Lactoperoxidase, Peroxide, Thiocyanate Antimicrobial System: Correlation of Sulfhydryl Oxidation with Antimicrobial Action

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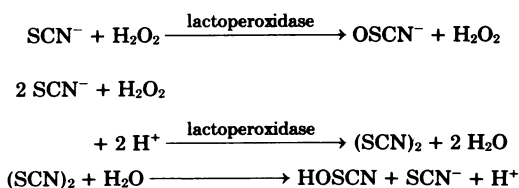
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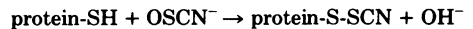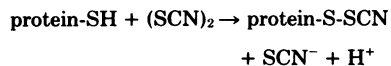
The antimicrobial activity of the lactoperoxidase, peroxide, thiocyanate system against *Escherichia coli* was directly related to the oxidation of bacterial sulfhydryls. Lactoperoxidase catalyzed the oxidation of thiocyanate, which resulted in the accumulation of hypothiocyanite ion, OSCN⁻. A portion of the bacterial sulfhydryls were oxidized by OSCN⁻ to yield sulfenic acid and sulfenyl thiocyanate derivatives. The remaining sulfhydryls were not oxidized, although OSCN⁻ was present in large excess. The oxidation of sulfhydryls to sulfenyl derivatives inhibited bacterial respiration. This inhibition could be reversed by adding sulfhydryl compounds to reduce the sulfenyl derivatives and the excess OSCN⁻. Also, this inhibition could be reversed by washing the cells so as to remove the excess unreacted OSCN⁻. After washing, the bacteria underwent a time-dependent recovery of their sulfhydryl content. This recovery resulted in recovery of the ability to respire. The inhibited cells were viable if diluted and plated shortly after the incubation with the lactoperoxidase, peroxide, thiocyanate system. On the other hand, long-term incubation in the presence of the excess OSCN⁻ resulted in loss of viability. Also, the inhibition of respiration became irreversible. During this long-term incubation, the excess OSCN⁻ was consumed and the sulfenyl derivatives disappeared.

Lactoperoxidase-catalyzed oxidation of thiocyanate (SCN⁻) in milk and saliva contributes to the antimicrobial activity of these fluids (4, 9–11, 13, 14, 16–18, 20, 22, 24, 25). The lactoperoxidase, H_2O_2, SCN⁻ system produces an inhibitor of bacterial growth (4, 10, 11, 17, 18). A number of metabolic activities are inhibited, including respiration and glycolysis (10, 14, 17, 18). The growth of certain bacteria is completely inhibited, whereas others recover and resume growth (4, 14, 20). Washing the cells or adding reducing agents reverses the inhibition of growth (11, 14, 17, 18).

Recently, lactoperoxidase-catalyzed oxidation of SCN⁻ was shown to result in accumulation of hypothiocyanite ion, OSCN⁻ (1, 11). The oxidation of SCN⁻ may yield OSCN⁻ directly or may yield thiocyanogen, $(SCN)_2$, which hydrolyzes rapidly to yield hypothiocyanous acid, HOSCN (12).

$$SCN^- + H_2O_2 \xrightarrow{\text{lactoperoxidase}} OSCN^- + H_2O_2$$

$$2\ SCN^- + H_2O_2$$

$$+\ 2\ H^+ \xrightarrow{\text{lactoperoxidase}} (SCN)_2 + 2\ H_2O$$

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

Lactoperoxidase catalyzes the incorporation of SCN⁻ into protein substrates (2, 3). Similar results are obtained by adding $(SCN)_2$ in carbon tetrachloride to aqueous solutions of the proteins. The reaction of $(SCN)_2$ or OSCN⁻ with proteins oxidizes the protein sulfhydryls to sulfenyl thiocyanate derivatives.

$$\text{protein-SH} + (SCN)_2 \rightarrow \text{protein-S-SCN}$$
$$+\ SCN^- + H^+$$

$$\text{protein-SH} + OSCN^- \rightarrow \text{protein-S-SCN} + OH^-$$

Sulfenyl thiocyanate derivatives can undergo further modifications, including reversible hydrolysis to yield sulfenic acids.

$$\text{protein-S-SCN} + H_2O \rightleftarrows \text{protein-S-OH} + SCN^- + H^+$$
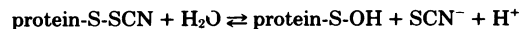
Also, sulfenyl derivatives can undergo slow oxidation to sulfonic acids or other forms. The SCN moiety can be displaced from sulfenyl thiocyanate by reduction with a sulfhydryl compound, such as dithiothreitol. When all the protein sulfhydryls are oxidized by $(SCN)_2$ or OSCN⁻, the tyrosine, tryptophan, and histidine residues are modified. The carbon portion of SCN⁻ or $(SCN)_2$ is incorporated to a greater extent than sulfur. This type of incorporation is not released upon incubation with dithiothreitol.

456

The aim of this study was to determine whether the lactoperoxidase system modifies bacterial sulfhydryls and aromatic amino acid residues and whether these modifications are relevant to the antimicrobial mechanism. In addition, the antimicrobial action of $OSCN^-$ was compared with that of the lactoperoxidase, H_2O_2, SCN^- system to determine whether $(SCN)_2$ or other short-lived intermediates contributed to the antimicrobial activity.

MATERIALS AND METHODS

Chemicals. All chemicals were of reagent grade. Lactoperoxidase purified from bovine milk (15) was provided by M. Morrison. Myeloperoxidase purified from human leukemic granulocytes was provided by M. Morrison and J. Naskalski. Horseradish peroxidase (type VI), dithiothreitol, 5,5'-dithiobis(2-nitrobenzoic acid), and catalase were obtained from Sigma Chemical Co., St. Louis, Mo.; [^{14}C]thiourea (55.7 Ci/mol) and $K^{35}SCN$ (15.6 Ci/mol) from Amersham Corp., Arlington Heights, Ill.; and $NaS^{14}CN$ (10 Ci/mol) from ICN Pharmaceuticals, Cleveland, Ohio. Sodium borohydride was used to reduce 5,5'-dithiobis(2-nitrobenzoic acid) to 5-thio-2-nitrobenzoic acid (1).

Bacteria. *Escherichia coli* ML 308-225 was grown aerobically at 37°C in medium A (6), modified by addition of 5 mM tris(hydroxymethyl)aminomethane (Tris)-chloride, pH 6.6, with 1% (wt/vol) disodium succinate as carbon source. Cells were harvested in late logarithmic phase of growth by centrifugation at 25°C for 10 min at 5,300 × g and then suspended in 0.1 M potassium phosphate buffer, pH 6.6, with 1 mM magnesium sulfate.

Peroxidase systems. Reaction mixtures contained 6×10^8 cells/ml, 0.1 μM lactoperoxidase or myeloperoxidase, and varying amounts of KSCN. At 1-min intervals, H_2O_2 was added with each addition sufficient to give a concentration of 30 μM. Incubations were continued after the last addition of H_2O_2 for a total of 25 min of exposure to the peroxidase system. Amounts of KSCN are given as the initial concentration, and amounts of H_2O_2 are given as the concentration that would have been obtained if H_2O_2 had not been consumed.

Determination of $OSCN^-$. The concentration of $OSCN^-$ was determined by the oxidation of 2 mol of the sulfhydryl compound, 5-thio-2-nitrobenzoic acid, to the disulfide compound, 5,5'-dithiobis(2-nitrobenzoic acid), by 1 mol of $OSCN^-$ (1). An excess of the sulfhydryl compound was added to reaction mixtures, and absorbance of the supernatant at 412 nm was measured after centrifugation at 5,300 × g for 5 min at 0 to 5°C. An ε of 13,600 was assumed for 5-thio-2-nitrobenzoic acid (7).

Sulfhydryl determination. Sulfhydryl concentration was measured by the reaction of 1 mol of sulfhydryl with 5,5'-dithiobis(2-nitrobenzoic acid) to yield 1 mol of 5-thio-2-nitrobenzoic acid (7). Reaction mixtures (2 ml) were centrifuged at 5,300 × g for 10 min at 0 to 5°C. The cells were resuspended to the original volume in the phosphate buffer with magnesium sulfate and then diluted with 4 ml of 0.1 M Tris-chloride buffer, pH 8.0, containing 10 mM ethylenediaminetet-

raacetic acid. Portions (0.1 ml) of 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) in 0.1 M potassium phosphate, pH 7.0, and 0.5 ml of 10% (wt/vol) sodium dodecyl sulfate were added. The mixtures were incubated at 37°C for 1 h and centrifuged at 18,000 × g for 10 min. Absorbance of 5-thio-2-nitrobenzoic acid was measured at 412 nm.

Sulfenyl determination. Sulfenyl derivatives were measured by the reaction of these derivatives with [^{14}C]thiourea to yield the radioactive mixed-disulfide derivative (19). Portions (0.1 ml) of reaction mixtures were incubated at 37°C for 15 min with 0.9 mM [^{14}C]thiourea and 1% (wt/vol) sodium dodecyl sulfate. The mixtures were precipitated with 1 ml of cold 10% (wt/vol) trichloroacetic acid, filtered through nitrocellulose filters (Millipore Corp., Bedford, Mass.), and washed with 5% (wt/vol) trichloroacetic acid. The filters were dissolved in scintillation fluid (5), and the radioactivity was determined in a liquid scintillation spectrometer.

Incorporation of SCN^-. Incorporation of $^{35}SCN^-$ or $S^{14}CN^-$ into bacterial components was measured by acid precipitation of 0.1-ml portions of reaction mixtures. The precipitation and determination of radioactivity were performed as described above.

Rates of respiration. Reaction mixtures were made 30 mM in disodium succinate, and then consumption of O_2 was measured at 25°C with an O_2 electrode (American Instrument Co., Silver Spring, Md.). Relative rates of O_2 consumption were determined from the linear portion of the continuous recording of O_2 concentration over 3 to 10 min.

Assay of viability. Serial 1:10 dilutions were prepared from 1-ml portions of reaction mixtures in sterile, modified medium A without a carbon source. One-milliliter portions of appropriate dilutions were spread on a solid medium containing modified medium A, 2% (wt/vol) agar (Difco Laboratories, Detroit, Mich.), and 1% glucose. Plates were incubated at 25°C, and the number of colonies was recorded after 2 to 3 days.

RESULTS

Sulfhydryl oxidation and inhibition of respiration. Figure 1 compares the amount of $OSCN^-$ detected when lactoperoxidase-catalyzed oxidation of SCN^- was carried out either in the absence or presence of *E. coli*. In the absence of the bacteria, and with excess SCN^-, about 1 mol of $OSCN^-$ was obtained per mol of H_2O_2. When H_2O_2 was in excess of SCN^-, the yield of $OSCN^-$ was lower than the amount of SCN^-, suggesting that $OSCN^-$ was oxidized by the excess H_2O_2. In the presence of the bacteria, the amount of $OSCN^-$ detected was only slightly smaller than the amount formed in their absence. Therefore, only a small part of the $OSCN^-$ was consumed by reaction with bacterial components.

The unreacted $OSCN^-$ could be removed from the bacteria by centrifugation (Fig. 1). Most of the $OSCN^-$ was recovered in the supernatant. Part of the $OSCN^-$ was lost from the supernatant during the time required for centrifugation,
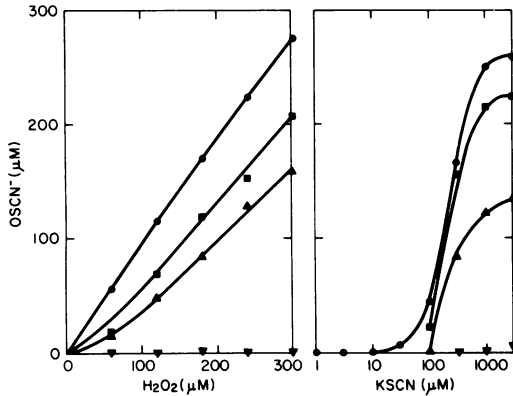
FIG. 1. *Formation of OSCN⁻. Lactoperoxidase-catalyzed oxidation of 1 mM KSCN with the indicated amounts of H_2O_2 (left) or of the indicated amounts of KSCN with 0.3 mM H_2O_2 (right) was carried out in the absence (●) or presence (■) of E. coli at 0 to 5°C. The concentration of OSCN⁻ was measured 15 min after the last addition of H_2O_2. When the oxidation of SCN⁻ was carried out in the absence of E. coli, 1 ng of catalase was added before measuring OSCN⁻. Also, the E. coli suspensions were centrifuged at 5,300 × g for 10 min at 0 to 5°C, and then the cells were suspended to the original volume. The amount of OSCN⁻ in the supernatant (▲) and the resuspended cells (▼) was measured.*

but little or no OSCN⁻ was bound to the bacteria.

Identification of the oxidizing agent formed in the presence of the bacteria as OSCN⁻ was based on recovery of SCN⁻ upon reduction, inability to extract the agent into organic solvents, and the reaction of 1 mol of the agent with 1 mol of added potassium cyanide, resulting in loss of ability to oxidize sulfhydryls (1).

Figure 2 shows the loss of bacterial sulfhydryls and formation of sulfenyl derivatives upon incubation *E. coli* with the lactoperoxidase system. About one-third to one-half of the sulfhydryls were oxidized. The remaining sulfhydryls were not oxidized even though OSCN⁻ was present in large excess. The yield of sulfenyl derivatives was about equal to the amount of oxidized sulfhydryls. Therefore, sulfhydryls were oxidized primarily to sulfenyl derivatives. However, in the range of SCN⁻ concentrations from 10 to 100 μM, the loss of sulfhydryls exceeded the formation of sulfenyl derivatives. Under these conditions, sulfhydryls may have been oxidized to disulfides, sulfonic acids, or other forms either by excess H_2O_2 or by products of the reaction of OSCN⁻ with excess H_2O_2.

Figure 3 shows incorporation of S¹⁴CN⁻ into bacterial components. In other experiments, similar incorporation of ³⁵SCN⁻ was observed. Also shown is the amount of incorporation mea-

sured after incubation with the sulfhydryl compound, dithiothreitol. The one-to-one incorporation of carbon and sulfur and the ability of exogenous sulfhydryl compounds to remove this incorporation indicated formation of the sulfenyl thiocyanate derivative of bacterial sulfhydryls. The yield of sulfenyl thiocyanate was lower than the amount of sulfenyl derivatives (compare with Fig. 2). Also, washing the bacteria by centrifugation at 0 to 5°C removed the incorporated radioactivity but did not lower the amount of sulfenyl derivatives. These results were consistent with hydrolysis of sulfenyl thiocyanate derivatives to form sulfenic acids.

In the range of SCN⁻ concentrations from 10 to 100 μM, the carbon portion of SCN⁻ was
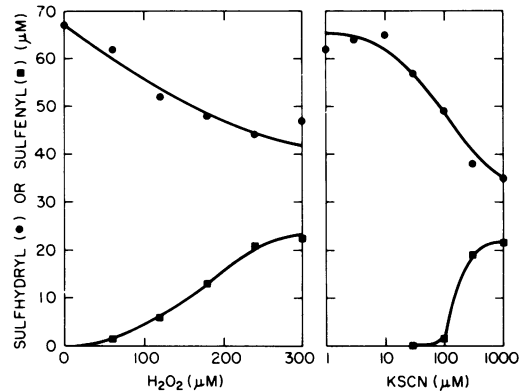

FIG. 2. *Oxidation of sulfhydryls to sulfenyl derivatives. Cells were exposed to the lactoperoxidase system with 1 mM KSCN and the indicated amounts of H_2O_2 (left) or 0.3 mM H_2O_2 and the indicated amounts of KSCN (right) at 0 to 5°C. The concentrations of sulfhydryls (●) and sulfenyl derivatives (■) were measured.*
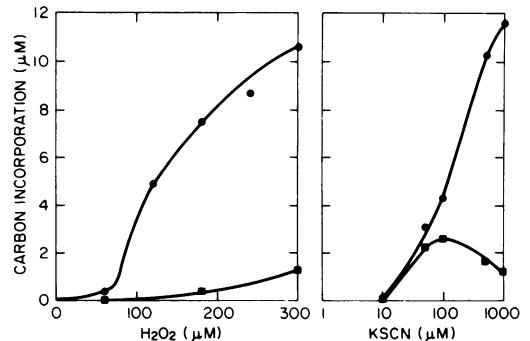

FIG. 3. *Incorporation of S¹⁴CN⁻. Cells were exposed to the lactoperoxidase system with 1 mM KS¹⁴CN and the indicated amounts of H_2O_2 (left) or 0.3 mM H_2O_2 and the indicated amounts of KS¹⁴CN (right) at 0 to 5°C. Incorporation of radioactivity was measured before (●) and after (■) addition of 10 mM dithiothreitol.*

incorporated into forms that were not released upon incubation with dithiothreitol. Much lower incorporation of the sulfur portion was observed. These results indicated incorporation of carbon into derivatives other than sulfenyl thiocyanate. The amount of this type of incorporation was much less than the amount of sulfenyl thiocyanate.

Exposure of the bacteria to the lactoperoxidase system inhibited their respiration (Fig. 4). Also shown are results obtained by adding dithiothreitol before measuring respiration. At high SCN⁻ concentrations, incubation with dithiothreitol almost completely relieved the inhibition of respiration. Therefore, inhibition was relieved by reduction of the excess OSCN⁻ and the sulfenyl derivatives.

In the range of SCN⁻ concentrations from 10 to 100 μM, dithiothreitol did not completely reverse the inhibition. This is the range in which sulfhydryls were oxidized to forms other than sulfenyl derivatives, and nonreversible incorporation of S¹⁴CN was obtained. These chemical modifications could be responsible for the nonreversible portion of inhibition.

Similar oxidation of sulfhydryls and inhibition of respiration were obtained by carrying out the oxidation of SCN⁻ in the absence of the bacteria and then adding the accumulated OSCN⁻ to the bacteria. Also, similar results were obtained when excess unreacted OSCN⁻ was removed from bacteria by centrifugation and then was added to previously untreated bacteria. Therefore, it made little difference whether bacteria were included directly with the lactoperoxidase

system or with OSCN⁻. However, one significant difference was observed: at low SCN⁻ concentrations, the amount of oxidized sulfhydryls was greater when the bacteria were incubated directly with the lactoperoxidase system.

The results shown in Fig. 1 through 4 were obtained by incubating *E. coli* with the lactoperoxidase system at 0 to 5°C. Similar results were obtained at 25 and 37°C, although it was not possible to demonstrate complete reversal of inhibition by dithiothreitol at the higher temperatures. Also, larger amounts of H₂O₂ or higher peroxidase concentrations were required to obtain the same yield of OSCN⁻, because the bacteria destroy H₂O₂ faster at high temperatures (23). At 0 to 5°C, the yield of OSCN⁻ was not increased by raising the lactoperoxidase concentration from 0.1 to 1 μM.

Similar results were obtained with either myeloperoxidase or lactoperoxidase. In contrast, horseradish peroxidase had no effect. Horseradish peroxidase does not catalyze the oxidation of SCN⁻ (21).

Recovery from inhibition. Removal of OSCN⁻ by centrifugation permitted recovery from inhibition (Fig. 5). About 15 to 30 min of incubation at 25°C was required for recovery after OSCN⁻ was removed. Recovery did not take place during washing at 0 to 5°C or when the washed cells were held at 0 to 5°C. Also, when unwashed cells were incubated with the excess OSCN⁻ at 25°C for 1 h and then washed, there was no recovery during subsequent incubation at 25°C.

After the cells were washed to remove OSCN⁻, the sulfhydryl content of the cells increased during incubation at 25°C (Fig. 6). Also, the content of sulfenyl derivatives decreased. Therefore, recovery appeared to result from the reduction of sulfenyl derivatives back to sulfhy-
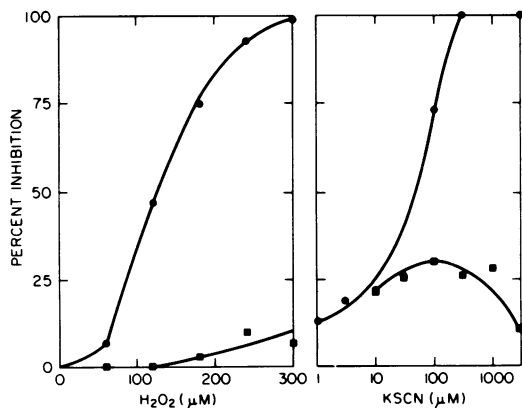
FIG. 4. *Inhibition of respiration. Cells were exposed to the lactoperoxidase system with 1 mM KSCN and the indicated amounts of H₂O₂ (left) or 0.3 mM H₂O₂ and the indicated amounts of KSCN (right) at 0 to 5°C. The cell suspensions were incubated at 25°C for 5 min in the absence (●) or presence (■) of 10 mM dithiothreitol, and then rates of O₂ consumption were measured.*
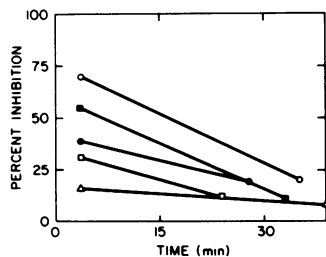
FIG. 5. *Recovery from inhibition. Cells were exposed to the lactoperoxidase system with 1 mM KSCN and 0.3 (○), 0.24 (■), 0.18 (●), 0.12 (□), or 0.06 (△) mM H₂O₂ at 0 to 5°C. The suspensions were centrifuged at 0 to 5°C for 10 min at 5,300 × g, and then the cells were suspended to the original volume and incubated at 25°C. Rates of O₂ consumption were measured at the indicated times after warming to 25°C.*

dryls. Alternatively, the sulfenyl derivatives may have been degraded, and new sulfhydryl components may have been synthesized. Under the same conditions, there was no increase in the sulfhydryl content of cells that had not been exposed to the lactoperoxidase system. Also, there was no increase in the number of viable cells. Therefore, the increase in sulfhydryl content was due to a repair process rather than to growth of the cells.

Bactericidal action. Figure 7 compares viability measured immediately after exposure to the lactoperoxidase system with viability measured after continued incubation at 25°C for 2 h. The bacteria were almost completely viable
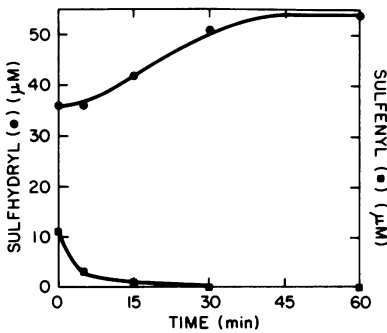

FIG. 6. Reduction of sulfenyl derivatives to sulfhydryls. Cells were exposed to the lactoperoxidase system with 0.3 mM H_2O_2 and 1 mM KSCN at 0 to 5°C. After centrifugation at 0 to 5°C for 10 min at 5,300 × g, the cells were suspended in cold buffer and then incubated at 25°C for the indicated times before determination of sulfhydryls (●) and sulfenyl derivatives (■).
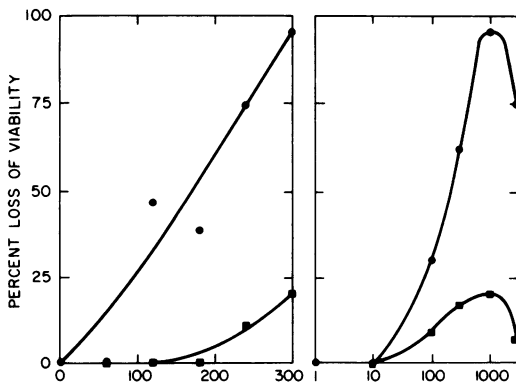

FIG. 7. Bactericidal action. Cells were exposed to the lactoperoxidase system with 1 mM KSCN and the indicated amounts of H_2O_2 (left) or with 0.3 mM H_2O_2 and the indicated amounts of KSCN (right) at 0 to 5°C. The cell suspensions were diluted and plated either without further incubation (■) or after a 2-h incubation at 25°C (●).

when diluted and plated immediately. Also, when the bacteria were held at 0 to 5°C for 2 h and then diluted and plated, there was only a small loss of viability. On the other hand, continued incubation at 25°C in the presence of excess OSCN⁻ caused the number of viable cells to decrease by about two orders of magnitude. Only a small part of this loss of viability could be reversed by dithiothreitol.

Inhibition of respiration did not increase or decrease greatly during incubation at 25°C with excess OSCN⁻ (Fig. 8). On the other hand, there was a qualitative change in the inhibition, as shown in the bottom portion of Fig. 8. After incubation at 25°C for 5 to 15 min, the inhibition could no longer be reversed by dithiothreitol.

The excess OSCN⁻ was consumed during continued incubation (Fig. 9). The rates of disappearance of OSCN⁻ were faster at higher temperatures. At all temperatures, the rate of disappearance of OSCN⁻ was much faster than the decomposition of OSCN⁻ observed in the absence of bacteria (1). There was no evidence for enzymatic decomposition of OSCN⁻. Similar rates of disappearance of OSCN⁻ were observed in the presence of heat-killed bacteria. Therefore, the disappearance of OSCN⁻ was probably due to reaction with cell components.
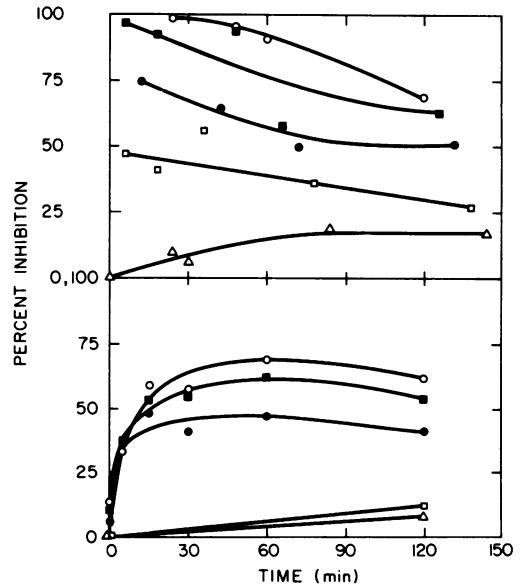

FIG. 8. Loss of ability of dithiothreitol to reverse inhibition of respiration. Cells were exposed to the lactoperoxidase system with 1 mM KSCN and 0.3 (○), 0.24 (■), 0.18 (●), 0.12 (□), or 0.06 (△) mM H_2O_2 at 0 to 5°C. The suspensions were incubated at 25°C for the indicated times, and then rates of O_2 consumption were measured in the absence (top) or presence (bottom) of 10 mM dithiothreitol.

Continued incubation with excess OSCN⁻ did not result in oxidation of the remaining bacterial sulfhydryls (Fig. 10). On the other hand, both sulfenic acid and sulfenyl thiocyanate were lost.
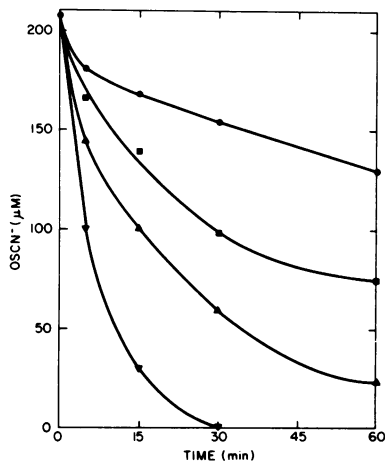

FIG. 9. *Consumption of OSCN⁻. Cells were exposed to the lactoperoxidase system with 0.3 mM H₂O₂ and 1 mM KSCN at 0 to 5°C. The suspensions were then incubated at 37 (▼), 25 (▲), 10 (■), or 0 to 5 (●) °C, and the amount of OSCN⁻ was measured at the indicated times.*
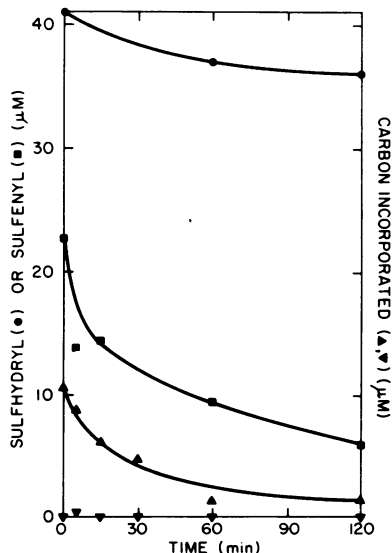

FIG. 10. *Modification of cell components upon extended incubation. Cells were exposed to the lactoperoxidase system with 0.3 mM H₂O₂ and 1 mM KSCN or KS¹⁴CN at 0 to 5°C. The suspensions were incubated at 25°C for the indicated times, and then sulfhydryls (●) and sulfenyl derivatives (■) were measured. Also, radioactive incorporation was measured before (▲) and after (▼) addition of 10 mM dithiothreitol.*

This loss may indicate oxidation of sulfenyl derivatives by the excess OSCN⁻. There was no increase in nonreversible incorporation of radioactivity from S¹⁴CN⁻ during continued incubation. Also, in other experiments, it was determined that there was no incorporation of ¹⁴C into lipid components or into non-acid-precipitable derivatives of cell components.

DISCUSSION

Lactoperoxidase or myeloperoxidase catalyzed the oxidation of SCN⁻ to OSCN⁻. In the presence of *E. coli*, a small part of the OSCN⁻ was consumed rapidly in the oxidation of bacterial sulfhydryls to sulfenyl thiocyanate and sulfenic acid derivatives. Oxidation of sulfhydryls to sulfenyl derivatives resulted in an immediate, reversible inhibition of respiration. Presumably, many sulfhydryl components of the cell were oxidized, including those components of the inner membrane that are essential to respiration of gram-negative bacteria.

Both the sulfenyl derivatives and the excess, unreacted OSCN⁻ could be reduced by exogenous sulfhydryl compounds. Reduction of the sulfenyl derivatives back to sulfhydryls reversed the inhibition of respiration. Similarly, when the excess OSCN⁻ was removed by centrifugation, the bacteria appeared to reduce the sulfenyl derivatives back to sulfhydryls. However, results presented here do not exclude the possibility that the sulfenyl derivatives were degraded and that new sulfhydryl compounds were synthesized to replace them. The recovery of sulfhydryl content restored the ability to respire.

Further studies will be required to establish the mechanism whereby *E. coli* recovers from inhibition. It has been proposed that oxidized bacterial sulfhydryls may be reduced at the expense of oxidation of NAD(P)H₂ (11). Possibly the recovery process could be due to reduction of sulfenyl derivatives by excess glutathione, followed by oxidation of NAD(P)H₂ and reduction of oxidized glutathione by glutathione reductase (8).

This recovery process accounts for the observation that the inhibited cells were viable. Presumably, recovery took place after the bacteria were diluted and plated for the assay of viability. Dilution would not remove OSCN⁻, but dilution may have lowered the OSCN⁻ concentration below that required for further oxidation of cell components.
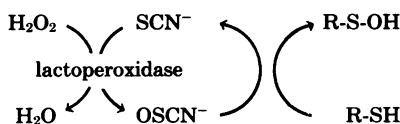
During continued incubation in the presence of excess OSCN⁻, the inhibition of respiration became irreversible and the bacteria lost their viability. These changes were accompanied by the consumption of the excess OSCN⁻ and the

loss of sulfenyl derivatives. The sulfenyl derivatives may have been oxidized by OSCN$^-$. The amount of OSCN$^-$ that was consumed exceeded the amount of sulfenyl derivatives, suggesting that OSCN$^-$ also reacted slowly with other cell components. These other components could be the oxidizable components, such as NAD(P)H$_2$, that may be required for the recovery process. On the other hand, more than 1 mol of OSCN$^-$ is consumed in the oxidation of 1 mol of sulfenyl derivatives of bovine serum albumin (2). The oxidation of a sulfenyl derivative to a sulfonic acid requires 4 oxidizing equivalents.

Lowering the temperature to 0 to 5°C slowed the recovery of cell sulfhydryl content and also slowed the reaction of OSCN$^-$ with sulfenyl derivatives or other cell components. Lowering the rates of these reactions permitted the demonstration of reversible inhibition, recovery, and irreversible inhibition as separate processes. When bacteria are exposed to the lactoperoxidase system in milk and saliva, these reactions may occur simultaneously. The extent of antimicrobial action would depend on the amount of OSCN$^-$ formed, the time of exposure to OSCN$^-$, and the ability of cells to repair oxidative damage.

At high ratios of H$_2$O$_2$ to SCN$^-$, the carbon portion of SCN$^-$ was incorporated into derivatives other than sulfenyl thiocyanate. This incorporation probably indicates modification of aromatic amino acid residues of proteins. This type of chemical modification did not appear to make a large contribution to antimicrobial action. However, such modifications may be significant in antimicrobial action in milk or saliva or against certain microorganisms.

Similar results were obtained when E. coli was exposed to preformed OSCN$^-$ or directly to the lactoperoxidase system. Therefore, it is not necessary to propose formation of short-lived intermediates, such as (SCN)$_2$, to account for antimicrobial action. On the other hand, at low SCN$^-$ concentrations, greater oxidation of sulfhydryls was obtained by incubating the bacteria with the lactoperoxidase system. Presumably, this difference was due to the turnover of SCN$^-$ (2).

$$\text{H}_2\text{O}_2 \quad \diagdown \diagup \quad \text{SCN}^- \quad \diagleftarrow \quad \diagrightarrow \quad \text{R-S-OH}$$

lactoperoxidase

$$\text{H}_2\text{O} \quad \diagup \diagdown \quad \text{OSCN}^- \quad \diagrightarrow \quad \text{R-SH}$$

As indicated in the diagram, the continuous oxidation of SCN$^-$ to OSCN$^-$ and reduction of OSCN$^-$ to SCN$^-$ would make the oxidation of sulfhydryls independent of SCN$^-$ concentration over a wide range. The reutilization of SCN$^-$

would be limited by the accumulation of unreacted OSCN$^-$, which would deplete the system of SCN$^-$.

The antimicrobial action of the SCN$^-$ system against E. coli differed from that of the peroxidase, H$_2$O$_2$, I$^-$ system (E. L. Thomas and T. M. Aune, unpublished work) in three ways. First, higher concentrations of SCN$^-$ than of I$^-$ were required for antimicrobial action. The I$_2$ that was produced by the peroxidase system reacted rapidly with E. coli cell components and did not accumulate in large excess. Therefore, the turnover of I$^-$ was not limited by the accumulation of unreacted I$_2$. Second, I$_2$ could oxidize all the bacterial sulfhydryls. It may be that OSCN$^-$ is less reactive than I$_2$ or too polar to have access to all the sulfhydryls. The sulfhydryls oxidized by OSCN$^-$ may be more reactive or located near the cell surface. Third, the cells did not recover even when the amount of I$_2$ was not sufficient to oxidize all the sulfhydryls. The extensive nonreversible incorporation of iodine atoms into tyrosine residues of proteins may have interfered with recovery.

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