# Lactoperoxidase-Catalyzed Oxidation of Thiocyanate by Hydrogen Peroxide: $^{15}N$ Nuclear Magnetic Resonance and Optical Spectral Studies

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*Received June 11, 1990; Revised Manuscript Received August 31, 1990*

ABSTRACT: To establish the agent(s) responsible for the activity of the lactoperoxidase (LPO)/$SCN^-/H_2O_2$ system, the oxidation of thiocyanate with hydrogen peroxide, catalyzed by lactoperoxidase, has been studied by $^{15}N$ NMR and optical spectroscopy at different concentrations of thiocyanate and hydrogen peroxide and at different pHs. The formation of hypothiocyanite ion ($OSCN^-$) as one of the oxidation products correlated well with the activity of the LPO/$SCN^-/H_2O_2$ system and was maximum when the concentrations of the $H_2O_2$ and $SCN^-$ were nearly the same and the pH was <6.0. At $[H_2O_2]/[SCN^-] = 1$, $OSCN^-$ decomposed very slowly back to thiocyanate. When the ratio $[H_2O_2]/[SCN^-]$ was above 2, formation of $CN^-$ was observed, which was confirmed by $^{15}N$ NMR and also by changes in the optical spectrum of LPO. The oxidation of thiocyanate by $H_2O_2$ in the presence of LPO does not take place at pH >8.0. Since thiocyanate does not bind to LPO above this pH, the binding of thiocyanate to LPO is considered to be prerequisite for the oxidation of thiocyanate. Maximum inhibition of oxygen uptake by *Streptococcus cremoris* 972 bacteria was observed when hydrogen peroxide and thiocyanate were present in equimolar amounts and the pH was below 6.0.

Lactoperoxidase (LPO, EC 1.11.1.7, donor-$H_2O_2$ oxidoreductase) is a metalloenzyme of approximately 78 000 molecular weight (Carlstrom, 1969; Morrison et al., 1966). It is found in milk, saliva, and tears. In common with other peroxidases, the enzyme catalyzes oxidation of a number of organic and inorganic substrates by hydrogen peroxide and is therefore a component of the biological defense mechanism of mammalians (Dunford & Stillman, 1976).

Thiocyanate ion/$H_2O_2$/LPO provides a potent nonspecific bacteriostatic or bacteriocidal system (Aune & Thomas, 1977, 1978; Jago & Morrison, 1962; Reiter et al., 1963, 1976; Wright & Tramer, 1958). This system operates in vivo to protect the gut of the calf from enteric pathogens (Reiter et al., 1980; Marshall et al., 1986) and has been used to preserve raw milk without refrigeration (Bjorck et al., 1979). Cell multiplication, lactic acid production in milk, and oxygen uptake by resting cells have been found to be inhibited by this system (Reiter et al., 1963). The maximum inhibition of both oxygen uptake and acid production by *Streptococcus cremoris* 972 is obtained when hydrogen peroxide and thiocyanate are present in equimolar amounts (Hog & Jago, 1970).

The chemical species responsible for the antimicrobial activity of the LPO/$SCN^-/H_2O_2$ system is believed to be one (or more) of the oxidation products of the thiocyanate (Chung & Wood, 1970; Hog & Jago, 1970; Hoogendoorn et al., 1977; Aune & Thomas, 1977; Marshall & Reiter, 1980; Thomas, 1981). It has been suggested that the antibacterial activity of this system may be due to cyanide ion (Chung & Wood, 1970), $(SCN)_2$ (Aune & Thomas, 1978), cyanosulfurous acid or cyanosulfuric acid (Hog & Jago, 1970), or $OSCN^-$ (Hoogendoorn et al., 1977; Aune & Thomas, 1977; Thomas, 1981). However, the mechanism of the action and oxidation of thiocyanate ion in this system is not yet well understood, although several studies have been reported to elucidate the nature of the active agent(s). Studies on the antimicrobial activity of the $SCN^-/H_2O_2$/LPO system have suggested formation of a relatively stable chemical species with antimicrobial activity (Aune & Thomas, 1977; Bjorck et al., 1975). Aune and Thomas (1977) showed that the yield of the oxidized species of $SCN^-$ in the $SCN^-/H_2O_2$/LPO system was maximum when the concentration ratio $[H_2O_2]/[SCN^-]$ approaches unity at physiological pH. A pathway of thiocyanate oxidation was proposed based on the observation that $SCN^-/H_2O_2$/LPO system is analogous to the halogenating system (Aune & Thomas, 1977, 1978; Chung & Wood, 1970; Morrison & Schonbaum, 1976; Thomas, 1981):

$$H_2O_2 + 2SCN^- + 2H^+ \rightarrow 2H_2O + (SCN)_2$$

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

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

Indirect evidence was obtained for LPO-catalyzed oxidation of $SCN^-$ to yield $(SCN)_2$ (Aune & Thomas, 1978). $(SCN)_2$ is however relatively unstable in aqueous solution and hydrolyzed to hypothiocyanous acid (HOSCN) (Hughes, 1975). Thus the major product of $SCN^-$ oxidation was suspected to be $OSCN^-$/HOSCN at physiological pH (Aune & Thomas, 1977; Hoogendoorn et al., 1977; Magnusson, 1984; Thomas, 1981). $OSCN^-$ is relatively stable at high pH and cannot be extracted into organic solvents (Thomas, 1981).

Several chemical methods for estimation of $OSCN^-$ have been reported (Aune & Thomas, 1977; Thomas et al., 1980; Hoogendoorn, 1977). These methods are based either on reduction of $OSCN^-$ to $SCN^-$ which can be assayed by the iron complex method (Aune & Thomas, 1977) or on the oxidation of 5-thio-2-nitrobenzoic acid by $OSCN^-$ to colorless disulfide compound (Aune & Thomas, 1977, 1978). It was observed that oxidation of thiocyanate by LPO/$H_2O_2$ gives rise to a rapid increase in the absorption at 235 nm, $A_{235}$ (Chung & Wood, 1970). Pruitt and Tenovuo (1982) have observed a linear correlation between the increase in $A_{235}$ and $OSCN^-$ concentration. It was however suggested that $OSCN^-$ may not be the only product of the LPO-catalyzed oxidation of thiocyanate ion and other products may include higher oxy

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0006-2960/91/0430-118$02.50/0 

acids such as $O_2SCN^-$ and $O_3SCN^-$ (Hog & Jago, 1970; Aune & Thomas, 1977, 1978; Bjorck et al., 1979). However, their contribution is expected to be negligible on the basis of absorption at 235 nm (Pruitt & Tenovuo, 1982). The increase at 235 nm can be used to estimate the concentration of $OSCN^-$ produced during oxidation (Pruitt & Tenovuo, 1982).

Oxidation products of the $SCN^-/H_2O_2$/LPO system reported so far all contain nitrogen atom derived from the thiocyanate substrate. In this paper the use of nuclear magnetic resonance (NMR) of $^{15}N$ nucleus and optical spectroscopy is emphasized to identify some of stable reaction products of $SCN^-/H_2O_2$/LPO system. Isotopically enriched (>99 atom %) $SC^{15}N$ was used for NMR measurements. The major stable product of $SCN^-$ oxidation has been identified to be $HOSCN/OSCN^-$. The formation of $HOSCN/OSCN^-$ was observed to be strongly pH dependent and was found to be maximum at pH ≤6. Its formation has been found to correlate with the activity of the $SCN^-/H_2O_2$/LPO system, the formation becoming maximum when the concentration of $H_2O_2$ approaches that of $SCN^-$ at pH below 6. Similarly maximum inhibition of oxygen uptake by *S. cremoris* 972 bacteria was observed when hydrogen peroxide and thiocyanate are present in equimolar amounts and pH of the solution is below 6.0.

## Materials and Methods

Lactoperoxidase was isolated from fresh raw unskimmed cow's milk by essentially the same procedure as previously described (Modi et al., 1989a,b, 1990a,b). After ion-exchange chromatography on a CM52 column, the LPO fractions were pooled and dialyzed against 5 mM phosphate bufer (pH = 6.8). After the dialyzed sample was centrifuged, it was concentrated on Amicon ultrafiltration cell on PM30 and applied to a Sephadex G-100 column. Fractions with Rz ($A_{412}/A_{280}$) = 0.85–0.91 were concentrated and lyophilized. Concentration of the enzyme was determined spectrophotometrically by using a molar extinction coefficient of $1.12 \times 10^5$ $cm^{-1}$ $M^{-1}$ at 412 nm for lactoperoxidase (Carlstrom, 1969). Deuterium oxide (>99.85%) was purchased from Aldrich. Enriched $^{15}N$ sodium thiocyanate ($NaSC^{15}N$, atomic % of $^{15}N$ >99) was purchased from MSD Isotopes. All other reagents were of analytical grade.

*Oxidation of Thiocyanate Catalyzed by LPO.* LPO (0.1 μM) was incubated with $H_2O_2$ (0–4 mM) and thiocyanate (1 mM) in 0.1 M phosphate buffer at 23 °C for 5 min, until the change in thiocyanate concentration was complete.

*Determination of Thiocyanate Concentration.* Thiocyanate concentration was determined essentially by the procedure described by Aune and Thomas (1977). A 1.6-mL aliquot of 0.1 M HCl and a 0.2-mL aliquot of 0.1 M $FeCl_3$ were added to 0.2-mL aliquots of reaction mixture, and the absorbance of the $FeSCN^{2+}$ complex ion (Betts & Dainton, 1953) was measured at 450 nm. The concentration of $SCN^-$ was determined from a standard curve of $A_{450}$ vs thiocyanate concentration.

*Determination of Extent of Oxidation.* The oxidation of thiocyanate by LPO and $H_2O_2$ was accompanied by an increase in absorbance at 235 nm ($A_{235}$), which has been assigned due to formation of $OSCN^-$ with a molar extinction coefficient $1.29 \times 10^3$ $M^{-1}$ $cm^{-1}$ (Pruitt & Tenovuo, 1982). Therefore, $A_{235}$ was used to estimate the extent of thiocyanate oxidation (Hog & Jago, 1970; Magnusson et al., 1984). $A_{235}$ was measured when the increase in $A_{235}$ was completed (~5 min) against a blank containing no $SCN^-$ or $H_2O_2$. Formation of $OSCN^-$ can also be monitored by reduction of $OSCN^-$ to $SCN^-$ with an excess of glutathione (Hoogendoorn et al., 1977; Marshall & Reiter, 1980). The difference (Δ) in thiocyanate concentration before and after addition of glutathione shows the formation of $OSCN^-$.

*Preparation of $(SCN)_2$ and $OSCN^-$ by Chemical Methods.* Synthesis of $(SCN)_2$ (either unlabeled or $^{15}N$ labeled) was carried out by reaction of lead thiocyanate with bromine (Wood, 1946). Lead thiocyanate was prepared by the addition of 4.5 g of lead nitrate in 10 mL of water to 2.98 g of NaSCN in 10 mL of water at 0 °C (Aune & Thomas, 1977). The concentration of $(SCN)_2$ in carbon tetrachloride ($CCl_4$) was determined by measuring absorbance at 295 nm with a molar extinction coefficient of 140 $M^{-1}$ $cm^{-1}$ (Bacon & Irwin, 1958).

Synthesis of $OSCN^-$ (either unlabeled or $^{15}N$ labeled) was carried out by treating 1 mL of 2 mM $(SCN)_2$ in $CCl_4$ with 10 mL of 0.1 M potassium phosphate. Concentration of $OSCN^-$ was determined by measuring absorbance at 235 nm with a molar extinction coefficient of $1.29 \times 10^3$ $M^{-1}$ $cm^{-1}$ (Pruitt & Tenovuo, 1982).

*NMR Measurements.* The $^{15}N$ NMR measurements were made on a Bruker FT NMR spectrometer at 50.68 MHz in a 10-mm NMR tube with $D_2O$ for frequency lock. The spectra were obtained by accumulation of 400–1000 transients at 16K data points. The quoted pHs are meter readings only, uncorrected for the isotope effect. The quoted chemical shifts are relative to $NO_3^-$ as external standard.

*Optical Spectroscopy.* Optical measurements were carried out on a Shimadzu 2100 spectrophotometer at 23 °C equipped with TCC-260 temperature contrller. Quartz cells of 10-mm path length were used.

*Bacterial Culture.* *S. cremoris* 972 was maintained by essentially the same procedure as reported earlier (Briggs & Newland, 1953) by periodic subculture on an enriched medium containing 0.5% glucose, 0.5% lactose, 0.6% liver extract, 0.6% sodium acetate, 0.6% yeast extract, 0.5 mL/100 mL salt A, (10 g of $KH_2PO_4$ and 10 g of $K_2HPO_4$, made up to 100 mL with water), 0.5 mL/100 mL salt B, (4 g of $MgSO_4 \cdot 7H_2O$, 200 mg of NaCl, 200 mg of $MnSO_4 \cdot 4H_2O$, and 200 mg of $FeSO_4 \cdot 7H_2O$, and made up to 100 mL with water), pH = 7.6, and 1.5% agar. The medium was autoclaved at 15 lb/in.$^2$ for 20 min.

Overnight cultures of *S. cremoris* 972 were diluted to 1 mL in 100 mL in fresh broth and grown at 35 °C for 16 h (Hog & Jago, 1970). The cells were collected in sterile tubes by centrifuging at 5000 rpm for 10 min at 4 °C, washed twice with 0.9% NaCl, and resuspended in 0.9% NaCl.

## Results

*Optical Studies on the $SCN^-/H_2O_2$/LPO System.* Figure 1 shows the loss of thiocyanate upon addition of different concentrations of $H_2O_2$ (0.0–0.9 mM) to LPO (0.1 μM) and $SCN^-$ (1.0 mM) at different pHs. LPO and $H_2O_2$ were incubated with 1 mM thiocyanate. The concentration of thiocyanate was determined from absorbance at 450 nm (see Materials and Methods). The depletion of thiocyanate increases with an increase in $H_2O_2$ concentration (Figure 1), and it was maximum for pH <6.0 and minimum for pH = 8.0.

Figure 2 shows the extent of oxidation of thiocyanate catalyzed by LPO at different pHs and $[H_2O_2]/[SCN^-]$ ratios. The extent of oxidation was measured in terms of change in the absorbance at 235 nm ($A_{235}$) as described under Materials and Methods. In the pH range of 4–8, the extent of oxidation was always observed to be high when hydrogen peroxide and thiocyanate were present in equimolar amounts (Figure 2). However, it was observed to be maximum at pH = 6 and below (up to pH = 4). The data in Figure 2 show that at pH = 6.1 and 5.0 the profiles of $A_{235}$ vs the $[H_2O_2]/[SCN^-]$ ratio are superimposable; this is also true for the data at pH = 4.0,

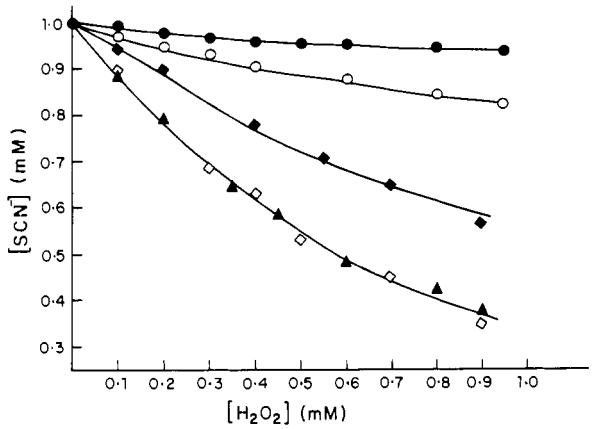


FIGURE 1: Loss of thiocyanate by oxidation at different pHs. LPO (0.1 $\mu$M) and $H_2O_2$ (0–0.9 mM) were incubated in 0.1 M phosphate buffer with 1 mM KSCN at pH = 8.0 (●), pH = 7.0 (○), pH = 6.5 (◆), pH = 6.0 (◇), and pH = 5.0 (▲).

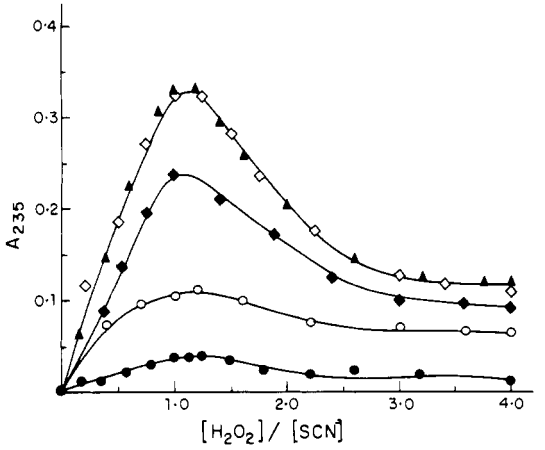


FIGURE 2: Change in the absorbance at 235 nm at different $[H_2O_2]/[SCN^-]$ ratios and pHs. This change in absorbance is related to the extent of oxidation of thiocyanate (see Materials and Methods). LPO (0.1 $\mu$M) and $H_2O_2$ (0–4 mM) were incubated in 0.1 M phosphate buffer with 1 mM KSCN at pH = 8.0 (●), pH = 7.0 (○), pH = 6.5 (◆), pH = 6.0 (◇), and pH = 5.0 (▲).

which are not shown for convenience. At pH >6.1, the extent of oxidation of thiocyanate decreases, though it remains high at an equimolar ratio of $H_2O_2$ and $SCN^-$. Since a rise in $A_{235}$ correlates with the concentration of oxidation product ($HOSCN/OSCN^-$) (Pruitt & Tenovuo, 1982), the production of $HOSCN/OSCN^-$ is maximum at pH ≤6 but decreases as pH is increased to 8. Recently, it has been shown by $^{15}N$ NMR studies (Modi et al., 1989a) that $SCN^-$ binds to LPO only in the acidic range (pH <6.1) and that the binding is facilitated by protonation of an amino acid residue on the enzyme with $pK_a$ = 6.1. The pH dependence of extent of oxidation suggests that oxidation is maximum when the enzyme-bound oxidizable substrate ($SCN^-$) species are predominantly present. The increase in the yield of oxidation product on lowering pH also suggests that binding of thiocyanate to LPO may be a prerequisite for its oxidation in the $SCN^-/H_2O_2/LPO$ system. To further confirm this, the hypothiocyanate concentration of this system was measured as a function of pH by reduction of $OSCN^-$ to $SCN^-$ using glutathione (Hoogendoorn et al., 1977; Marshall & Reiter, 1980). The concentration of $HOSCN/OSCN^-$ was determined from the difference ($\Delta$) in thiocyanate concentration before and after addition of glutathione (see Materials and Methods). Figure 3 shows the effect of pH on the formation of $HOSCN/OSCN^-$ at equimolar concentrations of $H_2O_2$ and

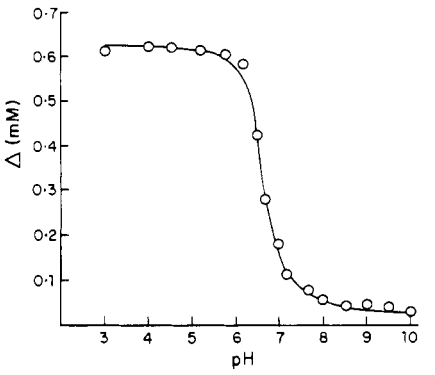


FIGURE 3: Effect of pH on the difference ($\Delta$) in thiocyanate concentration before and after addition of glutathione (3 mM). LPO (0.1 $\mu$M) and $H_2O_2$ (0.6 mM) were incubated in 0.1 M phosphate buffer with 0.6 mM KSCN at different pHs.

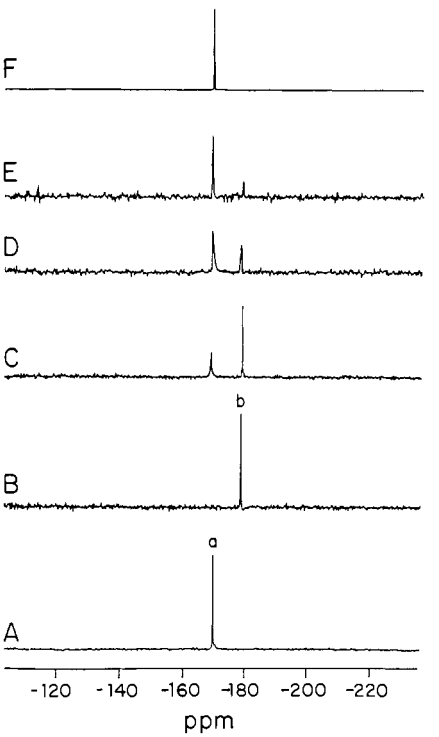


FIGURE 4: (A) $^{15}N$ NMR spectra of $SC^{15}N^-$ (1.0 mM) in the presence of LPO (0.1 $\mu$M) in 0.1 M phosphate buffer (pH = 6.1). (B) Effect of addition of $H_2O_2$ (1 mM) to (A). Panels C–F show changes observed with time. Time counting was started after addition of $H_2O_2$ (1 mM), and spectra were taken at different times, i.e., after 6 h (C), 24 h (D), 29 h (E), and 40 h (F). The resonances at −170 ppm (a) and −179 ppm (b) have been assigned to $SC^{15}N^-$ and $OSC^{15}N^-$, respectively.

$SCN^-$. $\Delta$ is almost constant between pH 3.0 and 6.0, and it decreases considerably when pH was increased to 7.0 (Figure 3). Since $\Delta$ represents the total amount of $HOSCN/OSCN^-$ formed, this result is consistent with those of Figure 2.

*$^{15}N$ NMR Studies on the $SCN^-/H_2O_2/LPO$ System.* Figure 4 shows $^{15}N$ NMR spectra of $SC^{15}N^-$ (1.0 mM) in the presence of LPO (0.1 $\mu$M) and the sequential effect after addition of $H_2O_2$ (1.0 mM) on the $LPO/SCN^-$ system at pH = 6.1. The addition of equimolar $H_2O_2$ caused complete disappearance of $^{15}N$ NMR resonance due to $SCN^-$ (resonance **a**, −170 ppm), and a new resonance **b** at −179 ppm was observed almost immediately after addition of equimolar $H_2O_2$ at pH = 6.1. This resonance **b** is therefore attributed to the formation of oxidation product of the $SCN^-/H_2O_2/LPO$ system. Resonance **b** was observed to be relatively stable and decomposed slowly over several hours. The decomposition

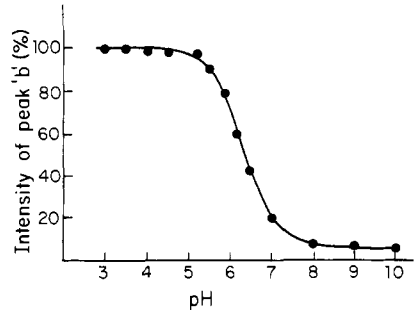


FIGURE 5: Effect of pH on the intensity of the $^{15}N$ NMR resonance of $OSC^{15}N^-$ (−179 ppm, resonance b). LPO (0.1 μM) and $H_2O_2$ (1 mM) were incubated with thiocyanate (1 mM) in 0.1 M phosphate buffer at different pHs. Measurements were done in each case on fresh solutions immediately after addition of $H_2O_2$. The intensity of the $^{15}N$ NMR resonance of $OSC^{15}N^-$ at pH = 3 was taken as 100%; the values at other pHs are scaled accordingly.

Table I: Chemical Shifts of $^{15}N$ NMR Resonances of Various Species

| species | solvent | pH | chemical shift (ppm) |
|---|---|---|---|
| $CN^-$ | 0.1 M phosphate buffer | 6.1 | −128 |
| $SCN^-$ | 0.1 M phosphate buffer | 6.1 | −170 |
| $(SCN)_2$[a] | $CCl_4$ | | −173 |
| $OSCN^-/HOSCN$[b] | 0.1 M phosphate buffer | 6.1 | −179 |

[a] Prepared chemically by reaction of lead thiocyanate with bromine (see Materials and Methods). [b] Prepared chemically by hydrolysis of $(SCN)_2$ (see Materials and Methods).

resulted in the reappearance of resonance **a** (due to $SCN^-$), which increased slowly in intensity with time.

Intensity of the product resonance **b** was found to be strongly pH dependent (Figure 5). The intensity of resonance b remains nearly constant in the pH range 3–5. Taking the intensity of resonance **b** at pH = 3 as 100%, we have plotted the intensity of resonance **b** at different pH values (Figure 5). The pH variation of the intensity of this resonance resembles closely the pH dependence of the difference of $A_{235}$ (Δ) before and after addition of glutathione (ie., formation of $OCSN^-$) (Figure 3). Since the intensity of the $A_{235}$ absorbance has been shown to correlate with $OCSN^-$ as the oxidation product in $SCN^-/H_2O_2$/LPO system (Pruitt & Tenevo, 1982), the present observation on $^{15}N$ NMR resonance of $SCN^-/H_2O_2$/LPO system strongly suggests that resonance b at −179 ppm can be assigned to $OSCN^-$. To confirm whether the $^{15}N$ NMR resonance at −179 ppm was indeed due to $OSC^{15}N^-$, we chemically prepared the $OSC^{15}N^-$ and monitored its $^{15}N$ NMR in aqueous solution at pH = 6.1 (see Table I). Since the $pK_a$ of HOSCN is 5.3 (Thomas, 1981), the oxidation product in the pH range of 4–6 is expected to be a mixture of $HOSCN/OSCN^-$. We however observe only a single resonance at −179 ppm. Since chemically prepared $OSCN^-/HOSCN$ shows a single $^{15}N$ resonance at −179 ppm, we ascribe the above resonance to a mixture of these two species. To determine if $CN^-$ and $(SCN)_2$ were produced in LPO-catalyzed oxidation at an $[H_2O_2]/[SCN^-]$ ratio of 1:1, we measured the $^{15}N$ chemical shift o free $CN^-$ and $(SCN)_2$ (the latter was chemically prepared), which are listed in Table I. Our $^{15}N$ NMR studies in Figure 4 show no evidence of the existence of these species as oxidation product in the LPO/$SCN^-/H_2O_2$ system. No attempt was however made to study $^{15}N$ NMR of cyanosulfurous or cyanosulfuric acid or their salts. These acids produced by nonenzymatic procedure have been shown to be very short-lived in aqueous medium (Jander et al., 1947). It has been shown that HOSCN decomposes

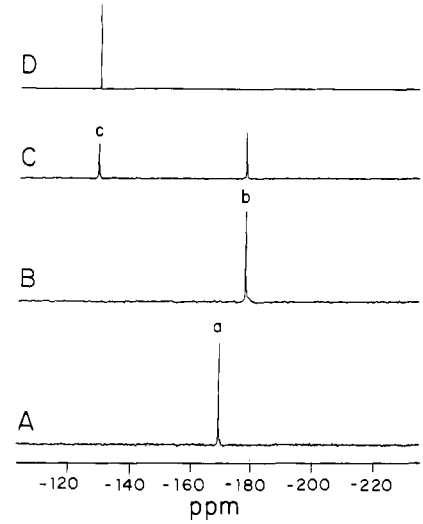


FIGURE 6: (A) $^{15}N$ NMR spectra of $SC^{15}N^-$ (1.0 mM) in the presence of LPO (0.1 μM) in 0.1 M phosphate buffer (pH = 6.0). (B) Effect of addition of $H_2O_2$ (4 mM) to (A). Spectra C and D were taken 1 and 10 min, respectively, after the addition of $H_2O_2$. The resonances at −170 ppm (a), −179 ppm (b), and −128 ppm (c) are assigned, respectively, to $SC^{15}N^-$, $OSC^{15}N^-$, and $C^{15}N^-$.

to $SCN^-$ with the formation of cyanosulfurous acid (Wood, 1946).

$$2HOSCN \rightarrow HO_2SCN + SCN^- + H^+ \quad (1)$$

The slow decomposition of oxidation product ($HOSCN/OSCN^-$) as observed in Figure 4 may be proceeding possibly through formation of cyanosulfurous acid ($HO_2SCN$). However, this species could not be detected by $^{15}N$ NMR since it decomposes very rapidly to sulfate, $CO_2$, ammonia, and $SCN^-$ in aqueous solution (Thomas, 1981).

To determine the effect of change in the $[H_2O_2]/[SCN^-]$ ratio on the oxidation of the LPO/$H_2O_2/SCN^-$ system, we carried out $^{15}N$ NMR at $[H_2O_2]/[SCN^-] = 4$. The results of this investigation is summarized in Figure 6. The resonance at −170 ppm refers to $SC^{15}N^-$ in the presence of LPO (pH = 6.1). On addition of $H_2O_2$ so as to have $[H_2O_2]/[SCN^-] = 4$, the characteristic $OSC^{15}N^-$ resonance appears immediately at −179 ppm. After a few minutes, a new resonance at −128 ppm appears and the intensity of the $OSC^{15}N^-$ resonance decreases. The −128 ppm $^{15}N$ NMR resonance can be easily assigned to $C^{15}N^-$ (see earlier and Table I). If the solution is left to stand for longer period (>15 min), the $OSC^{15}N^-$ resonance completely disappears and the $C^{15}N^-$ resonance gains in intensity. Therefore, the oxidized product ($OSCN^-$) is observed to be unstable in an excess of $H_2O_2$ and is further readily oxidized to cyanide. Since cyanide is known to bind to iron in LPO, the $C^{15}N^-$ signal due to cyanide bound to LPO (LPO–CN) was monitored but found to be very broad and downfield shifted at +533 ppm (Behere et al., 1985). When an excess of $SCN^-$ was added after cyanide formation, only an increase in intensity of the $^{15}N$ NMR resonance of $SCN^-$ (resonance **a**, −170 ppm) was observed due to addition of more $SC^{15}N^-$, and no change in its intensity and formation of $OSCN^-$ was observed with time on addition of $H_2O_2$. This is consistent with the known observation that LPO–CN is catalytically inactive.

The formation of cyanide was also confirmed by optical absorbtion spectra of LPO for diferent ratios of $[H_2O_2]$ to $[SCN^-]$ (Figure 7). At an equimolar ratio, no change in optical spectrum of LPO was observed on addition of $H_2O_2$ and thiocyanate. But high-spin LPO spectra changed to

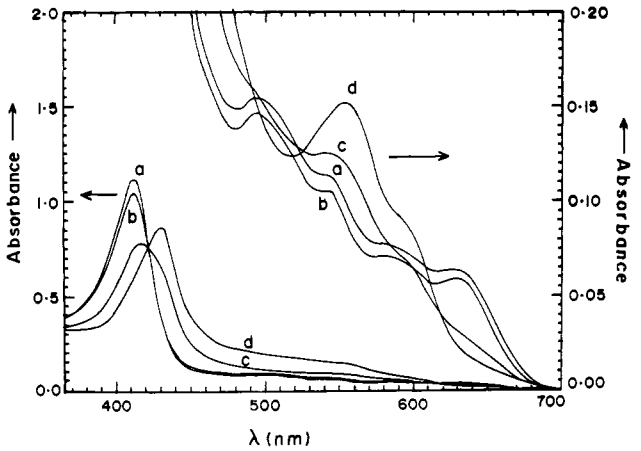


FIGURE 7: Effect of addition of thiocyanate and hydrogen peroxide on the optical spectra of LPO in 0.1 M phosphate buffer (pH = 6.0) at 23 °C. (a) LPO in the absence of $SCN^-$ and $H_2O_2$. (b, c, and d) Spectra recorded after incubation of LPO (0.1 $\mu$M) and $SCN^-$ (1 mM) with 1, 2, and 4 mM $H_2O_2$, respectively.

low-spin LPO–CN spectra when the ratio of $[H_2O_2]$ to $[SCN^-]$ was increased above 1 (Figure 7). This is consistent with our above observation that the $^{15}N$ NMR resonance due to free cyanide at −128 ppm was observed when the ratio of $[H_2O_2]$ to $[SCN^-]$ was more than 2.

*Study on the Bacteriocidal Action of the $SCN^-/H_2O_2$/LPO System on S. cremoris 972.* The bacteriocidal action of the $SCN^-/H_2O_2$/LPO system on *S. cremoris* 972 was independently monitored, optically by measuring the intensity of the 235-nm band, by $^{15}N$ NMR, and also by oxygen uptake by the bacteria in presence of $SCN^-/H_2O_2$/LPO. When 0.2 mL of *S. cremoris* 972 bacterial cell suspension was added to an incubated mixture of LPO (0.1 $\mu$M), $H_2O_2$ (1 mM), and $SCN^-$ (1 mM) in glucose (200 mM), $A_{235}$ was observed to be almost zero, suggesting the absence of $OSCN^-$. This result was also confirmed by $^{15}N$ NMR. On addition of 0.2 mL of bacterial suspension to LPO (0.1 $\mu$M), $SC^{15}N^-$ (1 mM), and $H_2O_2$ (1 mM) in glucose (200 mM), the $^{15}N$ NMR resonance due to $OSCN^-$ (which was present in the absence of the bacteria, see Figure 4) disappeared and the $^{15}N$ NMR resonance due to $SCN^-$ was observed in phosphate buffer (0.1 M, pH = 6.1). This suggests that, on addition of bacteria, $OSCN^-$ is converted to $SCN^-$. $A_{235}$ was therefore measured separately before and after addition of 0.2 mL of *S. cremoris* 972 to LPO (0.1 $\mu$M), $H_2O_2$ (0–4 mM), $SCN^-$ (1 mM), and glucose (200 mM). Figure 8 shows a plot of the difference in $A_{235}$ ($\Delta A_{235}$) vs the ratio of $[H_2O_2]$ to $[SCN^-]$ at different pHs. $\Delta A_{235}$ represents the difference in $OSCN^-$ concentration in the presence and absence of the bacteria. $\Delta A_{235}$ is observed to be maximum when $[H_2O_2]/[SCN^-]$ is 1 and pH is below 6.1 (Figure 8). This was also confirmed by monitoring the intensity of the $^{15}N$ NMR resonance of $OSCN^-$ in the incubation mixture of $LPO/H_2O_2/SCN^-$ under similar conditions in the absence and presence of 0.2 mL of *S. cremoris* 972 bacterial cell suspension at different pH. Variation in the difference in intensity of the $^{15}N$ NMR resonance of $OSCN^-$ with $[H_2O_2]/[SCN^-]$ ratio at different pH values shows a behavior similar to that in Figure 8.

Oxygen uptake by *S. cremoris* 972 was estimated by using an oxygen electrode. Figure 9 shows the effect of $[H_2O_2]/[SCN^-]$ ratio on the inhibition of oxygen uptake by *S. cremoris* 972 in the presence of LPO at different pHs. Incubation mixtures contained 1 mM $SCN^-$, $H_2O_2$ concentration as indicated in the figure, 0.1 $\mu$M LPO, 200 mM glucose in phosphate buffer (0.1 M), and 0.2 mL of bacterial cell suspension at 23 °C. As seen from Figure 9, maximum inhibition of oxygen uptake by *S. cremoris* 972 is obtained when hydrogen peroxide and thiocyanate were present in equimolar amounts and pH is below 6.0. At higher concentrations of $H_2O_2$, the inhibition was absent. Figure 9 also shows that inhibition of oxygen uptke by *S. cremoris* 972 was strongly pH dependent. The inhibition was maximum when the pH of the solution is 6.0. However, as the pH increases, inhibition decreases, and beyond pH = 8, there is no inhibition.

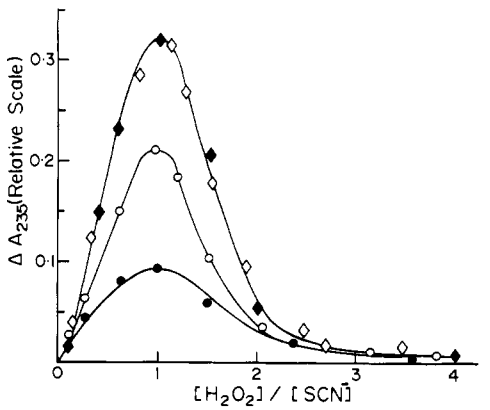


FIGURE 8: Plot of the difference in $A_{235}$ ($\Delta A_{235}$) when LPO (0.1 $\mu$M), $SCN^-$ (1 mM), $H_2O_2$ (as indicated in the figure), and glucose (200 mM) were incubated in the presence and absence of 0.2 mL of *S. cremoris* 972 bacteria vs the $[H_2O_2]/[SCN^-]$ ratio at different pHs. The solutions were prepared in phosphate buffer (0.1 M), and the pH of the final solution was 5.5 (◇), 6.1 (◆), 6.5 (○), or 7.0 (●).

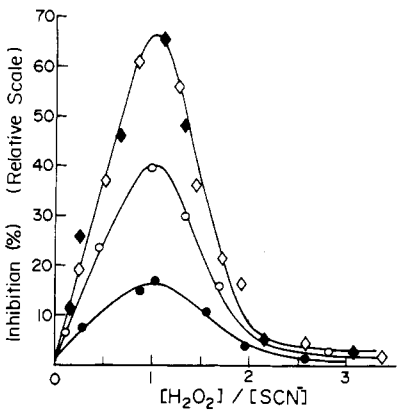


FIGURE 9: Effect of $[H_2O_2]/[SCN^-]$ ratio on inhibition of oxygen uptake by *S. crmeoris* 972 in the presence of LPO at different pHs. The pH of the final solution (0.1 M phosphate buffer) was 5.5 (◇), 6.1 (◆), 6.5 (○), or 7.0 (●).

To unambiguously confirm that the species responsible for the bacteriocidal action in $LPO/SCN^-/H_2O_2$ system is indeed $OSCN^-$/HOSCN, the inhibition of oxygen uptake by *S. cremoris* 972 was studied by using *chemically* prepared OSCN/HOSCN (≈1 mM). The results, summarized in Figure 10, show that the inhibition in the oxygen uptake achieved by the chemically prepared $OSCN^-$ is almost the same (≈70%) as obtained in the $LPO/SCN^-/H_2O_2$ system under comparable concentration (see Figure 9). Figure 10 also includes, for comparison, the effect of $CN^-$ and $SCN^-$ on the inhibition of the oxygen uptake, which is observed to be very low. These results clearly confirm that $OSCN^-$/HOSCN acts as a bacteriocidal agent in the $LPO/SCN^-/H_2O_2$ system.

## Discussion

The results of our independent measurements presented in the foregoing section show that at equimolar concentrations of $H_2O_2$ and $SCN^-$ the initial stable oxidation product of the

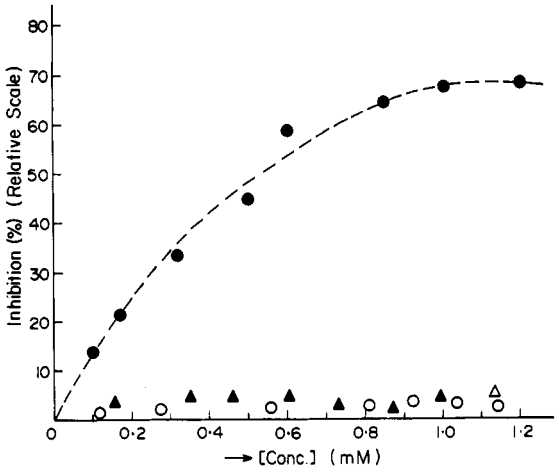


FIGURE 10: Effect of chemically prepared $OSCN^-/HOSCN$ (●), $CN^-$ (▲), and $SCN^-$ (○) on the oxygen uptake by *S. cremoris* 972 at pH = 6.1. Glucose (200 mM) and different concentrations of $OSCN^-$, $CN^-$, and $SCN^-$ were incubated in the presence and absence of 0.2 mL of bacteria.

$LPO/H_2O_2/SCN^-$ system is $HOSCN/OSCN^-$. As we have mentioned above, both these species are likely to exist in the pH range of 4–6 since the $pK_a$ of HOSCN is 5.3. The $^{15}N$ NMR chemical shifts of $HOSC^{15}N$ and $OSC^{15}N^-$ are expected to be very close, if not almost identical. This sets a limitation to their individual detection by $^{15}N$ NMR. The intermediate species, cyanosulfurous acid ($HO_2SCN$), involved in the decomposition of HOSCN could not also be detected since this species is highly unstable and decomposes rapidly (Thomas, 1981). Other products such $(SCN)_2$ and $CN^-$ proposed earlier (Chung & Wood, 1970; Aune & THomas, 1978) are ruled out on the basis of $^{15}N$ NMR results at an $[H_2O_2]/[SCN^-]$ ratio of 1 (Figure 4).

The formation of cyanide was observed only when the ratio of $[H_2O_2]$ to $[SCN^-]$ was increased above 1 (Figures 6 and 7). Therefore, further oxidation of $OSCN^-$ to $CN^-$ may account for the decrease in the yield of the $OSCN^-$ at higher ratio (>1). When the ratio of $[H_2O_2]$ to $[SCN^-]$ is above 4, a large excess of $CN^-$ is produced, which binds to the heme iron, making the high-spin LPO almost fully converted to low-spin LPO–CN (Behere et al., 1985). This was also confirmed by optical spectroscopy. Due to the presence of strongly bonded cyanide ion at the sixth coordination position of iron in LPO–CN it cannot now react with $H_2O_2$ to form compounds I and II (Dunford & Stillman, 1976), making LPO inactive. Due to the loss of its peroxidative activity LPO cannot oxidize $SCN^-$ to $OSCN^-$ any further, even if hydrogen peroxide is added in excess.

The yield of oxidation product is highly dependent on the pH of the system. The extent of oxidation is maximum when pH <6.0 and minimum when pH = 8.0. Thiocyanate binds to LPO at the distal histidine site with $pK_a$ = 6.1 (Modi et al., 1989a, 1990a). On the basis of line width of $^{15}N$ NMR resonance of $SC^{15}N^-/LPO$, it was suggested that the binding of thiocyanate to LPO is maximum when pH is below 6.0. Above pH = 8.0 the line width of thiocyanate in the presence of LPO is the same as in the absence of LPO, suggesting that thiocyanate does not bind to LPO above pH = 8.0 (Modi et al., 1989a, 1990a). Since the oxidation of $SCN^-$ by $H_2O_2$ is not also catalyzed by LPO at pH = 8.0, we suggest that the binding of thiocyanate to LPO may be a prerequisite for the oxidation of thiocyanate. The oxidation of thiocyanate which is believed to occur via two-electron transport is probably mediated by the histidyl imidazole in the distal site of the heme (Modi et al., 1989a, 1990a). The ferryl species $Fe^{IV}=O$ of compound I has been shown to be associated with the NH of the imidazole of distal histidine by hydrogen bonding (Hashimoto et al., 1986). The nitrogen atom of thiocyanate may form hydrogen bonding with distal histidine (Modi et al., 1989a, 1990a). Therefore, an electron-transfer path is formed by conjugated double and hydrogen bonds, which may facilitate the transport of electrons from thiocyanate to heme iron, as shown in case of oxidation of iodide (Sakurada et al., 1987). The pH dependence of the formation of $HOSCN/OSCN^-$ thus correlates with our earlier observation that thiocyanate binds to LPO only in the acidic range (pH <6.1). In this respect, the pH dependence of bacteriocidal action is also significant. The exact mode of the bacteriocidal action is not known. The fact that, in the presence of bacterial cells, only an $SCN^-$ signal is observed suggests that a two-electron oxidant (Magnusson et al., 1984) such as $HOSCN/OSCN^-$ transfers both the oxidizing equivalents by transferring the oxygen atom to the cell. It is not known whether such a transfer takes place by invasion of the bacterial cell or on the surface of the cell membrane. However, if the mode of action involves invasion of the cell, HOSCN is likely to be the active oxygen transferring agent which may, being neutral, penetrate through the hydrophobic lipid bilayer of the cell membrane, consistent with the earlier suggestions (Hog & Jago, 1970; Bjorck & Claesson, 1980).

Slow disappearance of resonance b in Figure 4 corresponds to degradation of $HOSCN/OSCN^-$. The degradation is expected to proceed through formation of cyanosulfurous acid (eq 1; Thomas, 1981); the nonobservance of an intermediate resonance due to cyanosulfurous acid suggests that these species if formed are present only in extremely small concentrations, besides being unstable in aqueous solution. In the present study we have mainly concentrated on the variation in the ratio of $H_2O_2$ to $SCN^-$ concentrations by keeping the enzyme concentration relatively low ($[SCN^-]/[LPO] = 10^4$). If much larger enzyme concentrations are used, then these higher oxy acids can also be formed by further oxidation of $OSCN^-$ by compound 1 (Pruitt & Tenovuo, 1982).

In the $LPO/H_2O_2/SCN^-$ system, $OSCN^-$ is stable for a considerably long period (see Figure 4). But if *S. cremoris* 972 is added, $OSCN^-$ converts immediately to $SCN^-$. This conversion to $SCN^-$ depends upon pH and the $[H_2O_2]/[SCN^-]$ ratio as we have seen from a plot of $\Delta A_{235}$ and $\Delta I$ vs the $[H_2O_2]/[SCN^-]$ ratio (Figures 8 and 9). This conversion is maximum when the pH of system is 6 and hydrogen peroxide and thiocyanate are present in equimolar amounts.

Activity of the $LPO/H_2O_2/SCN^-$ system is maximum when the ratio of $[H_2O_2]$ to $[SCN^-]$ is 1 at pH = 6.0 as seen from inhibition of oxygen uptake by *S. cremoris* 972 at different $[H_2O_2]/[SCN^-]$ ratios (Figure 9). The formation of $OSCN^-$ is also observed to be maximum when the ratio of $[H_2O_2]$ to $[SCN^-]$ is 1 for pH <6.0 (Figure 2). Moreover, activity of $LPO/H_2O_2/SCN^-$ is strongly pH dependent (Figure 10), which is similar to the pH dependence of $OSCN^-$ (Figures 3 and 5). Therefore, changes in $A_{235}$ parallel the changes produced in bacterial inhibition by the $[H_2O_2]/[SCN^-]$ ratio and the pH of the solution. Thus, the potential bacteriocidal or bacteriostatic activity of the $H_2O_2/LPO/SCN^-$ system may be related to the formation of $HOSCN/OSCN^-$ species rather than $CN^-$ and $(SCN)_2$, on the oxidation of thiocyanate since these species [$CN^-$ and $(SCN)_2$] as suggested earlier (Chung & Wood, 1970; Aune & Thomas, 1978) are not present in solution when the bacteriocidal activity of this system is maximum (i.e., when the $[H_2O_2]/[SCN^-]$ ratio is 1). The

formation of $CN^-$ (when the ratio $[H_2O_2]/[SCN^-] > 2$), and consequent binding of $CN^-$ to LPO, inactivates the enzyme and hence cannot account for the bacteriocidal activity. Therefore, the activity of the $SCN^-/H_2O_2/LPO$ system is due to $HOSCN/OSCN^-$ only, which is consistent with both $^{15}N$ NMR and optical spectroscopic results, where the profile of $HOSCN/OSCN^-$ formtion (Figure 2) correlates well with antibacterial activity profile (Figure 10). Our $^{15}N$ NMR studies also show that $OSCN^-$ is relatively stable under these conditions. It undergoes complete autoreduction to $SCN^-$ after several hours (Figure 4), which in turn is oxidized back to $OSCN^-$ on further utilization of $H_2O_2$. Moreover, on reacting with *S. cremoris* 972, $OSCN^-$ is converted to $SCN^-$. This oxidation–reduction cycle in the presence of LPO maintains a high level of $OSCN^-$ in the system and may be responsible for the preservation of milk (Bjorck et al., 1979) without refrigeration for several hours.

Our results clearly demonstrate that $^{15}N$ NMR can be used as an effective probe to study many properties of the oxidation product. These properties correlate very well with optical as well as bacteriocidal properties of the $SCN^-/H_2O_2/LPO$ system.

## ACKNOWLEDGMENTS

We thank Drs. G. K. Jarori and G. Krishnamurthy for many helpful suggestions and discussions. The NMR studies were carried out at the 500-MHz FT NMR National Facility, which is gratefully acknowledged.

**Registry No.** LPO, 9003-99-0; $SCN^-$, 302-04-5; $H_2O_2$, 7722-84-1; $OSCN^-$, 63296-34-4; $CN^-$, 57-12-5.

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