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Chimica. — *Free radical reactions in thioamino acids: cystine and cysteine.* Nota di MAURIZIO TAMBA (*), ROBERTO BADIELLO (*) e E. MARTIN FIELDEN (**), presentata (***) dal Socio G. SEMERANO.

RIASSUNTO. — Sono state irradiate con la tecnica della radiolisi ad impulsi soluzioni acquose diluite di cistina e cisteina. Si riportano gli spettri di assorbimento dei transienti nelle differenti condizioni sperimentali e si discute la natura delle specie radicaliche coinvolte. Si riportano dati cinetici sulla formazione e decadimento delle specie e si suggerisce il relativo meccanismo di reazione.

INTRODUCTION

The aqueous radiation chemistry of sulphur-containing compounds is of interest in radiobiology for the role played by these molecules in radioprotection and repair phenomena [1] and there is a wide literature on the subject [2]. In particular, Adams *et al.* [2] have shown that the —SH and —S—S— groups are respectively oxidized and reduced to give, in the end, a radical anion complex $RSSR^-$ and they reported the kinetics of formation and decay of these species in the cysteamine–cystamine system [3].

This paper reports the results obtained in a study of the properties of free radicals derived from the amino acids cystine and cysteine. Some preliminary results have been communicated already [4] but, while this work was in progress, other publications on the pulse radiolysis of disulphide and sulphydril compounds have appeared [5–7]. These are discussed in the text where necessary.

EXPERIMENTAL

DL-cystine, L-cysteine (Sigma) were used as supplied. Triply distilled water and phosphate buffer (5 mM) were used throughout and the pH's of the solutions were adjusted, where appropriate, by using small amounts of perchloric acid and potassium hydroxide.

The pulse radiolysis experiments have been carried out jointly with the 2 MeV Febetron 705 at Bologna and the 4.3 MeV Mullard SL 46 electron accelerator at Sutton. Details of the pulse radiolysis apparatus at Bologna and Sutton have been described elsewhere [8, 9].

Solutions were handled under specified gas compositions and pressure in either all glass flow system (Bologna) or all glass syringes (Sutton).

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Results and discussion

a) *Pulse radiolysis of cystine.*

An intense transient absorption with a maximum at 415 nm is produced when deaerated solutions of cystine are pulse irradiated (fig. 1).

This particular absorption is not affected by the presence of OH scavengers (methanol and *t*-butanol were used) but its appearance is prevented if the electron scavenger, nitrous oxide, is present in the solution. This absorption

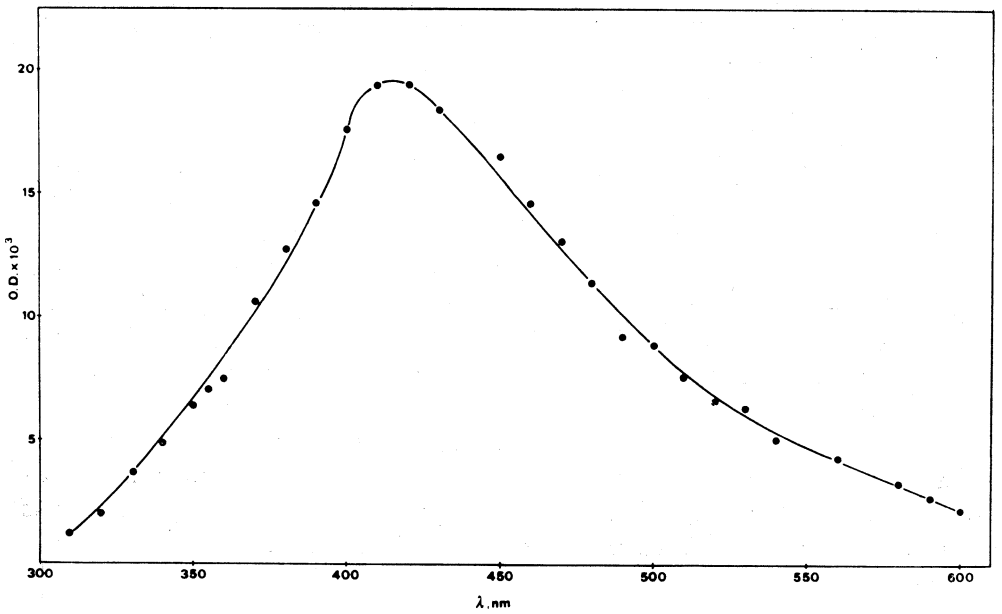

Fig. 1. – Absorption spectrum of 10^{-3} M deaerated solutions of cystine, 1 μsec after a single electron pulse. pH = 7.4, path cell = 2 cm, dose ≃ 750 rads.

has been noted previously in irradiated cystine solution [2, 5, 10] and similar transients absorbing in this spectral region have been found with other disulphide compounds [2, 5, 7]. These transients are ascribed to the disulphide anion $RSSR^-$ produced, in this case, by direct electron attachment:

(1) $$e^-_{aq} + \text{RSSR (Cystine)} \longrightarrow RSSR^-.$$

Under the conditions of these particular experiments, the radical anion is unstable and the transient absorption at 415 nm decays rapidly (in less than 20 μsec) by a first-order process leaving a much weaker absorption in the 320–360 nm region (fig. 2). This absorption decays in turn, but with a second-order kinetics.

The first-order decay of the $RSSR^-$ species has been ascribed to the reaction:

(2) $$RSSR^- \longrightarrow RS + RS^-$$

Our values for the extinction coefficient of $RSSR^-$ at 415 nm of 7.8×10^3 (based on $G e^-_{aq} = 2.7$) and of the rate constant $k_2 = 5.4 \pm 0.5 \times 10^5$ sec^{-1} (average of 10 determinations) are fair agreement with the values reported in the literature [5, 10].

The thiol radical, produced via reaction (2), is believed to be responsible for the weak absorption remaining after the decay of $RSSR^-$, but this is complicated by absorptions due to the radical product of the reaction between

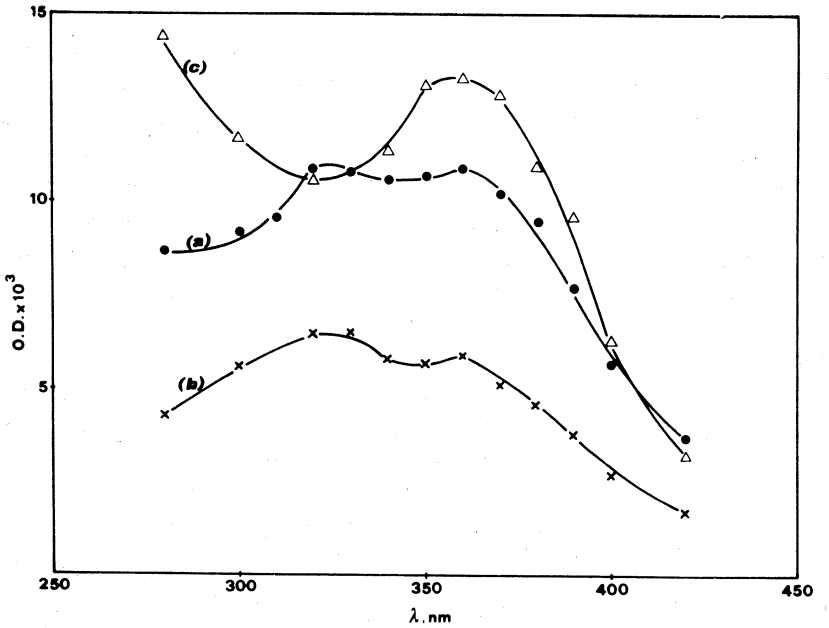

Fig. 2. – Absorption spectra of 3×10^{-4} M cystine: (*a*) Argon saturated, 20 μsec after the pulse; (*b*) Argon saturated in the presence of 0.1 *t*-butanol, 20 μsec after the pulse; (*c*) Nitrous oxide saturated, 1 μsec after the pulse. pH = 7.4, path cell = 7.5 cm, dose ≃ 800 rads.

OH and cystine. This is readily demonstrated by the fact that the decay kinetics of the 320–360 nm absorptions are not uniform throughout the spectral range and that the presence of OH scavengers in the solution reduces the size of the absorption. In order to assign the various components of this radical absorption, other experiments were carried out.

Fig. 2 shows the spectrum recorded by irradiating cystine in the presence of N_2O (*c*). This served to remove e^-_{aq} and produce an extra (double) yield of OH radicals by the reaction:

$$(3) \qquad e^-_{aq} + N_2O \xrightarrow{H_2O} N_2 + OH + OH^-.$$

Thus, in this solution, only OH and H radicals are present in the relative amounts of G, 5.4 and 0.5 respectively.

Also shown is the spectrum recorded after the decay (20 μsec) of $RSSR^-$ in a solution containing 0.1 M *t*-butanol (*b*).

This alcohol does not interfere with the e^-_{aq} and H atom species but will completely scavenge the OH radical. The resultant *t*-butanol radical is regarded as unreactive and presents a negligible absorption in this spectral region.

The only primary radicals available in this solution then are e^-_{aq} and H atoms in the relative proportion of G 2.7 and 0.5. These spectra can be compared with that produced after 20 μsec in deaerated solution (*a*). This solution initially contained all three primary radicals, e^-_{aq}, OH, H in the proportion G 2.7, 2.7 and 0.5 respectively. It is apparent that spectrum (*c*) is markedly different from (*b*) and (*a*). Thus spectrum (*a*) is composed of species produced from (Ge^-_{aq} + GH + GOH) whereas (*b*) and (*c*) are produced from (Ge^-_{aq} + GH) and (GH + 2 GOH) respectively. It should be possible to derive the radical spectra resulting from each separate primary radicals by suitable arithmetic combinations of (*a*), (*b*) and (*c*). For example, the OH product should be represented by (*a* — *b*) and the hydrogen atom product by $c - 2 \times (a - b)$. In practice such manipulations cannot be relied upon to give accurate spectra because of the assumptions involved, i.e. the precise radical yields and the effect of decay on spectra recorded at different times. These manipulations do show however that the H and OH radicals produced an absorption peaking at 360 nm and the species remaining after the decay of $RSSR^-$ has a single peak at 320 nm. It is likely that the radical absorbing at 360 nm is produced via abstraction of a hydrogen atom from the weakest C—H bond, and in particular at the more highly substituted position.

b) *Pulse radiolysis of cysteine.*

It is known that —SH groups react rapidly with hydrated electron, but they are also very sensitive to oxidation and the RS radical, produced initially by the reaction:

$$RSH + OH \longrightarrow RS + H_2O \tag{4}$$

can be converted into $RSSR^-$ by the reaction:

$$RS + RS^- \rightleftarrows RSSR^-. \tag{5}$$

In the pH range 5–6, cysteine is present in its unionized form, so that the RS radicals produced via reaction (4) should not be converted into $RSSR^-$. In fact, pulse irradiation of N_2O saturated solutions of cysteine at these pH values, yields an insignificant absorption at 415 nm, although a weak transient absorption is observed at 320 nm (fig. 3 a). The spectrum and its extinction coefficient (Table I) is similar to that derived from the rapid decomposition of $RSSR^-$ formed by reaction (1). We believe that the intermediate with λ_{max} at 320 nm is the thyl radical RS. The decay kinetics of this transient are always second-order under our experimental conditions.

In solutions at pH $\geq$ 7, cysteine is partially ionized:

$$RSH \rightleftarrows RS^- + H^+. \tag{6}$$

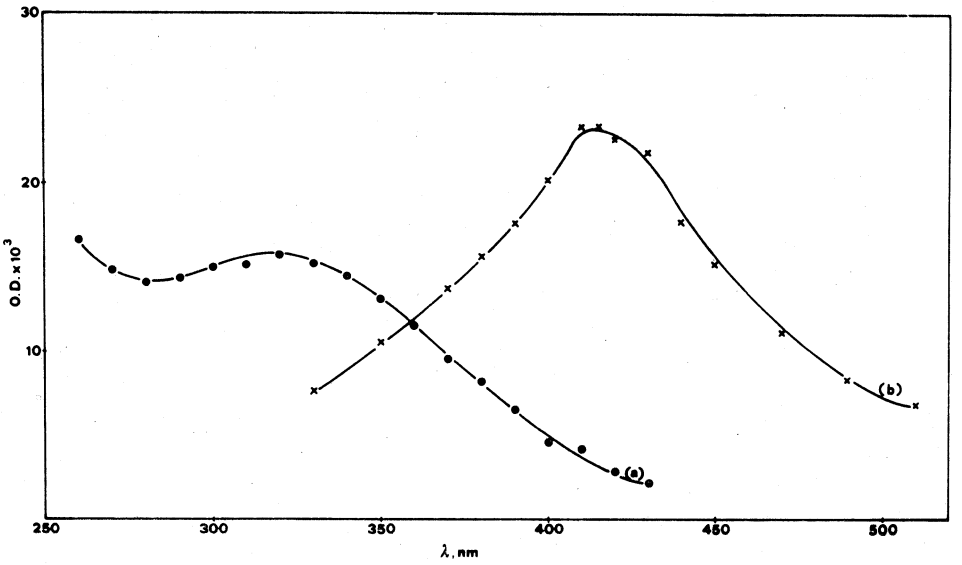

Fig. 3. – (*a*) Absorption spectrum of 3×10^{-4} M cysteine saturated with N_2O, 2 μ sec after a single pulse; pH = 5, path cell = 7.5 cm, dose ≃ 1 Krad. (*b*) Absorption spectrum of 10^{-3} M cysteine saturated with N_2O, 2 μ sec after the pulse; pH = 7.4, path cell = 2 cm, dose ≃ 1 Krad.

The fraction of cysteine in ionized form RS^-, was determined as a function of pH, by the method of Benesch and Benesch [11]. When cysteine is irradiated in N_2O saturated solutions at pH ≥ 7, the typical absorption of $RSSR^-$ is observed at 415 nm (fig. 3 b). The rate of formation of $RSSR^-$ was determined directly by the build-up of the transient at 415 nm at different pH values. The rate of growth of $RSSR^-$ showed first-order kinetics and exhibits an increasing rate with higher pH's. The rate constant k ($RS + RS^-$) was found to be $2.1 \times 10^9\ M^{-1}\ sec^{-1}$ and $1.3 \times 10^9\ M^{-1}\ sec^{-1}$ respectively at pH 9 and 8. These data are in agreement with the value reported by Hoffman and Hayon [6].

TABLE I

Absorption maxima, extinction coefficients and decay kinetics of radicals.

Compound	Radical	pH	λ_{max} (nm)	ε_{max}	Decay kinetics
Cystine . .	$RSSR^-$	7.4	415	7.8×10^3	$5.4 \pm 0.5 \times 10^5\ sec^{-1}$
	RS·	7.4	320	320	—
	$[-SCH_2\dot{C}(NH_3)^+COO^-]_2$	7.4	360	380	$3.4 \pm 0.3 \times 10^8\ M^{-1}\ sec^{-1}$
Cysteine . .	RS·	5.0	320	340	$1.4 \pm 0.1 \times 10^9\ M^{-1}\ sec^{-1}$

The second-order kinetic decay of $RSSR^-$ is dependent upon the pH and cysteine concentration (fig. 4 b and 4 a), in agreement with the equilibrium reactions (5) and (6). The amount of $RSSR^-$ formed is also a function of the pH, the 415 nm absorption having a maximum value at pH 8.5. At higher pH's the pH dependence can be related to the pK of the —SH group

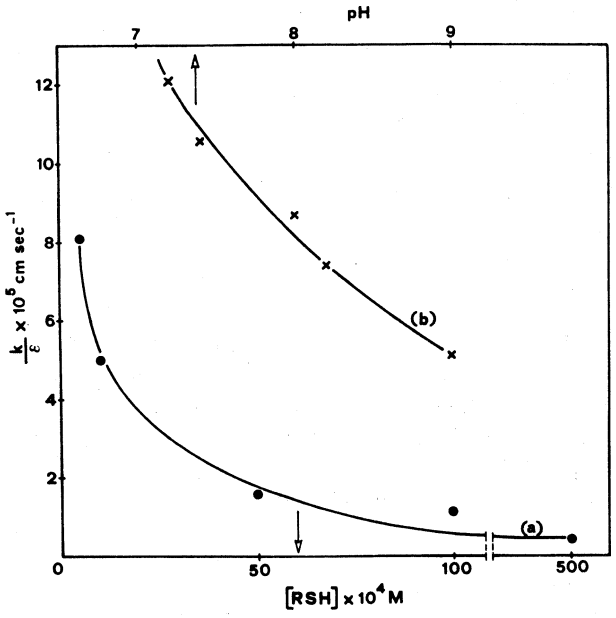

Fig. 4. – Effect of cysteine concentration on the slopes of second-order kinetic decay of $RSSR^-$ at pH 9 (*a*); effect of pH on the slopes of second-order kinetic decay of $RSSR^-$ at [RSH] = 1 mM (*b*).

and to the overall charge of the RS and RS^- species. Since on increasing the pH, the total charge of the sulphydril molecule changes from zero, in neutral solutions, to —2 in alkaline solutions, the coulombic interaction between the negatively charged RS and RS^- species at high pH's may account for the smaller yield of $RSSR^-$ found in these experiments. A similar pattern has been observed in other sulphydril compounds [6, 7].

c) *Determination of the equilibrium constant* K_5.

The equilibrium constant for reaction (5) has been calculated in the pH range 7.2–9.3 and for RSH concentrations from 5×10^{-5} M to 10^{-2} M. K_5 is defined as:

$$K_5 = \frac{[RSSR^-]_{eq}}{[RS]_{eq}\,[RS^-]} \cdot$$

The fraction of cysteine in the form RS^- was determined as a function of pH by the method of Benesch and Benesch. $[RSSR^-]_{eq}$ was calculated from the maximum absorption observed at 415 nm, on irradiating N_2O-saturated

solutions of cysteine. The value of ε_{415} used was obtained from the experiments in which $RSSR^-$ was produced directly from cystine. $[RS]_{eq}$ was determined from the stoichiometric relationship: $G(OH) = G(RS + RSSR^-)$. Corrections had to be made at the highest concentrations of RSH where some e_{aq}^- were not converted into OH by N_2O since RSH itself could compete for e_{aq}^-. Out of the fraction of e_{aq}^- that reacts with RSH, some, but not all, will also yield the RS radical:

$$RSH + e_{aq}^- \longrightarrow R + HS^- \quad (7)$$

$$R + RSH \longrightarrow RS + RH. \quad (8)$$

By comparing the yield of $RSSR^-$ produced in a 10^{-3} M cysteine solution with and without presence of N_2O, it was found that only 20 % of the e_{aq}^- that react with RSH subsequently produced RS and hence, ultimately, $RSSR^-$.

The values of the equilibrium constant at different pH's are reported in Table II. These values are of the same order of magnitude calculated for other sulphydril compounds [3, 6, 7, 10] from either the method here described and from the ratio of the rate constants k_5/k_{-5}. The marked and different dependence on pH's of the rate constants of formation and disappearance of $RSSR^-$ in several thio–compounds is strictly reflected in the values of the equilibrium constants, making a careful comparison among them difficult.

TABLE II

Equilibrium constant values obtained for reaction (5) *at different* pH.

Compound	pH	$K_5 \times 10^{-3}$ (M^{-1})	$\frac{[RSSR^-]_{eq}}{[RS]_0}$ (*)
Cysteine	7.2	4.6±0.30	0.18
	7.5	3.8±0.15	0.25
	8.0	1.7±0.30	0.28
	9.0	1.0±0.10	0.35
	9.3	0.8±0.04	0.33

(*) $[RS]_0 = [RS] + [RSSR^-]$ represents the total radical concentration. The ratio-values reported have been determined from [RSH] = 1 mM.

In experiments carried out at pH 8, 9 and 10 with different initial concentrations of RSH, so as to maintain the concentration of RS^- present in the solution constant, the absorption due to $RSSR^-$ at pH 9 and 10 is 86 % and 37 % respectively of that recorded at pH 8. This confirms that not only the protonation of the sulphydril group is involved in equilibrium (5), but also that

there is a marked dependence on the overall charge of the species which is affected by the degree of protonation of the $-NH_3^+$ groups. In addition the reaction:

$$RS + RS \longrightarrow RSSR \tag{9}$$

has to be considered. It was suggested that the step (9) is rate determining in the second-order decay of $RSSR^-$ [3]. The rate of this reaction will also depend on the charge of the species and would be expected to be slower at higher pH's. However, at relatively high pH's, the decay of $RSSR^-$ may also take place through the additional reaction:

$$RS + RSSR^- \longrightarrow \text{products} \tag{10}$$

since this has been observed with other sulphydril compounds [7, 10, 12].

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