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# ESR Studies of Free Radicals in Solution. I. Oxidation of Cysteine and Related Thiols with Ceric Ion

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A system has been developed for the study of sulfur free radicals in solution by oxidation of a variety of thiols with $Ce^{4+}$ ion. This system generates at least four free radical species, labelled "A," "B," "C," and "D," with g-values at $2.0106 \pm 0.0002$, $2.0186 \pm 0.0002$, $2.0032 \pm 0.0002$, and $2.0151 \pm 0.0002$, respectively. Free radical "A" has been identified as the RS· free radical and when the thiol oxidized is a primary mercaptan, such as cysteine, the signal is a 1 : 2 : 1 triplet with $a_{C_1H}$ of 9.5 G and 3.5 gauss line width. With thiolactic acid the expected 1 : 1 doublet is observed with $a_{C\ H}$ of 8.5 G and 3.0 gauss line width, whereas the tertiary thiols give the expected singlet with a 2.5 gauss line width. The experimental conditions for studying such systems are fully described, as well as the conditions under which "B," "C," and "D" are formed. The possible identity of such species, and the potential of this technique for the study of RS· free radicals and their role in radioprotection and/or radiation damage is discussed.

Free radicals produced by oxidation of cysteine and related thiols have long been considered of interest from the point of view of radiation damage and radiation protection. Accordingly, a number of studies have been published discussing the nature of such divalent aliphatic sulfur radicals (*1–15*). All of these studies were conducted in the solid phase, either in a frozen (polycrystalline, amorphous or glassy) matrix (*7–15*) or in single crystals (*1–6*).

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The spectra reported were broad lines, and had been assigned to various radical species with high electron density on sulfur. The model of Kurita and Gordy, in which the electron spin density is concentrated in a nonbonding $3p$ orbital of the sulfur atom, accounted for the basic features (*10*). However, it was not clear whether the spectra obtained from the solid state reflected the nature of cysteinyl free radicals in solution, because no study of ESR spectra of such aliphatic divalent sulfur radicals in solution had been published when the present work was undertaken. As biological systems are usually in a liquid and semi-colloidal phase at 37°C, it was felt that a systematic study of thiyl and related free radicals in solution would give a better understanding of the significance of free radicals in biological (both pathological and physiological) processes. The present work, dealing with the chemical oxidation of cysteine and related thiols in solution is the first of such studies. Some preliminary results of this work have been published (*16*) and also presented at scientific conferences (*17*).

## EXPERIMENTAL

The flow system used was constructed similar to the model used by previous authors (*18–21*) and is illustrated in Fig. 1. The central unit is a polypropylene block, $6 \times 8 \times 2\frac{1}{4}$ inches, containing two drilled cylindrical reservoirs, having a capacity of 170 ml each. A gas inlet leads to the top of each reservoir allowing the solutions to be pressurized under flow conditions. Reservoir refill is accomplished by gravity from storage bottles set above the central block. The reservoir outlets lead to teflon body Valcor #51C19T34–1 solenoid valves. These normally closed valves theoretically require a minimum operating pressure of 4 psi. However, due to a chronic sluggishness in opening response and an incorrigible variation in flow rates between the two valves, a pressure of 9 psi was utilized instead. To correct for flow differences, each fluid line was then passed through a stainless steel Nupro SS–4M fine metering valve equipped with a micrometer handle. These valves were periodically recalibrated and as such allow equal and consistent flow rates. The two fluid lines then connect directly to a Varian V–4549 four jet liquid flow mixing chamber and a quartz flat cell. The mixed liquids subsequently pass through an exit solenoid valve (Skinner #V52 DA 2100) before disposal. This exit valve prevents back flow and pressure in the fluid leaving the vertical flat cell assembly.

The flow system allowed flow rates from 0.2 to 5.5 ml/sec under these conditions. Perfect mixing, characterized by turbulent flow, was observed in this whole range. No undesirable laminar flow could be detected at the lower flow rates. These characteristics were determined by the method of Borg (*22*). When aqueous bromphenol blue, which is a yellow solution at pH 2, was mixed in the flow cell with dilute NaOH, the immediate development of the blue color, which appears at a pH greater than 4.6, was observed.

The "dead volume" between the point of mixing of the flow jets and the bottom of the flat cell in the cavity was 27 $\mu$l, this giving us a "dead time" of 135 msec and 5 msec at the two extreme flow rates used.

Due to the limitations imposed by the combined volume of the reservoirs (see Fig. 1) of 340 ml, typical runs were performed with two minute scan times, using both 50 and 100 G scan ranges. Longer scan times could be used at the slower

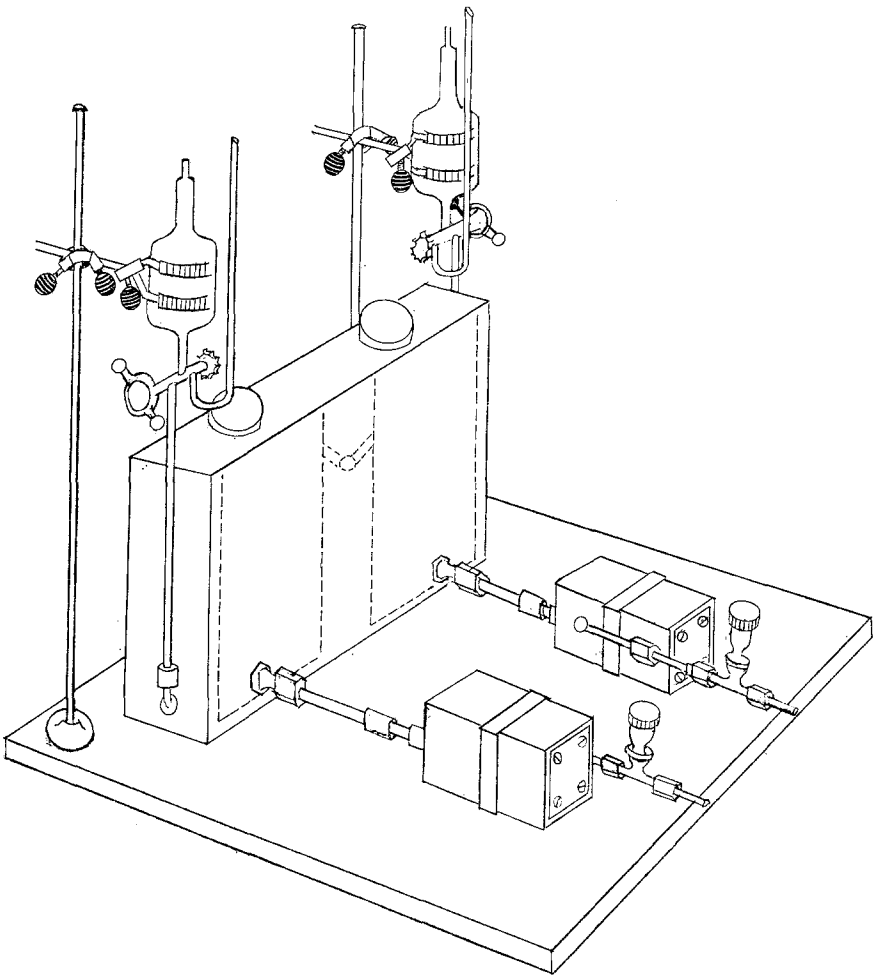


FIG. 1. Schematic representation of the flow system.

flow rates, but not at the faster flow rates, as reservoir refill during a run was unfeasible.

Two types of spectrometers were used: a Varian Model E–3 ESR spectrometer with associated 4-inch magnet, and a Varian Model V–4500–10A EPR spectrometer with associated 9-inch magnet, which utilized the rectangular $TE_{102}$ mode E–4531 and V–4531 multi-purpose cavities, respectively. Both instruments operate at an X-band microwave frequency of 9.5 GHz and a modulation frequency of 100 KHz. To assess line widths each sample was systematically scanned at modulation amplitudes of 0.5–6 G, and in some instances modulation amplitudes as low as 80 mG were utilized to detect possible further hyperfine splitting.

Most spectra were recorded at a microwave power of 200 mW. There was no evidence of power saturation of the spectra studied in this work. The other controls are the standard ones of the Varian E–3; they include a 3 KHz bandpass at 100 KHz,

and filters varying from 100 msec to 100 sec. A 1 sec filter was routinely used for scanning experiments.

All reagent solutions were prepared in distilled water. No attempts were made to de-gas the solutions. The cysteine used was cysteine ·HCl monohydrate, A grade (Calbiochem). D-penicillamine was obtained from Merck, Sharp, and Dohme Research Laboratories. Other mercapto compounds used were high grade reagents from similar commercial sources. The ceric salts used included ceric potassium nitrate, $CeK_2(NO_3)_6$, (K and K Laboratories); ceric ammonium nitrate, $Ce(NH_4)_2(NO_3)_6$ (Matheson, Coleman, and Bell); ceric bisulfate, $Ce(HSO_4)_4$ (G. Frederick Smith Chemical Company), and ceric ammonium sulfate, $Ce(NH_4)_4(SO_4)_4 \cdot 2H_2O$ (Allied Chemical Company, B and A quality). All of these materials were used without further purification. It is of interest to note that no signals could be obtained when the latter ceric salt, $Ce(NH_4)_4(SO_4)_4 \cdot 2H_2O$, was used as the oxidizing agent in place of the others. No explanation for this difference can be given here. All thiol oxidations were performed by two different ceric ion systems. The $CeK_2(NO_3)_6$ was readily water soluble yielding a pH of 2.0 at a concentration of $5 \times 10^{-3}$ *M*. However, the pH decreased slightly with increasing $CeK_2(NO_3)_6$ concentrations. The $Ce(HSO_4)_4$ was soluble only in dilute sulfuric acid. Reagent grade 1 *N* $H_2SO_4$ was used. The thiols were generally prepared in 0.1 *N* HCl at pH 1.3. All solutions were freshly prepared and used shortly thereafter.

The g-values were determined by two methods. The symmetrical doublet (*18*) of ascorbic free radical (g = 2.0052) (*23*), produced by dissolving ascorbic acid (0.1 *M*) in 0.1 *N* NaOH, was utilized as a standard in the replacement method. The solution was purged with oxygen in order to maintain the stability of the radical and was then flowed into the flat cell. The spectrum was recorded and the flow system rinsed with an ample quantity of distilled water. The thiol–ceric ion reaction mixture was then flowed through and its spectrum recorded.

The 2,2,6,6-tetramethyl-4-hydroxycyclohexyl nitroxide (g = 2.0057, $\Delta H$ = 16.35 G) (*19*) was also used as a standard in this replacement method. Alternately, in the capillary method, a sample of diphenylpicrylhydrazyl free radical (DPPH), g=2.00354 (*24*) dissolved in benzene was drawn up into a fine capillary. It was sealed with wax and placed directly on the flat cell, so that standard and thiyl free radical spectra could be recorded simultaneously.

## RESULTS AND DISCUSSION

Upon reaction of a $5 \times 10^{-3}$ *M* solution of $CeK_2(NO_3)_6$, pH 2.0, and a $2 \times 10^{-2}$ *M* solution of cysteine hydrochloride in 0.1 *N* HCl, pH 1.2, at a mixing flow rate of 1.1 ml/sec, we observed an ESR first derivative spectrum represented in Fig. 2. It consists of a 1 : 2 : 1 triplet with a line width of 3.5 G and a hyperfine splitting of 9.5 G. The isotropic g-value of this free radical species was determined as $2.0106 \pm 0.0002$, when compared with DPPH and ascorbic acid standards.

It became apparent, however, that this triplet (which we shall call "A" from now on) was not the only free radical species formed, but that under slightly different concentrations and flow rates, a singlet "B" was observed at lower field. The isotropic g-value for "B" is $2.0186 \pm 0.0002$ and this singlet has a line width of 3.5 G (Fig. 3).

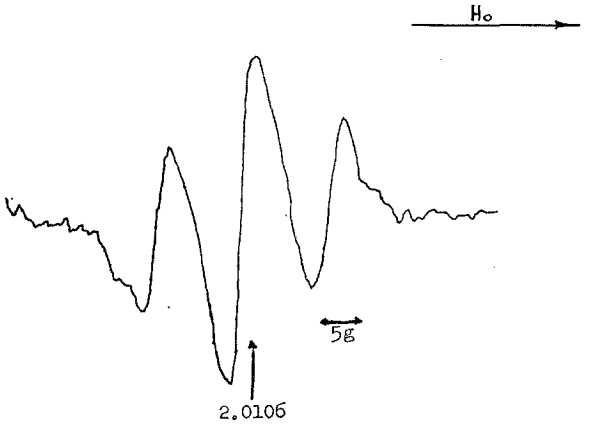



FIG. 2. First derivative ESR spectra of a solution obtained by mixing cysteine, $2 \times 10^{-2}$ *M* in 0.1 *N* HCl and $1 \times 10^{-2}$ *M* of $CeK_2(NO_3)_6$ at room temperature. Flow rate, 2.1 ml/sec.

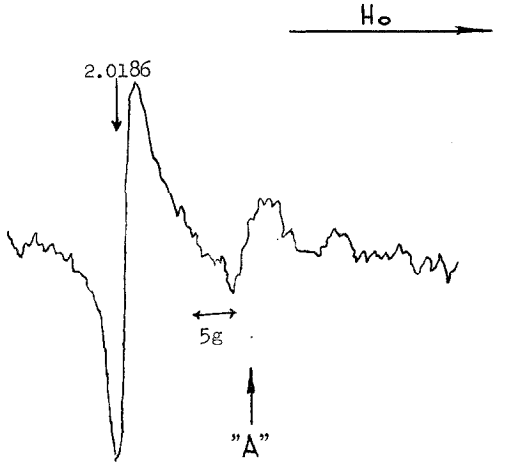



FIG. 3. First derivative ESR spectra of a solution obtained by mixing cysteine, $2 \times 10^{-2}$ *M* in 0.1 *N* HCl and $2.5 \times 10^{-3}$ *M* of $CeK_2(NO_3)_6$ at room temperature. Flow rate, 2.7 ml/sec.

In the course of analyzing the conditions under which either the "A" or the "B" species were formed, we observed that a third free radical species, labeled "C" (Fig. 4), appears upon reacting a $2 \times 10^{-2}$ *M* solution of cysteine in 0.1 *N* HCl with $5 \times 10^{-3}$ *M* $Ce(HSO_4)_4$ in 1 *N* $H_2SO_4$. The isotropic g-value of this third singlet was determined as $g = 2.0032 \pm 0.0002$, based both on the 2,2,6,6-tetramethyl-4-hydroxycyclohexyl nitroxide and the internal "A" free radical as standards. The line width of this species was found to be 1.5 G.

Thus, oxidation of cysteine at acidic pH, using ceric ion, gives rise to at least three different free radical species, "A," "B," and "C." Their ESR characteristics are outlined in Table 1.

In order to characterize the nature of these free radicals, we proceeded to oxidize under similar conditions a number of related primary, secondary, and tertiary

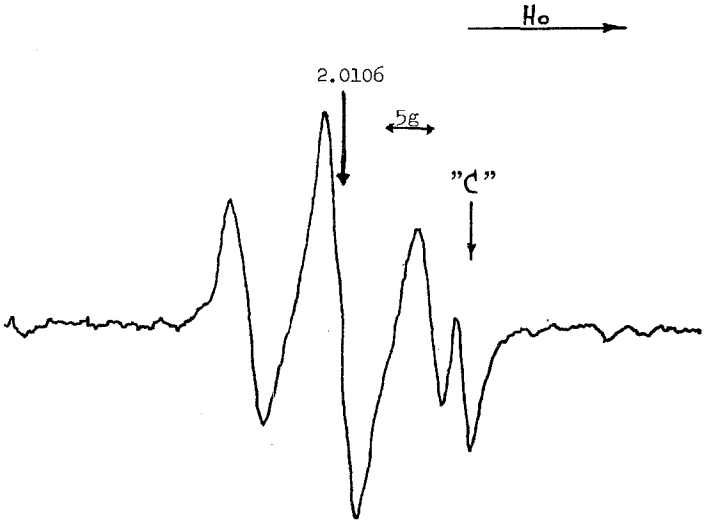



FIG. 4. First derivative ESR spectra of a solution obtained by mixing cysteine, $4 \times 10^{-2}$ *M* in 0.1 *N* HCl and $4 \times 10^{-2}$ *M* $Ce(HSO_4)_4$ in 1 *N* $H_2SO_4$ at room temperature. Flow rate 2.0 ml/sec.

TABLE 1

ESR CHARACTERISTICS OF FREE RADICAL SIGNALS PRODUCED UPON $Ce^{4+}$ OXIDATION OF CYSTEINE IN SOLUTION

| Designation | Possible structure | g-value, $\pm 0.0002$ | Line width (G) | Type of signal | HFS (G) |
|---|---|---|---|---|---|
| "A" | RS· | 2.0106 | 3.5 | 1 : 2 : 1 triplet | 9.5 |
| "B" | $(RSO_2)$· | 2.0186 | 3.5 | singlet | — |
| "C" | — | 2.0032 | 1.5 | singlet | — |

aliphatic thiols. Such results are condensed in Tables 2, 3, and 4. In addition, some sulfur substituted cysteine derivatives and aromatic thiols were oxidized.

As can be readily seen from these data, all of the aliphatic thiols studied gave ESR spectra indicating free radical species having isotropic g-values practically identical ($\pm 0.0002$) to that of the cysteinyl species "A" and "B." In most cases the same was true of species "C," with several exceptions, particularly amongst the secondary thiols. The relative proportions and intensities of each signal was a function of the conditions used. The major variations were produced by differences in flow rate and in concentration ratio of thiol to $Ce^{4+}$ ion within each of the two ceric oxidizing systems used. The most detailed survey of these variables, among all the thiols studied, was performed using cysteine and penicillamine.

By comparing the aqueous $CeK_2(NO_3)_6$ and the $Ce(HSO_4)_4$–1 *N* $H_2SO_4$ oxidizing systems, it was observed that the latter, more acidic reagent, generally yielded signals of species "A" of slightly higher intensity. This comparison was based on the usual minimum thiol/$Ce^{4+}$ ratio of 4 to 1 ($2 \times 10^{-2}$ *M* thiol and $5 \times 10^{-3}$ *M* $Ce^{4+}$) at flow rates from 0.2–3.5 ml/sec.

TABLE 2

ESR CHARACTERISTICS OF FREE RADICALS PRODUCED UPON $Ce^{4+}$ OXIDATION OF PRIMARY ALIPHATIC THIOLS IN SOLUTION

| Thiol[a] | Thiol/$Ce^{4+}$ ratio[b] | Signals observed at g-value of | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 2.0106 "A" | | | 2.0186 "B" | | 2.0032 "C" | |
| | | Spectrum | HFS(G) | L.W. | Spectrum | L.W. | Spectrum | L.W. |
| L-Cysteine-HCl·$H_2O$ | 4–1 | Triplet | 9.5 | 3.5 | Singlet | 3.5 | Singlet | 1.5 |
| L-Cysteine methyl ester·HCl | 4–1 | Triplet | 9.5 | 3.5 | Singlet | 3.5 | Singlet | 1.5 |
| L-Cysteine ethyl ester·HCl | 4–1 | Triplet | 9.5 | 3.5 | Singlet | 3.5 | Singlet | 1.5 |
| *N*-Acetyl-L-cysteine | 4–1 | Triplet | 9.5 | 3.5 | Singlet | 3.5 | Singlet | 1.5 |
| β-Mercaptopropionic acid | 12–3 | Triplet | 10.5 | 4.5 | Singlet | 2.5 | Not seen as yet | |
| Cysteamine·HCl | 4–4 | Triplet | 9.5 | 4.0 | Not seen as yet | | Singlet | 1.5 |
| Cysteamine-*N*-acetic acid·HCl | 4–1 | Triplet | 10.5 | 4.0 | Singlet | 2.5 | Not seen as yet | |
| Mercaptoethanol | 4–1 | Triplet | 10.5 | 4.0 | Singlet | 2.0 | Complex spectrum | |
| Thioglycolic acid | 4–1 | Triplet | 9.0 | 3.5 | Singlet | 2.0 | Complex spectrum | |
| Gluthathione | 4–4 | Triplet | 9.0 | 2.5 | — | | Singlet | 1.5 |

[a] Each spectrum was most easily observed over the range of flow rates 1.1–3.5 ml/sec.

[b] In this column, 4 represents a concentration of $2 \times 10^{-2}$ *M* and 1, a concentration of $5 \times 10^{-3}$ *M*. These ratios represent the minimum concentrations necessary to produce a reasonable spectrum.

HFS = hyperfine splitting
L.W. = line width (gauss)

TABLE 3

ESR CHARACTERISTICS OF FREE RADICALS PRODUCED UPON $Ce^{4+}$ OXIDATION OF SECONDARY ALIPHATIC THIOLS IN SOLUTION

| Thiol[a] | Thiol/$Ce^{4+}$ ratio[b] | Signals observed at g-value of | | | | | |
|---|---|---|---|---|---|---|---|
| | | 2.0106 "A" | | | 2.0186 "B" | | 2.0032 "C" |
| | | Spectrum | HFS(G) | L.W. | Spectrum | L.W. | Spectrum |
| Thiolactic acid | 4–1 | Doublet | 8.5 | 3.0 | Singlet | 2.0 | Complex signals |
| α-Mercaptopropionyl glycine | 4–1 | Doublet | 8.5 | 3.0 | Not seen as yet | | Complex signals |
| Thiomalic acid | 4–1 | Doublet | 8.75 | 3.0 | Singlet | 2.5 | Complex signals |

[a] Each spectrum was most easily observed over the range of flow rates 1.1–3.5 ml/sec.

[b] In this column, 4 represents a concentration of $2 \times 10^{-2}$ *M* and 1, a concentration of $5 \times 10^{-3}$ *M*. These ratios represent the minimum onc entrations necessary to produce a reasonable spectrum.

HFS = hyperfine splitting
L.W. = line width (gauss)

TABLE 4

ESR CHARACTERISTICS OF FREE RADICALS PRODUCED UPON $Ce^{4+}$ OXIDATION OF TERTIARY ALIPHATIC THIOLS IN SOLUTION

| Thiol[a] | Thiol/$Ce^{4+}$ ratio[b] | Signals observed at g-value of | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 2.0106 "A" | | 2.0186 "B" | | 2.0032 "C" | | 2.0151 "D" | |
| | | Spectrum | L.W. | Spectrum | L.W. | Spectrum | L.W. | Spectrum | L.W. |
| D-Penicillamine | 4–1 | Singlet | 2.5 | Singlet | 2.5 | Singlet | 1.5 | Singlet | 4.0 |
| *N*-Acetyl-DL-penicillamine | 4–1 | Singlet | 3.0 | Singlet | 2.5 | Singlet | 2.5 | Singlet | 4.0 |
| α-Mercaptoisobutyic acid | 4–1 | Singlet | 2.5 | Singlet | 3.0 | Singlet | 2.0 | Singlet | 3.5 |

[a] Each spectrum was easily obtained at a flow rate of 0.2 ml/sec and studied over the 0.2–3.5 ml/sec.

[b] In this column, 4 represents a concentration of $2 \times 10^{-2}$ *M* and 1, a concentration of $5 \times 10^{-3}$ *M*. These ratios represent the minimum concentrations necessary to produce a reasonable spectrum.

L.W. = line width (gauss)

However, species "B" was only formed by aqueous $CeK_2(NO_3)_6$ oxidation and was never observed in the $Ce(HSO_4)_4$–1 *N* $H_2SO_4$ system. Its formation was favored by an 8 to 1 thiol/$Ce^{4+}$ ratio ($2 \times 10^{-2}$ *M* thiol versus $2.5 \times 10^{-3}$ *M* $Ce^{4+}$) and faster flow rates. Thus, in the aqueous $CeK_2(NO_3)_6$ system, the intensity of species "B" grew and that of species "A" decreased with increasing flow rates, while in the $Ce(HSO_4)_4$–1 *N* $H_2SO_4$ system, species "B" was not seen and the intensity of species "A" rose with increasing flow rates.

Species "C" could be produced in both oxidizing systems, but at different reagent concentration ratios. This species could generally be obtained in weak intensity (i.e., relative to the simultaneously observed species "A") at the usual minimum 4 to 1 thiol/$Ce^{4+}$ ratio. However, a "C" signal of comparable intensity was observed in the aqueous $CeK_2(NO_3)_6$ system only when the $CeK_2(NO_3)_6$ concentration was considerably greater. Thiol/$Ce^{4+}$ ratios of 4 to 4 or 4 to 6 [$2 \times 10^{-2}$ *M* or $3 \times 10^{-2}$ *M* $CeK_2(NO_3)_6$] were necessary, depending on the particular thiol in question. In both oxidizing systems "C" signal intensity increased both with faster flow rates and greater $Ce^{4+}$ ion concentrations. In a number of cases to be discussed below, the use of the $Ce(HSO_4)_4$–1 *N* $H_2SO_4$ system resulted in a complex "C" spectrum.

*Primary Aliphatic Thiols*

Considering cysteine as the basic structural model, it can be seen from Table 2 that "A," "B," and "C" signals are obtained regardless of whether:

1. the carboxyl group is blocked (methyl and ethyl esters),
2. the amino group is substituted (*N*-acetyl derivatives),
3. the $-\overset{|}{C}HNH_2$ unit is removed (thioglycolic acid), or,
4. both carboxyl and amino groups are removed (mercaptoethanol, etc.).

Cysteamine appears to be one of the thiols used which is the most difficult to oxidize. The "A" and "C" signals have been observed in the $Ce(HSO_4)_4$–1 $N$ $H_2SO_4$ system only at four times the usual ceric ion concentration. However, the $N$-acetic acid derivative of cysteamine was readily oxidized by the minimum $5 \times 10^{-3}$ $M$ aqueous $CeK_2(NO_3)_6$ reagent, resulting in the observation of the usual "B" signal.

$\beta$-mercaptopropionic acid was also more difficult to oxidize, requiring a higher concentration of ceric ion in both oxidizing systems for signal observation.

The tripeptide glutathione has also been oxidized by aqueous $CeK_2(NO_3)_6$, yielding the "A" triplet at the usual g-value. However, while the line width and hfs are similar to those of cysteine, there is indication of another free radical species overlapping the central peak of "A". Whether these are one or several carbon radicals produced by proton abstraction from the cysteinyl, glycyl, or $\gamma$-glutamyl residues, is to be determined.

*Secondary Aliphatic Thiols*

When oxidized by the aqueous $CeK_2(NO_3)_6$ system, all three secondary thiols (Table 3) yielded the expected 1 : 1 doublet ("A" signal) at $g = 2.0106 \pm 0.0002$. The oxidation of thiolactic (Fig. 5) and thiomalic acids also resulted in the "B"

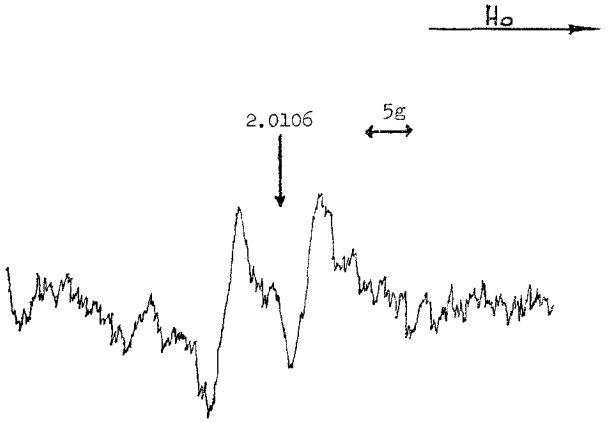



FIG. 5. First derivative ESR spectra of a solution obtained by mixing thiolactic acid $2 \times 10^{-2}$ $M$ in 0.1 $N$ HCl and $5 \times 10^{-3}$ $M$ $CeK_2(NO_3)_6$ at room temperature. Flow rate, 1.0 ml/sec.

singlet at $g = 2.0186 \pm 0.0002$ in good intensity under the usual minimum concentrations used [$2 \times 10^{-2}$ $M$ thiol and $5 \times 10^{-3}$ $M$ $CeK_2(NO_3)_6$]. The oxidation of $\alpha$-mercaptopropionyl glycine resulted in the expected 1 : 1 doublet for "A," but the limited quantity of this thiol available at the time precluded optimizing flow conditions for observing "B."

The signals arising at high field values (in the region corresponding to "C") were no longer those of the previously noted singlet, but were of a much more complex nature, as will be discussed below.

*Tertiary Aliphatic Thiols*

The three tertiary thiols used (Table 4) all yielded the expected singlet ("A" signal) at $g = 2.0106 \pm 0.0002$, with a line width of 2.5 G in both ceric ion oxidizing

systems. This signal was obtained in good intensity, even at very slow flow rates (0.2 ml/sec) under the usual minimum concentrations of $2\times10^{-2}$ *M* thiol and $5\times10^{-3}$ *M* $Ce^{4+}$. The "B" singlet (g = 2.0186 ± 0.0002) was also obtained in good intensity with all three compounds in the $CeK_2(NO_3)_6$ system, but not at all in the $Ce(HSO_4)_4$–1 *N* $H_2SO_4$ system. The "C" spectrum was obtained as an unresolved singlet (g = 2.0032 ± 0.0002), both in the $Ce(HSO_4)_4$–1 *N* $H_2SO_4$ system at $5\times10^{-3}$ *M* $Ce^{4+}$ concentration and in the $CeK_2(NO_3)_6$ system at $Ce^{4+}$ ion concentrations of $3\times10^{-2}$ *M* and above.

Since the field between species "B" and "A" was now uncovered, and since "A" appears here as a singlet rather than a doublet or a triplet, a fourth free radical species, labelled "D" was also observed upon $CeK_2(NO_3)_6$ oxidation of all three tertiary thiols (Fig. 6). The isotropic g-value of this fourth singlet was determined

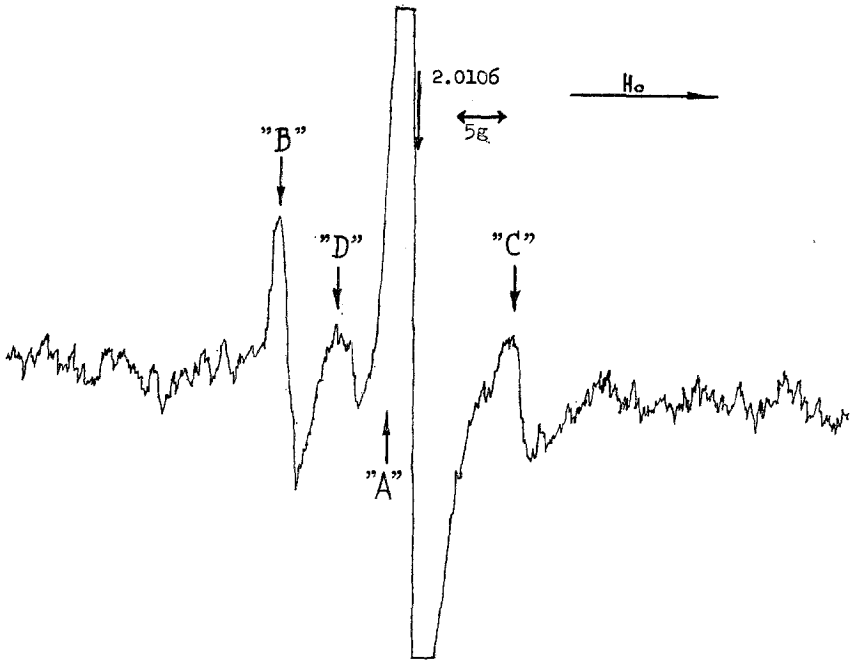



FIG. 6. First derivative ESR spectra of a solution obtained by mixing D-penicillamine, $2\times10^{-2}$ *M* in 0.1 *N* HCl and $7.5\times10^{-2}$ *M* $CeK_2(NO_3)_6$ at room temperature. Flow rate, 0.2 ml/sec.

as g = 2.0151 ± 0.0002 based on the internal "A" free radical as standard. The line width of this species is 4.0 G. The intensity of this singlet increases with increasing flow rate. Thus, singlets "B," "D," and "A" appear at 4 to 1 thiol/$Ce^{4+}$ ratio, while at 4 to 6 thiol/$Ce^{4+}$ ratio, all four singlets "B," "D," "A," and "C" are observed together. (See Fig. 6.) In the $Ce(HSO_4)_4$–1 *N* $H_2SO_4$ system, "D" has been obtained along with "A" and "C" only in the case of *N*-acetyl-DL-penicillamine, at which time "C" was relatively weak in intensity.

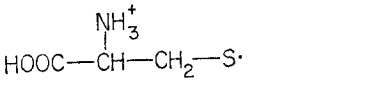
*Aromatic Thiols*

A number of aromatic compounds, including *o*-mercaptobenzoic acid, 2-thiobarbituric acid, 6-mercaptopurine, 2-thiolhistidine, and triphenylmethyl mercaptan, have been oxidized by ceric ion. Of these, only triphenylmethyl mercaptan displayed signals upon oxidation by both the $CeK_2(NO_3)_6$ and $Ce(HSO_4)_4$–1 *N* $H_2SO_4$ systems. Signals were obtainable with the other compounds only upon $CeK_2(NO_3)_6$ oxidation (i.e., when comparable concentrations were used).

The spectra were completely different from those of the aliphatic thiols, e.g., no "A," "B," "C," or "D" signals were obtained as such, but rather singlets were observed at the minimum $2\times10^{-2}$ *M* thiol and $5\times10^{-3}$ *M* $CeK_2(NO_3)_6$ concentrations. These singlets were resolvable into hyperfine patterns at ceric ion concentrations some twenty times greater (0.1 *M*). The g-values for these species ranged from 2.0003 to 2.010 and are thus in agreement with previous studies on ESR of aromatic thiols in solution (*25–26*).

*Identification of Signal "A" as the RS · Free Radical*

On the basis of the preceding considerations, it can be readily seen that the ESR signal we have so far called "A" can be readily assigned to the thiyl free radical I,



I

based on the following considerations:

(a) Its g-value is in the general region to be expected for sulfur radicals (e.g., the spin orbit coupling constant of sulfur is 382 $cm^{-1}$). It is higher than that of the aromatic thiyl free radicals and free radical anions (g = 2.007–2.009) (*25*a,b), probably because of the higher unpaired electron density on the sulfur atom. On the other hand, the g-value of "A" is lower than that of the sulfur free radical of Bennett (*27*), whose 2,2,6,6-tetramethylpiperidyl-1-thiyl radical has an isotropic g-value of $2.0173 \pm 0.0005$ when observed in DMF solution. As Bennett's species has an $-\overset{|}{N}-S\cdot$ system, the heavy electron density in the vicinity of the unpaired electron is likely to produce a low field shift of such a free radical signal. Thus, the g-value of "A" being intermediate between these two extreme cases is quite consistent with the assigned structure.

(b) Species "A" from cysteine is a 1 : 2 : 1 triplet. This hfs pattern suggests two adjacent protons, obviously on the α-carbon. All other primary thiols studied give a similar triplet, while secondary thiols (Table 3) give a 1 : 1 doublet, to be expected from RR'CHS· free radicals. Finally, tertiary thiols give the expected singlet at the same g-value.

(c) Oxidation of a number of substituted thiols (Table 2) always leads to species "A" when the thiol function is free. When the SH function itself is substituted, as in methionine, *S*-methyl-L-cysteine, and *N*-acetyl-DL-homocysteine thiolactone, no free radicals are detectable with the usual minimum concentrations in the $CeK_2(NO_3)_6$ system. However, upon $Ce(HSO_4)_4$–1 *N* $H_2SO_4$ oxidation, *S*-methyl-L-cysteine and *N*-acetyl-DL-homocysteine thiolactone exhibit very weak "A" triplets.

This is not surprising in view of the highly acidic nature of the $Ce(HSO_4)_4$–1 $N$ $H_2SO_4$ system, which may result in acid-catalyzed cleavage of the S–$CH_3$ bond (*28*). Other examples of cleavage of sulfur–sulfur bonds under the type of conditions used in this work (*29–30*), have also been reported. All these considerations lead us to conclude that species "A" is the thiyl free radical I in solution. This is further substantiated by the very interesting work of Armstrong (*29*), who studied the oxidation of amino acids by OH· radicals generated in the $Ti^{3+}$–$H_2O_2$ system. His results and ours are in excellent agreement, thus confirming the above assignment of the RS· free radical.

It is of interest to compare the properties of the thiyl radical in solution to those of the "sulfur" free radicals observed and studied in the solid phase by previous authors using ESR techniques. In the previous solid phase studies (*1–16*) the nature of the ESR spectrum obtained strongly depended on the temperature of observation, the time and temperature of annealing, etc. The signals obtained in such studies were usually asymmetric with relatively broad line width, and because of their "trapped" nature certainly do not lend themselves to dynamic studies.

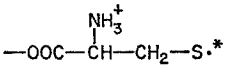
Only in single crystal studies can hyperfine couplings be obtained, but unfortunately this approach requires the preparation and use of such single crystals. The use of randomly oriented solid systems do not lend themselves to detailed ESR interpretations, and therefore the experiments undertaken here suggest a simpler method for extracting information about thiol containing intermediates.

The system described in this work, on the contrary, generates free radical species where one can readily distinguish between primary, secondary and tertiary thiols. Also, as can be seen from Table 2, reproducible and detectable differences exist in the ESR first derivative spectra of thiyl free radicals generated from different primary aliphatic thiols. This leads us to suggest that this system will be able to detect and identify the specific thiyl free radical sites in proteins and their environment, whereas with the exception of single crystal work, solid state studies only allow gross identification of the presence or absence of "sulfur" free radical sites.

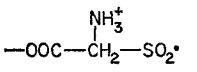
Furthermore, this system examines thiyl free radicals under dynamic conditions. It seems reasonable to suggest that species "B," "C," and "D" arise from the thiol, either by reaction of the RS· free radical with $O_2$ or $H_2O$, or by its internal rearrangement, or by an independent reaction of the thiol with the oxidizing agent. A few possible structures of such species are represented by structures II–VIII. Attempts to establish a genetic relationship based on kinetic studies (*30*) have so far been unsuccessful. The only species with which it has been possible, within the limitations of the experimental technique, to observe a buildup after stopping the mixing of cysteine and the oxidizing agent, is species "C."

The nature of this last species appears to be difficult to assign at this time. The low g-value, $2.0032 \pm 0.0002$, strongly suggests a high electron density on carbon. Possible species are represented by structures VII and VIII.

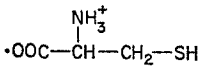
Whether the unpaired electron would be localized on $C_1$ or $C_2$ in structure VII, would vary with the nature of the substituents $R_1$ and $R_2$, since the reactivity and lability of the C–H bond are dependent on the inductive and field effects of these groups. Carboxylate radical ions of the type in structure VIII have been described (*31*) in studies at 77°K, the g-values found ranging from 2.0026 to 2.0032. The absence



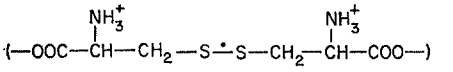
II



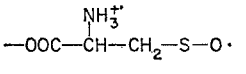
III



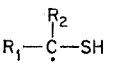
IV



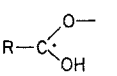
V



VI



VII



VIII

of observable hfs speaks against structures such as VII or VIII, but may also be due to lack of resolution resulting from oxygen broadening, solvent effects, etc. (*32*). Thus, it is not possible at this time to assign the structure of signal "C," other than to suggest that a carbon radical is the most likely because of its g-value.

On the same basis, the high g-value, $2.0186 \pm 0.0002$, of species "B" precludes either a carbon or a nitrogen free radical, and favors a free radical with the unpaired electron mainly localized on either sulfur or oxygen. Structures III–VI would be formed by reaction or rearrangement of the cysteinyl free radical I, whereas II is a possible excited precursor.

The predominance of the singlet "B" at faster flow rates would suggest that "B" is either a precursor, or at best a side product formed simultaneously with I. However, the flow rates used indicated free radical lifetimes of the order of $10\text{–}300 \times 10^{-3}$ sec and thus make it unlikely that we would be observing an excited species such as II. Carboxyl radicals such as structure IV with an unpaired electron on oxygen are very unstable (*33*) and rearrange very rapidly to the decarboxylation products. No evidence for such a process has been observed here.

Another possibility would result from the addition of I to cysteine, which might lead after hydrogen ion removal, to the semi-resolved ionized molecule V. Such a species has been observed by Adams (*34*) in spectrophotometric studies of the pulse radiolysis of cysteamine, but we failed to observe any evidence for such a species (*35*) under our conditions. Finally, the possibility of formation of species III, produced by the addition of $O_2$ to I, is suggested as likely, as the solutions used have not been de-aerated, and have thus a considerable amount of dissolved oxygen.

Several authors (*36–38*) have correlated ESR signals in $\gamma$-irradiated proteins and in other solid biological material with known "sulfur" spectra. Henriksen, in particular, has shown that upon annealing solid irradiated samples from 77°K to 296°K, migrations occur to the "sulfur" free radical species. Such results would tend to agree with the general contention that thiyl (RS·) free radicals are stable, terminal free radicals (*39*). Such behavior has been used to explain the mechanism of action of sulfur-containing radio-protective agents.

The present results suggest that there is little, if any, difference between the primary

thiyl free radicals produced by oxidation of a variety of simple thiols. While it is certainly true that our conditions (pH 1–2, concentrations $10^{-2}$–$10^{-3}$ $M$) are still very far removed from biological conditions, the fact that we are able to generate and study such radicals under liquid phase and dynamic conditions, is certainly a step in the right direction to try to gain a more accurate picture of the processes occurring upon radiation induced cellular damage. As it has been shown that oxidation by both the $Ti^{3+}$–$H_2O_2$ (*29*) and the $Ce^{4+}$ (*40*) systems give free radicals and products similar to those induced by radiation, it is clear that the system described in the present work should be particularly valuable in assessing the molecular basis of radiation damage and/or protection.

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