Lactoperoxidase-Catalyzed Oxidation of Thiocyanate: Equilibria between Oxidized Forms of Thiocyanate†

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ABSTRACT: Lactoperoxidase-catalyzed oxidation of thiocyanate ion (SCN^-) was studied in the pH range 5–8 so as to obtain either hypothiocyanous acid (HOSCN) or hypothiocyanite ion ($OSCN^-$) as the major observed product. A pK_a value of 5.3 was calculated for HOSCN, from the effect of pH on two independent parameters: (1) the extent of extraction of HOSCN into organic solvents from aqueous mixtures of HOSCN and $OSCN^-$ and (2) the rate of decomposition of HOSCN–$OSCN^-$ mixtures. Partition coefficients of 5.0, 2.1, and 2.3 were calculated for extraction of HOSCN into ethyl acetate, 1-octanol, and 2-octanol. Identification of HOSCN was confirmed by the 2:1 stoichiometry for extraction of oxidizing equivalents and the SCN moiety. Decomposition of HOSCN–$OSCN^-$ was accelerated by SCN^-, which also changed the apparent kinetic mechanism of decomposition. A second-order rate constant of 3 M^{-1} s^{-1} was calculated for decomposition of HOSCN in the presence of 0.1–0.2 mM SCN^-, assuming the rate-limiting step to be the dismutation of HOSCN. A first-order constant of 2.6×10^{-3} s^{-1} was calculated at 10 mM SCN^-, assuming the reaction of HOSCN with SCN^- to be rate limiting. A number of pH buffering agents also accelerated the decomposition of HOSCN–$OSCN^-$. These agents or SCN^- had no effect on extraction of HOSCN into organic solvents. Certain nitrogenous compounds stabilized the oxidizing activity of HOSCN–$OSCN^-$ mixtures, apparently by lowering the concentration of free HOSCN. Stabilization by sulfonamides and aromatic imines was consistent with formation of derivatives containing the nitrogen–thiocyanate (N–SCN) linkage (thiocyanatosulfonamides and thiocyanatimines). These N–SCN derivatives retained the oxidizing equivalents of HOSCN or $OSCN^-$, but differed from HOSCN in their solubility in organic solvents. The results indicate that HOSCN is formed in significant amounts and can be relatively stable in the pH range consistent with peroxidase-catalyzed oxidation of SCN^- in exocrine secretions, leukocytes, and the thyroid gland. The neutral HOSCN molecule may be responsible for biological activities that have been attributed to the $OSCN^-$ anion. Also, the biological activity of HOSCN–$OSCN^-$ may be much greater at low pH, provided that the concentrations of SCN^- and other components of the medium favor the stability of HOSCN.

The mammalian hemoprotein peroxidases lactoperoxidase, myeloperoxidase, and thyroid peroxidase catalyze the oxidation of thiocyanate ion (SCN^-) by hydrogen peroxide (H_2O_2). Lactoperoxidase, H_2O_2, and SCN^- form an antimicrobial system in milk, saliva, and perhaps also in tears (Wright & Tramer, 1958; Zeldow, 1963; Klebanoff et al., 1966; Morrison & Allen, 1966; Thomas et al., 1981). This system contributes to the antimicrobial activity of exocrine secretions. Oxidation of SCN^- may also contribute to the myeloperoxidase-mediated antimicrobial activity of leukocytes. Also, SCN^- competes with iodide ion as a substrate for thyroid peroxidase (Wood, 1975). High levels of SCN^- are toxic to the thyroid gland, though it has not been established that oxidation of SCN^- is reponsible for the antithyroid effect.

A pathway of SCN^- oxidation was proposed, based on the observation that the peroxidase–H_2O_2–SCN^- system is analogous to a halogenating system (Morrison & Schonbaum, 1976; Aune et al., 1977; Aune & Thomas, 1978).

$$H_2O_2 + 2SCN^- + 2H^+ \xrightarrow{\text{peroxidase}} 2H_2O + (SCN)_2$$

$$(SCN)_2 + H_2O \rightarrow HOSCN + H^+ + SCN^-$$

$$HOSCN \rightleftharpoons H^+ + OSCN^-$$

These equations illustrate the relationship between $(SCN)_2$ (thiocyanogen), HOSCN (hypothiocyanous acid), and $OSCN^-$ (hypothiocyanite ion). The equations do not necessarily indicate that all of these agents are produced or that $(SCN)_2$ is the agent released from the peroxidase active site.

Indirect evidence was obtained for lactoperoxidase-catalyzed oxidation of SCN^- to yield $(SCN)_2$ or some other agent more reactive than $OSCN^-$ (Aune & Thomas, 1978). Nevertheless, $OSCN^-$ is the major observed product of SCN^- oxidation at neutral pH (Hoogendoorn et al., 1977; Aune & Thomas, 1977). Production of $OSCN^-$ is consistent with the equation

$$H_2O_2 + SCN^- \rightarrow H_2O + OSCN^-$$

Identification of $OSCN^-$ was based on the oxidation of 2 mol of a sulfhydryl compound (RSH) to the disulfide (R–S–S–R), with recovery of 1 mol of SCN^-.

$$OSCN^- + 2R\text{–}SH \rightarrow SCN^- + R\text{–}S\text{–}S\text{–}R + H_2O$$

The same stoichiometry would be obtained with HOSCN or a mixture of HOSCN and $OSCN^-$. However, the stoichiometry does rule out $(SCN)_2$. Identification of $OSCN^-$ rather than HOSCN as the product was based on the greater stability of the product at high pH, and on the inability to extract the product into organic (nonpolar) solvents from aqueous solutions at neutral pH.

Two modes of decomposition of $(SCN)_2$, HOSCN, or $OSCN^-$ in aqueous solutions have been described. At low concentrations (<0.5 mM), the rate of disappearance of oxidizing equivalents follows second-order kinetics (Walden & Audrieth, 1928; Wood, 1946). The rate-limiting step was proposed to be the dismutation of two HOSCN molecules, with formation of cyanosulfurous acid (HO_2SCN) as an intermediate.

$$2HOSCN \rightarrow HO_2SCN + SCN^- + H^+$$

Decomposition of HO_2SCN and the remaining steps in decomposition were rapid and yielded sulfate, carbon dioxide,

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ammonia, and SCN^-. Second-order kinetics were also observed when $OSCN^-$ was the predominant form (Aune & Thomas, 1977), whereas others have reported first-order kinetics.[1]

At high concentrations of $(SCN)_2$, HOSCN, or $OSCN^-$, or in strong acid, another mode of decomposition has been observed which yields cyanide (HCN) (Betts & Dainton, 1953; Hughes, 1975; Aune & Thomas, 1977). The HCN in turn reacts rapidly with HOSCN or $OSCN^-$, with the resulting loss of oxidizing equivalents. One of several proposed pathways of decomposition involves formation of NC–SCN (cyanogen thiocyanate, sulfur dicyanide) as a short-lived intermediate (Wilson & Harris, 1960, 1961; Kitching et al., 1962; Chung & Wood, 1970). Whatever the mechanism, this mode of decomposition is much faster than that observed at low concentrations of HOSCN or $OSCN^-$.

This rapid decomposition of HOSCN or $OSCN^-$ at high concentrations prevents many experimental approaches to the characterization of these substances. Attempts to determine the pK_a of HOSCN by pH titration have been unsuccessful.

Such characterization may be relevant to understanding the biological effects of SCN^- oxidation. For example, it has been proposed that the antimicrobial activity of $OSCN^-$ is limited by its anionic character (Thomas, 1978; Thomas & Aune, 1978). Biological membranes are impermeable to anions, except for anions that are substrates for specific transport systems. According to the proposal, the antimicrobial action of $OSCN^-$ is limited to oxidation of exposed microbial components, such as membrane-transport systems and electron-transport components. This proposal also implies that HOSCN would be a more potent antimicrobial agent than $OSCN^-$, because the uncharged HOSCN could diffuse through the hydrophobic membrane barrier and could oxidize intracellular components. On the other hand, the biological activity of HOSCN might be limited by its rapid rate of decomposition.

The aim of this study was to determine whether significant amounts of HOSCN are obtained in the pH range consistent with peroxidase-catalyzed oxidation of SCN^- in vivo. Also, parameters that determine the stability and reactivity of HOSCN–$OSCN^-$ mixtures were studied to determine whether HOSCN can be sufficiently stable to exert a biological effect. Finally, the solubility of HOSCN in organic solvents was studied to determine whether this agent has sufficient nonpolar character to diffuse through biological membranes.

Materials and Methods

Lactoperoxidase, purified from bovine milk (Morrison & Hultquist, 1973), was provided by Dr. M. Morrison and was dialyzed against 0.2 M KCl. Catalase, dithiothreitol, and 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2)[2] were obtained from Sigma. A 1 mM solution of Nbs_2 in 0.1 M Tris-HCl, pH 7, was reduced to Nbs with excess sodium borohydride, shaken at 37 °C for 15 min, and then stored overnight under nitrogen (N_2) to permit decomposition of excess borohydride. The Nbs concentration was calculated from the molar extinction coefficient of 13 600 at 412 nm (Ellman, 1959). Ethyl acetate (spectrophotometric grade) obtained from Fisher was washed with 0.1 M NaCl containing 0.1 mg/mL sodium borohydride for 30 min, briefly with 0.1 M NaOH in 0.1 M NaCl, and then repeatedly with 0.1 N NaCl. This procedure was necessary to remove oxidizing substances and acids. The 1- and 2-octanols were washed with 0.1 M NaCl. The $K^{35}SCN$ (46 Ci/mol) was from New England Nuclear, and $NaS^{14}CN$ (8 Ci/mol) was from ICN.

Synthesis of HOSCN–$OSCN^-$. Reaction mixtures contained 0.1 μM lactoperoxidase, calculated from the extinction coefficient of 11 400 M^{-1} cm^{-1} at 412 nm. Lactoperoxidase, H_2O_2, and SCN^- were incubated 5 min, and then 1 μg/mL catalase was added to remove any unreacted H_2O_2. Alternatively, $(SCN)_2$ was synthesized in CCl_4 by reacting excess lead thiocyanate with bromine as described previously (Aune et al., 1977). A 2-mL portion of the $(SCN)_2$ solution was diluted to 50 mL with CCl_4 and then added dropwise to 100 mL of rapidly swirled, cold 0.1 N NaOH. The aqueous phase was decanted and adjusted to pH 8 with 0.1 M HCl. Portions were diluted 1:5 with the desired solution, and the pH was adjusted with dilute HCl. Each experiment was carried out with HOSCN–$OSCN^-$ mixtures obtained enzymatically, and with mixtures obtained by alkaline hydrolysis of $(SCN)_2$. Essentially identical results were obtained, so that only the enzymatic experiments are reported.

The choice of pH buffering agent was critical to the stability of HOSCN–$OSCN^-$. For this reason, each experiment was performed in unbuffered 0.1 M NaCl carefully adjusted to the desired pH with dilute HCl or NaOH. At the HOSCN–$OSCN^-$ concentrations used in these experiments, complete decomposition resulted in a pH change of less than 0.1 unit. The experiments were repeated in the presence of Tris-HCl buffers, which were found to have no effect on extraction or decomposition. In the experiments reported here, all reaction mixtures contained 0.1 M NaCl and 0.1 M Tris-HCl buffers, which were added to assist in maintaining the desired pH in the range of 7–8. For consistency, Tris-HCl was also added in the pH range 5–7. Because Tris-HCl provides little buffering capacity in the acid range, the pH was carefully adjusted even when Tris-HCl was added. All solutions such as those of buffers or sulfonamides were adjusted to the desired pH prior to addition. All experiments were at 25 °C unless otherwise noted.

Determination of HOSCN, $OSCN^-$, and N–SCN Derivatives. An excess of the Nbs solution was added to the sample, the pH was adjusted to 7 with 1 M Tris-HCl buffer, and the absorbance at 412 nm was measured. The concentration of the oxidizing agents was calculated from one-half the difference between the amount of Nbs added and the amount remaining (Aune & Thomas, 1977).

Extraction. Portions (3 mL) of reaction mixtures were extracted with 3 mL of organic solvent equilibrated with 0.1 M NaCl. A 2-mL portion of the organic phase was transferred to 2 mL of 0.1 M NaCl–0.1 M Tris-HCl, pH 7, and Nbs was added. Also, Nbs was added to the remaining portion of combined aqueous and organic phases. The absorbance of the resulting aqueous phases was measured. The amount of oxidizing equivalents in the organic phase was calculated as 1.5 times that in the 2-mL portion. The amount in the aqueous phase was calculated as that in the remaining aqueous and organic phases minus half that in the 2-mL portion of the organic phase. Extraction and quantitation were performed rapidly (<5 min) so as to minimize decomposition. Values were corrected for incomplete phase separation by measuring transfer of $S^{14}CN^-$ from the aqueous to the organic phase. The percent extracted was 0.5% for ethyl acetate and 0.1% for octanol and was independent of pH.

Determination of SCN^-. Portions of solutions were adjusted to 0.5 mL with water and mixed with 0.5 mL of 20 mM

[1] K. M. Pruitt (1980) A.A.D.R. Symposium.

[2] Abbreviations used: Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); Nbs, 5-thio-2-nitrobenzoic acid; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

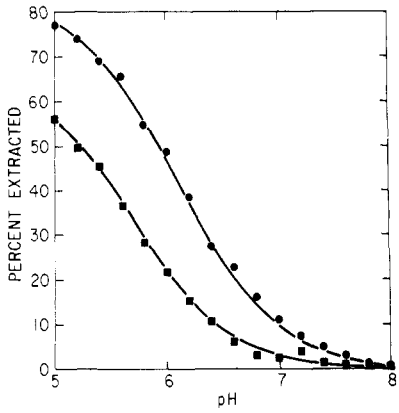

FIGURE 1: Effect of pH on extraction. Lactoperoxidase, 0.1 mM H_2O_2, and 0.3 mM SCN^- were incubated at the indicated pH, and then the reaction mixture was extracted with ethyl acetate (●) or 1-octanol (■). Extraction of oxidizing equivalents into the organic phase was calculated as a percentage of the equivalents recovered in the organic and aqueous phases. Total recovery varied by less than 5% over this pH range.

$FeCl_3$–1 M HCl, and SCN^- was quantitated from the absorbance of the $Fe(SCN)^{2+}$ ion at 450 nm (Betts & Dainton, 1953).

Determination of $^{35}SCN^-$ and $^{35}SO_4^{2-}$. Separation and quantitation of $^{35}SCN^-$ and $^{35}SO_4^{2-}$ by chromatography on cellulose sheets with *tert*-amyl alcohol–2,6-lutidine–water, 19:19:2, were performed as described previously (Aune et al., 1977).

Results

Extraction. Figure 1 shows that low pH favored extraction of oxidizing equivalents into the organic phase, and a larger percentage was extracted into ethyl acetate than into 1-octanol. In other experiments, only about 2% of the oxidizing equivalents could be extracted into chloroform from the aqueous phase at pH 5.

To account for the effect of pH, it was assumed that only HOSCN and not $OSCN^-$ is sufficiently nonpolar to be extracted into organic solvents. Because extraction depletes the aqueous phase of HOSCN, the $OSCN^-$ becomes protonated until the concentration of HOSCN in the aqueous phase ($HOSCN_{aq}$) is in equilibrium with HOSCN concentration in the organic phase ($HOSCN_{ex}$) and with $OSCN^-$ concentration in the aqueous phase ($OSCN^-_{aq}$).

$$HOSCN_{ex} \rightleftarrows HOSCN_{aq} \rightleftarrows H^+_{aq} + OSCN^-_{aq}$$

The ratio of ($HOSCN_{ex}$) to ($HOSCN_{aq}$) at a 1:1 volume ratio of the organic and aqueous phases is given by the partition coefficient, P.

$$P = (HOSCN_{ex})/(HOSCN_{aq})$$

The ratio of ($HOSCN_{aq}$) to ($OSCN^-_{aq}$) is determined by the equilibrium constant for dissociation (K_{eq}) and by the proton concentration (H^+_{aq}).

$$K_{eq} = (H^+_{aq})(OSCN^-_{aq})/(HOSCN_{aq})$$

Combining these equations, and substituting the two measured quantities

$$E = (HOSCN_{ex})$$

$$R = (HOSCN_{aq}) + (OSCN^-_{aq})$$

yields

$$P = E[K_{eq} + (H^+_{aq})]/[(H^+_{aq})R]$$

Because P should be invariant with respect to pH

$$P = E_i[K_{eq} + (H_i)]/[(H_i)R_i] = E_j[K_{eq} + (H_j)]/[(H_j)R_j]$$

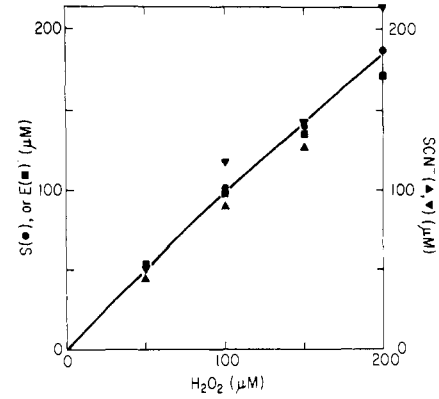

FIGURE 2: Stoichiometry of extraction of oxidizing equivalents and the SCN moiety. Lactoperoxidase, 1 mM SCN^-, and the indicated concentrations of H_2O_2 were incubated at pH 7. Portions of the reaction mixtures were taken for determination of the sum (S, ●) of HOSCN and $OSCN^-$ concentrations. Portions were adjusted to pH 5 and extracted twice with an equal volume of ethyl acetate. The amount of HOSCN extracted (E, ■) was calculated. Portions were acidified and extracted, and then the SCN^- concentration of the aqueous phase was measured. The decrease in SCN^- concentration of the aqueous phase was calculated (▲). The combined organic phase was added to 0.1 M NaCl–0.1 M Tris-HCl, pH 7, with 0.2 mM dithiothreitol, the SCN^- concentration of the aqueous phase was determined, and the amount of SCN^- recovered from the organic phase was calculated (▼).

where i and j denote values obtained at two different H^+_{aq} concentrations [(H_i) and (H_j)]. Solving for K_{eq}

$$K_{eq} = (H_i)(H_j)(R_iE_j - R_jE_i)/[(H_j)R_jE_i - (H_i)R_iE_j]$$

This equation was solved for each of the 136 possible pairs of results obtained with ethyl acetate shown in Figure 1. A median value for K_{eq} of 4.78 μM (pK_a = 5.32) and a median value for P of 5.0 were obtained. The same analysis was performed for results obtained with 1-octanol. Median values for K_{eq} of 6.71 μM (pK_a = 5.17) and for P of 2.1 were obtained. The curves drawn through the points in Figure 1 are plots of $100E/(E + R)$ vs. pH, using the median values of K_{eq} and P to calculate E and R. In other experiments, median values for K_{eq} of 5.56 μM (pK_a = 5.26) and for P of 2.3 were obtained from extraction of HOSCN into 2-octanol.

Figure 2 shows that extraction of HOSCN provided a method to confirm the identity of HOSCN (or $OSCN^-$). The addition of H_2O_2 to lactoperoxidase and SCN^- resulted in formation of about 1 mol of combined HOSCN and $OSCN^-$ (S) per mol of H_2O_2. Upon acidification and repeated extraction, about 1 mol of HOSCN (E) per mol of H_2O_2 was recovered in the ethyl acetate phase. The amount of SCN^- in the aqueous phase decreased by about 1 mol per mol of H_2O_2 upon oxidation and extraction, and this SCN^- was recovered upon reduction of the extracted HOSCN. These results confirm that HOSCN rather than $(SCN)_2$ was the extracted species. Also, no HCN was obtained upon reduction, indicating that NC–SCN was not present.

Decomposition. Figure 3 shows that SCN^- accelerated the decomposition of HOSCN–$OSCN^-$. The effect of SCN^- on stability was most pronounced at low pH. Oxidation of SCN^- was carried out at the indicated SCN^- concentration, and then the sum (S) of HOSCN and $OSCN^-$ was measured after 1 h. Similar results were obtained when SCN^- oxidation was carried out with 0.3 mM SCN^-, and increasing concentrations of SCN^- were added after SCN^- oxidation was complete.

At high concentrations (0.1–1 M), SCN^- lowered the "ceiling" at which HOSCN–$OSCN^-$ underwent instantaneous decomposition. At intermediate SCN^- concentrations (1–10

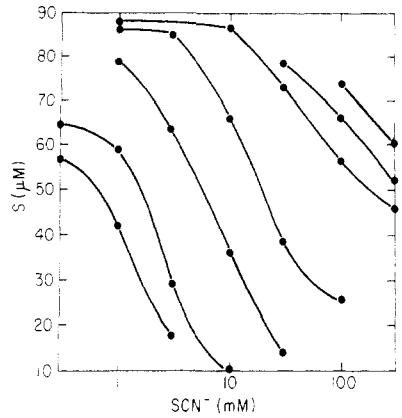

FIGURE 3: Effect of SCN^- concentration on decomposition. Lactoperoxidase, 0.1 mM H_2O_2, and the indicated concentrations of SCN^- were incubated at pH 5, 5.4, 6, 6.4, 7, 7.4, and 8 (curves from left to right). The HOSCN–$OSCN^-$ concentration was determined after 1 h.

mM), decomposition appeared first order with respect to HOSCN–$OSCN^-$ concentration:

$$\ln S = \ln S_0 - k_{app_e} t$$

where S_0 is the initial concentration, k_{app_e} is the apparent first-order rate constant, and t is time. At low SCN^- concentrations (0.2–1 mM), decomposition appeared second order:

$$1/S = 1/S_0 + k_{app}' t$$

where k_{app}' is the apparent second-order rate constant.

Figure 4 (top) shows plots of log S vs. t at pH values of 5–6, in the presence of 10 mM SCN^-. The slope of the lines [k_{app}, or $k_{app_e}/(\ln 10)$] decreased with increasing pH. Figure 4 (bottom) shows a plot of k_{app} vs. pH. To account for the effect of pH, it was assumed that the rate of decomposition of S is proportional to that portion of S which is in the form of HOSCN.

$$dS/dt \propto (HOSCN)$$

Substituting for (HOSCN) from the equations

$$S = (HOSCN) + (OSCN^-)$$

$$K_{eq} = (H^+)(OSCN^-)/(HOSCN)$$

yields

$$dS/dt \propto S[1 + K_{eq}/(H^+)]^{-1}$$

Integrating this equation yields

$$\ln S = \ln S_0 - [1 + K_{eq}/(H^+)]^{-1} kt$$

$$k_{app_e} = [1 + K_{eq}/(H^+)]^{-1} k$$

When $(H^+) \gg K_{eq}$, $k_{app_e} = k$ and $S = (HOSCN)$, so that k is the first-order rate constant for decomposition of HOSCN, at a particular concentration of SCN^-.

Because k should be invariant with respect to pH

$$k = k_i[K_{eq} + (H_i)]/(H_i) = k_j[K_{eq} + (H_j)]/(H_j)$$

Solving for K_{eq}

$$K_{eq} = (H_i)(H_j)(k_i - k_j)/[(H_i)k_j - (H_j)k_i]$$

This equation was solved for each of the 15 possible pairs of values shown in Figure 4 (bottom). A median value for K_{eq} of 6.71 μM ($pK_a = 5.17$) and a median value for $k/(\ln 10)$ of 1.15×10^{-3} s^{-1} were obtained ($k = 2.6 \times 10^{-3}$ s^{-1}). The curve drawn through the points in Figure 4 (bottom) is a plot of $k(H^+)/[K_{eq} + (H^+)](\ln 10)$ vs. pH, calculated from the median values of K_{eq} and k.

The same analysis was performed from results obtained by adding 0.1 mM H_2O_2 to 0.2 mM SCN^-, plotting $1/S$ vs. t to obtain values of k_{app}'. Figure 5 shows a plot of the apparent second-order rate constants vs. pH. Substituting for (HOSCN) and integrating the equation

$$dS/dt \propto (HOSCN)^2$$

yield

$$k_{app}' = [1 + K_{eq}/(H^+)]^{-2} k'$$

where k' is the second-order rate constant for HOSCN, and

$$[k_i(H_i)^{-2} - k_j(H_j)^{-2}]K_{eq}^2 + 2[k_i(H_i)^{-1} - k_j(H_j)^{-1}]K_{eq} + (k_i - k_j) = 0$$

Median values for K_{eq} of 4.27 μM ($pK_a = 5.37$) and for k' of 2.96 M^{-1} s^{-1} were obtained. The curve drawn through the points in Figure 5 is a plot of $[1 + K_{eq}/(H^+)]^{-2}k'$ vs. pH, calculated from the median values of K_{eq} and k.

Effect of pH Buffering Agents. A number of pH buffering agents also accelerated decomposition. The same results were obtained whether the buffers were present during SCN^- oxidation or were added after SCN^- oxidation was complete.

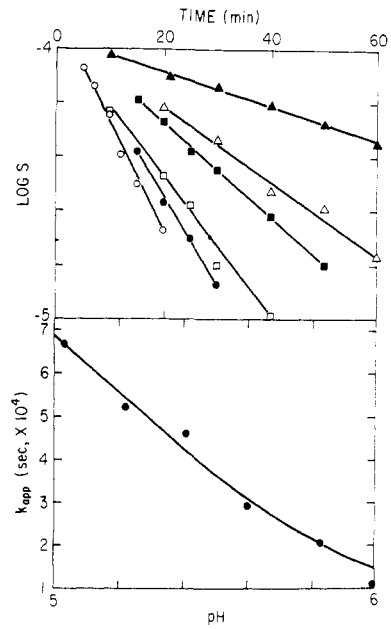

FIGURE 4: Effect of pH on decomposition at high SCN^- concentrations. Lactoperoxidase, 0.1 mM H_2O_2, and 10 mM SCN^- were incubated at pH 5.03 (○), 5.28 (●), 5.41 (□), 5.6 (■), 5.83 (△), and 5.96 (▲), and HOSCN–$OSCN^-$ (S) was measured after the indicated intervals. In the lower figure, the slopes of the lines in the upper figure are plotted vs. pH.

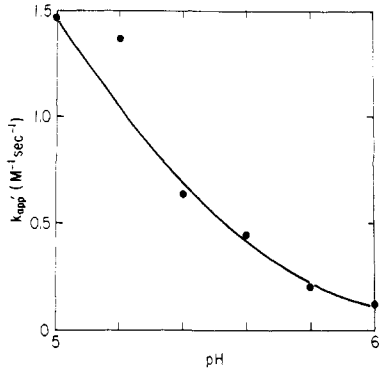

FIGURE 5: Effect of pH on decomposition at low SCN^- concentrations. Lactoperoxidase, 0.1 mM H_2O_2, and 0.2 mM SCN^- were incubated at pH 5, 5.2, 5.4, 5.6, 5.8, and 6, the concentration of HOSCN–$OSCN^-$ (S) was determined after varying intervals, and plots of $1/S$ vs. time were constructed. The slopes of these lines are plotted vs. pH.

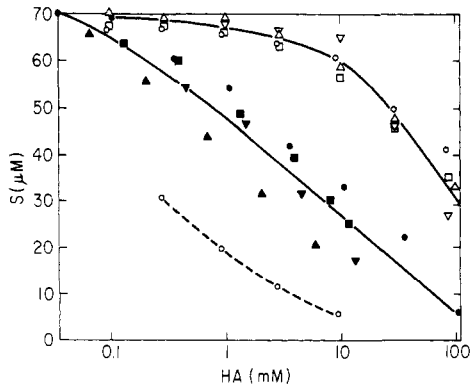

FIGURE 6: Effect of buffers on decomposition. Lactoperoxidase, 0.1 mM H_2O_2, and 1 mM SCN^- were incubated at pH 5 with the indicated concentrations of the protonated form (HA) of the following buffers: dimethylarsenic (--O--), aspartic (▲), glutamic (▼), benzoic (■), acetic (●), Mes (○), Pipes (□), $H_2PO_4^-$ (△), and boric (▽).

The buffers that accelerated decomposition were primarily weak acids that exist to a significant extent in the protonated form in the pH range 5–8. Stronger acids including lactic (pK_a = 3.1) and sulfanilic (pK_a = 3.2) had no detectable effect on decomposition during 1 h. Accelerated decomposition was also obtained with Mes and Pipes, in which the secondary amine moiety rather than the sulfonic acid moiety is considered to be protonated (Good et al., 1966).

Figure 6 compares the effect of the buffers on the amount of $HOSCN–OSCN^-$ remaining after 1 h at pH 5, in the presence of 1 mM SCN^-. Results are plotted vs. the concentration of the protonated form, (HA), calculated from published pK_a values, rather than vs. the nominal concentration, (HA + A^-). On this basis, weak acids with primary carboxyls (acetic, benzoic, aspartic, and glutamic) were nearly equal in their effect, suggesting that the protonated form was involved in decomposition. The most effective of the acids tested was dimethylarsenic (cacodylic), whereas the least effective were phosphoric (calculated as $H_2PO_4^-$) and boric (H_3BO_3). In other experiments, both dissolved carbon dioxide (as H_2CO_3 or HCO_3^-) and hydrofluoric acid (HF) accelerated decomposition, but the volatility of these agents made it difficult to compare their effects with those of other agents.

Comparing acetate and phosphate buffers at pH 5 as a function of the concentration of the protonated forms (CH_3CO_2H and $H_2PO_4^-$), we determined that phosphate was about an order of magnitude less effective in accelerating decomposition. Phosphate buffer at a nominal concentration of 20 mM did not significantly accelerate decomposition. Phosphate has a small buffering capacity at pH 5 relative to that at pH 7, but no agent with a higher buffering capacity at pH 5 was without an effect on the rate of decomposition. Figure 6 shows that Mes (pK_a = 6.2) and Pipes (pK_a = 6.8) were similar to phosphate. Either phosphate or Mes appears to be the buffer of choice for experiments at low pH in which stability of HOSCN is desired. Nevertheless, because the pK_a of $H_2PO_4^-$ (7.2) is higher than that of acetic acid (4.8), high concentrations of phosphate (0.1–1 M) accelerated decomposition even at pH 8, whereas acetate buffer at 1 M nominal concentration had no effect during 1 h at pH 7.

The action of HCN (pK_a = 9.3) was different from that of other weak acids. The addition of HCN resulted in stoichiometric and nearly instantaneous loss of $HOSCN–OSCN^-$ regardless of pH.

The effect of weak acids could not be interpreted as due to a shift in the pK_a of HOSCN, because the rate of decomposition could exceed that calculated for a pure solution of HOSCN. Also, SCN^- or weak acids did not promote extraction into organic solvents. No effect on extraction was obtained with acids that are either soluble (acetic) or insoluble ($H_2PO_4^-$) in the organic phase.

One possible explanation for the effect of SCN^- or weak acids is that they favor formation of $(SCN)_2$ from HOSCN.

$$HOSCN + SCN^- + H^+ \rightleftarrows (SCN)_2 + H_2O$$

Exchange between the SCN moiety of SCN^- and HOSCN was examined to test for such an effect. Oxidation of $S^{14}CN^-$ was followed by addition of unlabeled SCN^- and vice versa, and then the HOSCN (or $HOS^{14}CN$) was extracted into ethyl acetate. According to the equilibrium expression above, SCN^- should dilute the label in $HOS^{14}CN$, or label from $S^{14}CN^-$ should be introduced into HOSCN. However, no evidence for exchange was obtained at any combination of pH and SCN^- concentration, in the presence or absence of weak acids. Therefore, $HOSCN–OSCN^-$ mixtures were not in equilibrium with $(SCN)_2$.

These results do not rule formation of $(SCN)_2$ from HOSCN but do indicate that if $(SCN)_2$ is formed the rate of decomposition of $(SCN)_2$ is much faster than the rate of hydrolysis to yield HOSCN. For determination of whether $(SCN)_2$ might be formed as an intermediate in the decomposition of HOSCN, oxidation of $^{35}SCN^-$ at pH 5 was followed by addition of unlabeled SCN^- and vice versa. The HOSCN (or $HO^{35}SCN$) was allowed to decompose completely (24 h at 25 °C), and then the radioactivity in SCN^- and the decomposition product SO_4^{2-} were measured. Addition of 0.1 mM H_2O_2 to 0.3 mM $^{35}SCN^-$, followed by complete decomposition of the $HO^{35}SCN–O^{35}SCN^-$, yielded 0.034 mM $^{35}SO_4^{2-}$. Adding 1, 10, or 100 mM unlabeled SCN^- after $^{35}SCN^-$ oxidation was complete resulted in no detectable decrease in the amount of radioactivity appearing in $^{35}SO_4^{2-}$. Similarly, addition of 0.1 mM H_2O_2 to 0.3 mM SCN^-, followed by addition of tracer amounts of $^{35}SCN^-$ or 1 mM $^{35}SCN^-$, resulted in about 1% or 0.3% incorporation of radioactivity into $^{35}SO_4^{2-}$. These results show that at least 90% of the SO_4^{2-} was derived from $HOSCN–OSCN^-$, and less than 10% was derived from SCN^-. Therefore, the SO_4^{2-} was not derived from a symmetrical intermediate such as $(SCN)_2$.

Effect of Nitrogenous Compounds. Hypohalous acids (HOX) react with nitrogenous compounds including amines and amides to yield derivatives containing the nitrogen–halide (N–X) bond. These N–X derivatives retain the 2 oxidizing equivalents of HOX but differ from HOX in stability, solubility, and oxidation potential. From the analogy between HOX and HOSCN, the effect of nitrogenous compounds on $HOSCN–OSCN^-$ was investigated. No evidence was obtained for any interaction with ammonia or aromatic or aliphatic amines and amides, except for Mes and Pipes (cf. Figure 6). Two classes of nitrogenous compounds did interact with HOSCN–OSCN: sulfonamide compounds ($R–SO_2–NH_2$), which contain an acidic nitrogen moiety, and histidine and imidazole, which are heterocyclic aromatic imines.

Figure 7 shows the effect of concentration of the sulfonamide compound sulfamide ($NH_2–SO_2–NH_2$) on the amount of oxidizing equivalents measured after 1 h. The experiments were carried out under conditions that accelerate the decomposition of $HOSCN–OSCN^-$. Sulfamide had a stabilizing effect at all the pH values and protected against the destabilizing effect of SCN^- or weak acids. High concentrations of sulfamide relative to $HOSCN–OSCN^-$ were required. In other experiments, sulfamide did not prevent the rapid, stoichiometric loss of oxidizing equivalents observed upon addition of HCN.

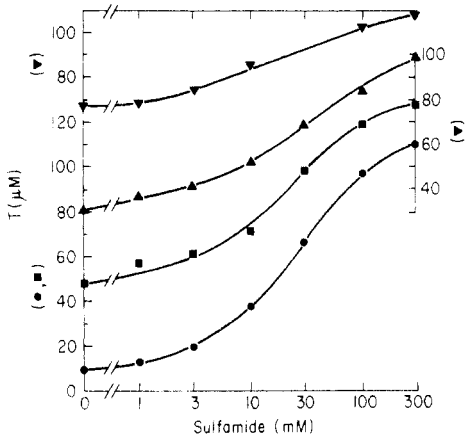

FIGURE 7: Effect of sulfamide on decomposition. The indicated concentrations of sulfamide were incubated with lactoperoxidase and 0.1 M H_2O_2 at pH 8 with 1 mM SCN^- and 0.7 M phosphate buffer (▼), at pH 7 with 0.1 M SCN^- (▲), at pH 6 with 1 mM SCN^- and 0.7 M acetate buffer (■), and at pH 5 with 1 mM SCN^- (●). The total concentrations (T) of the N–SCN derivative, HOSCN, and $OSCN^-$ were measured after 1 h.

Similar results were obtained with other sulfonamide compounds. However, those compounds with an aromatic amine group in addition to the sulfonamide group (e.g., sulfanilamide) interfered with SCN^- oxidation, apparently by serving as an alternative substrate for lactoperoxidase-catalyzed oxidation. Such compounds were effective when added after SCN^- oxidation was complete, or when added to chemically synthesized HOSCN–$OSCN^-$.

Some evidence was obtained that histidine or imidazole at 0.1 M had a stabilizing effect. However, the experiments were complicated by the loss of oxidizing equivalents in side reactions, and by the slow rate of oxidation of Nbs in the presence of these compounds.

Figure 8 compares the extraction of oxidizing equivalents into ethyl acetate from aqueous solutions at pH 6, in the presence of increasing concentrations of sulfonamides. The percent extracted was increased in the presence of *p*-toluenesulfonamide or sulfanilamide, and decreased in the presence of benzenesulfonamide or sulfamide. Extraction was also decreased in the presence of histidine or imidazole, but the concentrations required were about 10-fold higher than those of the sulfonamides. Only *p*-toluenesulfonamide significantly increased the extraction of oxidizing equivalents into 1-octanol (59%, 32%, 14%, and 9% extracted at pH 5, 6, 7, and 8 with 10 mM *p*-toluenesulfonamide, as compared to results in Figure 1).

The effect of sulfonamides on extraction was not proportional to their partition coefficients. Under the extraction conditions used in these experiments, about 100%, 92%, 69%, and 5% of *p*-toluenesulfonamide, benzenesulfonamide, sulfanilamide, and sulfamide were extracted into the ethyl acetate phase. These results suggest that HOSCN–$OSCN^-$ reacts with benzenesulfonamide to yield a derivative more polar than benzenesulfonamide, whereas the corresponding derivative of sulfanilamide is less polar than the parent compound. These differences may be due to modification of the sulfonamide moiety to yield a mixture of thiocyanatosulfonamide and -sulfonamidate derivatives ($R–SO_2–NH–SCN$ and $R–SO_2–N–SCN^-$).

By extension of the mathematical analysis described above, it was possible to obtain two independent measures of the dissociation constant (K_D) for the interaction of HOSCN–$OSCN^-$ with sulfamide. It was assumed that a thiocyanatosulfonamide derivative is formed, that this N–SCN derivative

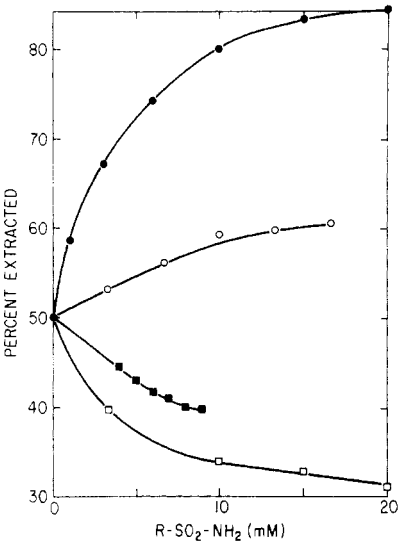

FIGURE 8: Effect of sulfonamides on extraction. Lactoperoxidase, 0.1 mM H_2O_2, and 0.3 mM SCN^- were incubated with the indicated concentration of the sulfonamide compound at pH 6. In the case of sulfanilamide, this compound was added 5 min after the addition of H_2O_2. The percent extracted into ethyl acetate is plotted vs. the concentration of the sulfonamide in the aqueous phase prior to extraction: R = p-CH_3–C_6H_4– (●); R = p-NH_2–C_6H_4– (○); R = C_6H_5– (■); R = NH_2– (□).

retains the 2 oxidizing equivalents of HOSCN–$OSCN^-$, and that the derivative is in equilibrium with both HOSCN and $OSCN^-$.

$$HOSCN + R–SO_2–NH_2 \rightleftarrows R–SO_2–NH–SCN + H_2O$$

$$OSCN^- + R–SO_2–NH_2 \rightleftarrows R–SO_2–N–SCN^- + H_2O$$

$$K_D = [(HOSCN) + (OSCN^-)] \times (R–SO_2–NH_2)/[(R–SO_2–HN–SCN) + (R–SO_2–N–SCN^-)]$$

Also, it was assumed that the concentration of the N–SCN derivative is small relative to that of sulfamide, that the derivative does not participate in decomposition, and that sulfamide and the derivative are not extracted into ethyl acetate. The relevant equations are

$$K_D = (R–SO_2–NH_2)[1 + K_{eq}(H)^{-1}]/[RPE^{-1} - K_{eq}(H)^{-1} - 1]$$

$$K_D = (R–SO_2–NH_2)/\{kk_{app}^{-1}[1 + K_{eq}(H)^{-1}] - 1\}$$

A median value for K_D of 16 mM was obtained from the effect of sulfamide concentration at pH 5 and 6 on extraction and on k_{app}, in the presence of 10 mM SCN^-. Similar values of 5–20 mM were obtained, assuming that the derivative was in equilibrium only with HOSCN, or only with $OSCN^-$. A value for K_D of 4 mM was obtained from the effect of *p*-toluenesulfonamide on k_{app} at pH 6.

On the basis of these studies, experiments on the long-term stability of 0.1 mM HOSCN–$OSCN^-$ mixtures were carried out. Maximum stability was obtained at pH 7. Freezing the solutions decreased stability. The addition of Tris-HCl, pH 7, and 0.1 M NaCl increased the stability in the frozen state. Solutions prepared in this way have retained at least 90% of their oxidizing activity after 2 months at −80 °C. Solutions were also prepared which contained very low levels of SCN^-, by extracting HOSCN into ethyl acetate at pH 5 and then back-extracting at pH 7. The dissolved ethyl acetate was removed by bubbling N_2 through the solutions. These solutions have retained at least 95% of their oxidizing activity after 2 months at −80 °C and 50% after 3 weeks at 5 °C. Solutions

containing 0.1 M sulfamide have retained 100% of their oxidizing activity at −80 or 5 °C and may be stable indefinitely.

Discussion

Similar values for the pK_a of HOSCN were obtained by two experimental approaches, one based on physical properties (partition coefficients) and the other on chemical properties (rates of decomposition). Also, the values did not depend on which of two kinetic descriptions was applied to the decomposition phenomenon. It was necessary to assume only that decomposition of HOSCN–$OSCN^-$ was due to reactions involving HOSCN and that $OSCN^-$ did not participate. From the calculated pK_a values of 5.2–5.4, HOSCN is a stronger acid than the analogous hypohalous acids HOCl (pK_a = 7.5), HOBr (pK_a = 8.7), and HOI (pK_a = 10.6).

The results indicate that significant amounts of HOSCN would be formed during peroxidase-catalyzed oxidation of SCN^- in vivo, at the lower pH values. With pK_a = 5.3, the percentage of HOSCN–$OSCN^-$ that would be in the form of HOSCN is 67% at pH 5, 17% at pH 6, 2% at pH 7, and 0.2% at pH 8. This observation may be relevant to the antimicrobial action of the lactoperoxidase–H_2O_2–SCN^- system in milk and saliva, in which products of bacterial metabolism can lower the pH to 5 or below. Also, myeloperoxidase functions within phagolysozomes of leukocytes at pH values as low as 4.5.

The effect of pH on the composition of HOSCN–$OSCN^-$ mixtures could be significant, in that HOSCN would be expected to be more reactive than $OSCN^-$. The uncharged HOSCN would diffuse more readily through the hydrophobic barrier of biological membranes and would not be subject to charge repulsion at reactive sites of proteins or other biological materials. With the assumption that pK_a = 5.3 and that the rate of reaction of HOSCN–$OSCN^-$ with biological materials is proportional to HOSCN concentration, the reaction would be 335 times faster at pH 5 than at pH 8.

The effect of pH on reactivity of HOSCN–$OSCN^-$ would be significant only when the biological action is determined by the rate of reaction. Because HOSCN and $OSCN^-$ are in equilibrium, consumption of HOSCN in reactions with biological materials would result in protonation of $OSCN^-$ to replenish the supply of HOSCN. Therefore, the extent of reaction would not be influenced by pH, provided that adequate time is available for the reaction to go to completion.

The proposal that HOSCN could diffuse through biological membranes is supported by the observation that HOSCN has a greater solubility in a water-saturated ethyl acetate or octanol phase than in the aqueous phase saturated with the organic solvent. Partition coefficients in octanol have been used to estimate the distribution of compounds between the extracellular compartment and the exterior portion of cell membranes (Leo et al., 1971). The partition coefficient of 2.3 for HOSCN in 2-octanol is comparable to those of certain diazene derivatives that oxidize intracellular glutathione of human erythrocytes (Kosower et al., 1975).

Extraction of HOSCN into organic solvents along with determination of SCN^- provides a method for identification of HOSCN–$OSCN^-$. This method was used to demonstrate the presence of $OSCN^-$ in human saliva (Thomas et al., 1980). The characteristic pH profile for extraction provides a method for distinguishing between HOSCN–$OSCN^-$ and similar oxidizing agents.

These results support and extend the observations of Hogg & Jago (1970). These workers proposed that the agent formed during lactoperoxidase-catalyzed oxidation of SCN^- was in an acid–base equilibrium and reported that SCN^- accelerated the decomposition of the agent. They determined a pK_a of 5.1 ± 0.1 by a polarographic technique, and a pK_a of 5.2 from the effect of pH on absorbance at 235 nm. At that time, methods had not been developed for quantitating the agent, or for identifying the agent based on the stoichiometry of SCN^- oxidation. The authors considered HOSCN, HO_2SCN, and HO_3SCN as possible structures. Either of the latter two structures was considered more likely, in that the highest yield of substances absorbing at 235 nm was obtained at high ratios of H_2O_2 to SCN^-. However, there does not appear to be a direct quantitative relation between the agent and substances absorbing at 235 nm (Oram & Reiter, 1966; Chung & Wood, 1970). On the basis of the similarity of the results of Hogg & Jago (1970) and those presented here, it is likely that the agent characterized by those workers was HOSCN–$OSCN^-$.

One possible limitation to the action of HOSCN–$OSCN^-$ in biological systems is the pH-dependent decomposition. If the rate of decomposition exceeded the rate of reaction with biological components, then low pH might decrease rather than increase the action of HOSCN–$OSCN^-$. Nevertheless, the results indicate that HOSCN can be relatively stable at low pH, depending on the composition of the medium.

Both SCN^- and a number of pH buffering agents accelerated the decomposition of HOSCN–$OSCN^-$. This effect raises questions as to the mechanism of decomposition. If, as proposed previously, the rate-limiting step is the dismutation of two HOSCN molecules, then SCN^- would not be involved in the rate-limiting step and should have no effect. Although the pK_a value calculated with the assumption that dismutation is rate limiting is consistent with the other calculated values, this result does not necessarily indicate that dismutation is the chemical mechanism of decomposition. Second-order kinetics indicate only that the two rate-limiting reactants are present in nearly equal concentrations. Peroxidase-catalyzed oxidation of SCN^- at a 1:2 ratio of H_2O_2 to SCN^- yields a mixture of HOSCN–$OSCN^-$ and SCN^- in nearly equal amounts, as does the hydrolysis of $(SCN)_2$. Therefore, second-order kinetics could result from the reaction of HOSCN with SCN^-. At high levels of SCN^-, decomposition would appear first order with respect to HOSCN–$OSCN^-$ concentration, because SCN^- concentration would greatly exceed that of HOSCN and would not change significantly during the reaction. Therefore, despite the apparent change in the kinetics of decomposition, the reaction of HOSCN with SCN^- may be rate limiting at both low and high SCN^- concentrations.

Although it is unlikely that HOSCN could be prepared in aqueous solutions completely free of SCN^- or other substances that accelerate decomposition, it might be that in the absence of these substances HOSCN would show no tendency to decompose. Solutions containing primarily $OSCN^-$ have been prepared which decompose slowly or not at all, depending on temperature. If HOSCN–$OSCN^-$ solutions or $OSCN^-$ salts with sufficient stability can be prepared, they might be useful as mild oxidants or antiseptic agents.

The stability of HOSCN–$OSCN^-$ in vivo might also be influenced by aromatic imines (such as histidine, either free or peptide linked) and by pharmacological agents such as sulfonamides (including the "sulfa" compounds). The stability of the oxidizing activity of HOSCN–$OSCN^-$ was enhanced by these compounds. Stabilization appears due to the reaction of HOSCN and/or $OSCN^-$ with the nitrogenous compounds to yield N–SCN derivatives (thiocyanatimines and thiocyanatosulfonamides). This reaction lowers the concentration of free HOSCN, suppressing the decomposition reaction. The N–SCN derivatives retain the 2 oxidizing equivalents of HOSCN–$OSCN^-$ and rapidly oxidize Nbs or other sulfhydryl

compounds to the corresponding disulfides, as illustrated with a thiocyanatosulfonamide.

$$R\text{-}SO_2\text{-}NH\text{-}SCN + 2R'\text{-}SH \rightarrow R\text{-}SO_2\text{-}NH_2 + R'\text{-}S\text{-}S\text{-}R' + H^+ + SCN^-$$

The reaction of $(SCN)_2$ with nitrogenous compounds in organic solvents to yield thiocyanatamines (R–NH–SCN) and related N–SCN derivatives has been described previously (Wood, 1946; Bacon, 1961; Hughes, 1975). In this report, no evidence was obtained for reaction of HOSCN–$OSCN^-$ with amines in aqueous solutions, suggesting that the equilibrium for hydrolysis lies far in the direction of the free amine and HOSCN–$OSCN^-$.

$$R\text{-}NH\text{-}SCN + H_2O \rightleftarrows R\text{-}NH_2 + HOSCN$$

Only with the aromatic imines and sulfonamides does the equilibrium lie sufficiently toward formation of the N–SCN derivative to permit its demonstration.

In addition to their effect on stability, the sulfonamides yield N–SCN derivatives that can have either high or low partition coefficients, depending on the structure of the parent sulfonamide. The ability of *p*-toluenesulfonamide to stabilize oxidizing activity and to promote solubility of oxidizing equivalents in nonpolar media suggests that such a compound could be used to enhance the antimicrobial activity of the lactoperoxidase–H_2O_2–SCN^- system. High concentrations of *p*-toluenesulfonamide were required, but it might be possible to identify sulfonamide or related compounds with more favorable properties. The N–SCN derivatives of *p*-toluenesulfonamide and benzenesulfonamide are analogues of the dichlorosulfonamide derivatives chloramine-T and -B, which were developed as antiseptic agents (Challenger, 1961). The reaction of HOSCN–$OSCN^-$ with sulfanilamide represents an interaction of two substances both of which have antimicrobial activity. The N–SCN derivatives of sulfanilamide and other "sulfa" compounds might provide a useful class of bifunctional antimicrobial agents.

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