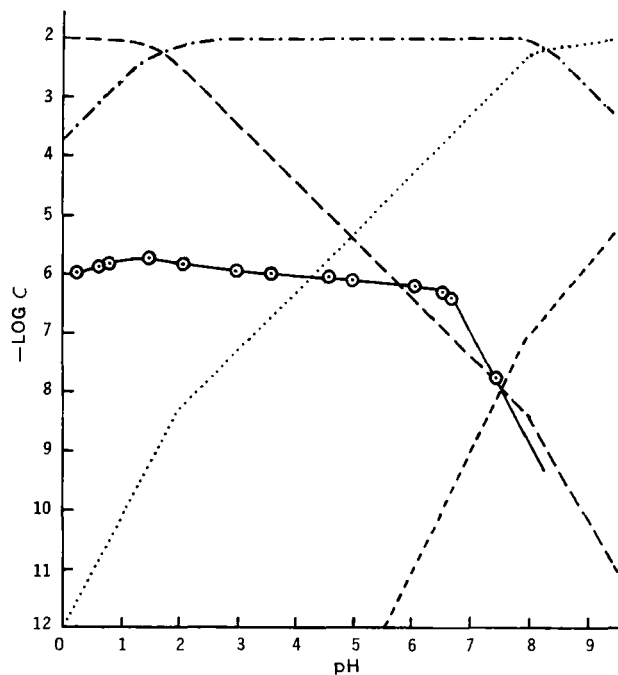
Figure 2—*Relation of the thiyl free radical concentration observed during ceric-ion oxidation of cysteine hydrochloride to the concentration of the different ionic forms of cysteine as a function of pH. Key:* C, *concentration of free radical or ionic species:* ——, *RS·;* – – –, *HSCH₂CH(NH₃⁺)COOH;* –·–·–·, *HSCH₂CH(NH₃⁺)COO⁻;* ········, *⁻SCH₂CH(NH₃⁺)COO⁻; and* - - - - -, *⁻SCH₂CH(NH₂)COO⁻.*

tions of cysteine and ceric ion employed in this study. This correlation suggests that the rate of formation (Scheme I) decreases with increasing pH. Studies on the rate of disappearance of the thiyl free radical generated by pulse radiolysis techniques (28) suggest that decay of RS· by dimerization (Scheme II) also decreases with increasing pH. (At pH 1.0, a K_d of $1.0 \times 10^{10}\ M^{-1}$ sec^{-1} was indicated; at pH 6.4, a K_d of $3.4 \times 10^9\ M^{-1}$ sec^{-1} was indicated.)

Effect of Ionic Strength—The decrease in RS· spin concentration at low pH's from 1.5 to 0.25 is apparently due to the influence of increased ionic strength on the rate of formation, although the high H$_3$O$^+$ concentration may also shift Scheme I to the left, according to the law of mass action. The ionic strength represents the contribution to the electrostatic forces of the ions of all types. In this pH range the prevalent form of cysteine is the positively charged species. The oxidation potential of the Ce^{+4}–Ce^{+3} couple is known (29) to be ligand dependent. Oxidation potentials of -1.44 and -1.28 have been measured when ceric ion is in 1 N H$_2$SO$_4$ or 1 N HCl, respectively. The chloride ion forms a complex (17) with ceric ion, which lowers the oxidation potential. A greater complexation of this nature in 3 N HCl, the highest acid concentration studied, may thus contribute to diminution of the observed RS· signal. The behavior of both ceric ion and cysteinyl species is affected significantly by the nature of their solvation shells at high ionic strength. The relationship between the ionic strength of the reaction mixtures and RS· concentration is illustrated in Fig. 3. The thiyl radical concentration rises rapidly at ionic strengths between 0.0675 and 0.0375 at the higher pH's and reaches a maximum at 0.29 at the optimum pH of 1.5. At higher ionic strengths up to 3.04 M, thiyl radical concentration gradually declines.

The influence of ionic strength on the rates of reactions between ions has been described by the Brönsted-Bjerrum equation:

$$\log\ k = \log\ k_0 + 1.02\ z_A z_B \mu^{1/2} \qquad \text{(Eq. 1)}$$

where k_0 is the rate constant in an infinitely dilute solution. Unfortunately, predictions made from this expression are not valid above an ionic strength of 0.01 (30), which is below that of the solutions of this study. Other electrolyte theories have been devel-
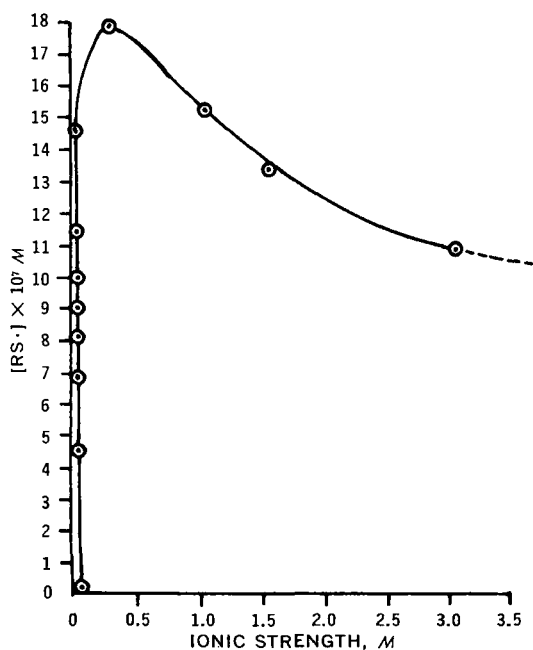
Figure 3—*Concentration of thiyl free radicals observed upon oxidation of 0.02 M cysteine hydrochloride by 0.005 M CeK₂(NO₃)₆ as a function of ionic strength.*

oped which include higher terms and additional parameters for ion sizes and repulsive forces, but they are not yet capable of making *a priori* predictions of ion–ion interactions for the higher valence ions or in the more concentrated solutions (31).

The present system may best be explained in terms of interactions between the ion clusters formed about the two reacting ions. The extent of this interaction is determined by the concentrations and charges of all the ions in solution. At high ionic strength the ceric ions are surrounded by clouds of many counterions, including nitrate ions and solvent molecules, and associate with chloride ions similarly surrounded by potassium-ion and hydronium-ion clouds. As the ionic strength increases, this association becomes greater and electrostatic repulsion between cationic groups increases, so the approach of ceric ions toward the —SH function to affect reaction is increasingly deterred. In the pH 2–7 region, where the ionic strength is much lower, ranging from 0.0375 to 0.0675, electrostatic forces still appear to predominate over the inductive effect of the —COOH group, which would enhance reaction with ceric ion. As the cysteinyl zwitterion is prevalent in this range, the —COO⁻ function tends to attract the ceric ions away from —SH, thus lowering the rate of reaction and the observed RS· spin concentration.

CONCLUSIONS

This study verified that thiyl free radicals observed at acidic pH's can also be produced and observed at physiological pH. The steady-state level of radicals necessary for ESR detection is governed by the rates of formation and decay of the species. When cysteine is oxidized by ceric ion, the rate of appearance of the thiyl free radical is strongly dependent on pH, which determines the ionic form of the parent molecule. The sensitivity of the production of spin concentration to the ionization of acidic functional groups makes ESR a direct method for studying acid–base chemistry at the molecular level. This reaction is most favored by a positive charge on the thiol. The RS· concentration is depressed by solutions of ionic strength greater than 0.29, due to ionic associations. Since thiyl free radicals are important intermediates in radiobiological processes, a comparison of the behavior of cysteinyl free radicals in the physiological pH range and at ionic strengths similar to those of biological fluids with that of structurally related radioprotective or sensitizing thiols would be particularly valuable in assessing their role in these processes.

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