Antibacterial Action of Lactoperoxidase-Thiocyanate-Hydrogen Peroxide on Streptococcus agalactiae

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Antibacterial activity of lactoperoxidase (LP)-thiocyanate (SCN)-hydrogen peroxide (H₂O₂) on Streptococcus agalactiae requires that the three reactants must be in contact with the cells simultaneously. Small but assayable amounts of LP adsorb to the cell surface and are not removed by washing. A diffusible antibacterial product of LP-SCN-H₂O₂ reaction was not found under our experimental conditions. Incubation of S. agalactiae cells with LP-H₂O₂ and ¹⁴C-labeled sodium SCN resulted in the incorporation of SCN into the bacterial protein. Most of the LP-catalyzed, incorporated SCN was released from the bacterial protein with dithiothreitol. Cells that had their membrane permeability changed by treatment with Cetab or 80% ethanol incorporated more SCN than did untreated cells, i.e., approximately 1 mol of SCN for each mol of sulfhydryl group present in the reaction mixture. Alteration of membrane permeability caused protein sulfhydryls, normally protected by the cytoplasmic membrane, to become exposed to oxidation. The results suggest the LP-H₂O₂-catalyzed incorporation of SCN into the proteins of S. agalactiae by a mechanism similar to that reported for bovine serum albumin. Removal of reactive protein sulfhydryls from a functional role in membrane transport and in glucolysis is a likely cause of the antibacterial effect for S. agalactiae.

The mode of action of the lactoperoxidase (LP)-thiocyanate (SCN)-hydrogen peroxide (H₂O₂) antibacterial system for the streptococci has been of interest since the three components of the system were identified (9, 19, 24). The reversibility of the antibacterial effect with reducing agents such as thioglycolate, glutathione, and catalase suggested an oxidative function for LP-H₂O₂ and the possible involvement of sulfhydryl groups in the antibacterial effect (11). A role for SCN was unexplained, although studies on the LP-catalyzed oxidation of SCN suggested that a "short-lived" intermediate oxidation product might be involved in growth inhibition (16). Reports on the LP-catalyzed incorporation of SCN into sulfhydryl groups of bovine serum albumin to form sulfenylthiocyanate derivatives of the protein sulfhydryls offered, for the first time, an explanation of a definite role for SCN in the antibacterial action (3; T. M. Aune and E. L. Thomas, Fed. Proc. 35:1386, 1976). The further studies on LP-catalyzed SCN oxidation showed an accumulation of hypothiocyanate (OSCN⁻) ion, which was stable in conditions existing in bacterial growth media and would oxidize protein sulfhydryl groups to sulfenylthiocyanate derivatives (1). Hoogendoorn et al. (8) suggested that OSCN⁻ was the growth inhibitor.

This present report deals with studies to show the LP-catalyzed incorporation of SCN into the proteins of Streptococcus agalactiae. It also deals with experiments to show the need for the simultaneous presence of all components in immediate contact with the bacteria for an antibacterial effect.

MATERIALS AND METHODS

Culture and medium. The culture of S. agalactiae and the chemically defined culture medium used were described in an earlier report (12).

For growth inhibition studies, the inoculum was grown in the chemically defined medium containing 5 µg of L-cystine per ml. Four milliliters of medium in screw-capped tubes (13 by 100 mm) was inoculated with 0.1 ml of the stock broth culture, maintained in 1% yeast extract-0.5% Casitone-0.5% glucose-0.1 M phosphate (pH 7), and incubated for 3 to 3.5 h at 37°C until the optical density at 610 nm in a Bausch & Lomb Spectronic 20 colorimeter was 0.55 to 0.60. The cells were sedimented by centrifugation, washed once, and suspended in 4 ml of sterile water. This suspension was diluted 1 to 10, and 0.2 ml (equivalent to about 20 µg of dry cells) was used to inoculate 4 ml of culture medium.

In tests to show binding of LP to bacteria, cells (equivalent to about 40 µg of cells [dry weight]) were suspended in 4 ml of medium containing (per milliliter) 1 U of LP, 0.7 µmol of sodium SCN (NaSCN), and 0.25
μmol of H₂O₂ or in medium containing LP without NaSCN or H₂O₂. After incubation for 30 min at 24°C, the cells were recovered by centrifugation and resuspended to 4 ml in distilled water. The supernatant medium was carefully removed to avoid any loss of bacterial cells. Portions of the suspension and supernatant liquor were assayed for LP activity (15).

Experiments were conducted to determine the following: (i) whether incubation of LP with bacteria was necessary for growth inhibition, (ii) whether H₂O₂ without LP would oxidize a cell component(s) so that subsequent reaction with SCN would cause growth inhibition, and (iii) whether a low-molecular-weight (diffusible) toxic product of the LP-SCN-H₂O₂ reaction would inhibit growth.

For (ii), the inoculum was incubated for 20 min at 25°C in 2 ml of medium containing 1 μmol of H₂O₂, followed by the addition of 2 ml of medium containing SCN. For (iii), the LP complex was separated from the inoculated portion of the medium with a semipermeable membrane reported to retain substances with a molecular weight in excess of 50,000. LP has a molecular weight of about 70,000. No LP activity has been detected in filtrates from these membranes. The membrane permitted rapid transfer of LP-free filtrate from the LP-SCN-H₂O₂ mixture into the inoculated medium with the use of centrifugal force. About 10 min elapsed from the mixing of the reactants until the filtrate had been transferred into the inoculated portion of the medium. The membrane cone, membrane cone support, and centrifuge tube assembly were sterilized before use, the membrane cone with ethylene oxide and the cone support and centrifuge tube by exposure for 15 min at 100°C in an autoclave. The inoculum in 2 ml of medium was incubated for 20 min at 25°C with 1 μmol of H₂O₂, and the other 2 ml of medium containing the LP-SCN-H₂O₂ mixture was rapidly filtered through the membrane cone and into the inoculated medium.

**Measurement of LP-catalyzed incorporation of SCN into proteins of S. agalactiae.** The effect of LP-H₂O₂ on the incorporation of SCN into the proteins of *S. agalactiae* cells was measured with the procedure described by Aune et al. (3) for the incorporation of SCN into bovine serum albumin.

Cells were grown in the stock culture medium. Phosphate buffer solution was sterilized separately and combined with the medium after cooling. A 4-ml culture was grown at 37°C for 3 to 4 h, until it reached an optical density at 610 nm of about 0.90. This culture, held overnight at 4°C, was used to inoculate 50 ml of medium in a 125-ml, loosely capped Erlenmeyer flask and incubated on a rotary shaker (New Brunswick Scientific Co., New Brunswick, N. J.) at 120 oscillations per min for about 2 h or until the optical density was about 0.90. The cells were harvested by centrifugation (yield, 0.36 to 0.38 g of wet paste), washed twice in distilled water, and then resuspended to a concentration of about 33 to 35 mg of dry cells per ml. Sulphydryl, protein, and cell dry weight were determined on the suspension before the cells were used. Cell dry weight was determined by drying 0.2 ml of suspension at 105°C to a constant weight on tared, stainless-steel planchets (300-mm diameter by 2 mm deep). Cell protein was measured on 3-μl samples of the suspension after digestion at 100°C for 10 min with 0.05 ml of 1 N NaOH (10). Sulphydryl groups were estimated by the procedure of Ellman (5) as applied to bacterial cells by Thomas and Aune (22).

After a 15-min incubation of the reaction mixture (see Table 3) at 37°C, 10 ml of cold 20% trichloroacetic acid was added, and the precipitate was removed by centrifugation. The precipitated cell material was washed further by centrifuging and resuspending five times in 5-ml portions of 5% trichloroacetic acid; each time, the supernatant liquor was filtered through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.), and the precipitate was finally transferred to the filter with four successive portions of 2.5 ml of 5% trichloroacetic acid. The Millipore disks were transferred to scintillation vials and dried overnight at 73°C. The vials were then filled with Cab-O-Sil and 15 ml of XDC scintillation fluid (4), and the radioactivity was determined in a Packard 3380 scintillation counter. The 14C counting efficiency was 90%.

Glutathione content of the cells was measured with the procedure described by Fahey et al. (6).

Sulfenyl groups were estimated by their reaction with [14C]thiouria to yield a radioactive mixed disulfide (18). The SCN incorporation reaction was carried out with unlabeled SCN, LP, and H₂O₂ as described in Table 3. Two micromoles of [14C]thiouria containing 0.5 μCi of radioactivity and 10 mg of sodium dodecyl sulfate were then added. After incubation for 1 h at 37°C, the cells were precipitated with trichloroacetic acid, washed, and filtered, and their radioactivity was determined as described above.

**Chemicals and materials.** LP was prepared from bovine skim milk by the procedure of Morrison and Hultquist (14); further details relating to purity and potency have been published previously (12). 5,5'-Dithiobis(2-nitrobenzoic acid) was purchased from Sigma Chemical Co., St. Louis, Mo.; reduced nicotinamide adenine dinucleotide phosphate, dithiothreitol (DTT), and glutathione reductase were from P-L Biochemicals, Inc., Milwaukee, Wis.; Na₂SO₄ was from Tridom Chemicals, Inc., Hauppauge, N. Y.; Cetab (cetyltrimethylammonium bromide) was from Mann Research Laboratories, New York, N. Y.; 14C-labeled NaSCN (NaS⁻¹⁴CN) and 14Cthiouria were from ICN Isotope and Nuclear Div., Cleveland, Ohio; Cab-O-Sil was from Packard Instrument Co., Inc., Downers Grove, Ill.; and the CF₅₀A membrane cones, supports, and centrifuge tubes were from Amicon Corp., Lexington, Mass.

**RESULTS**

*S. agalactiae* cells suspended in a culture medium containing LP or LP-SCN-H₂O₂ adsorbed LP, and the adsorbed enzyme was not removed by successive washing with enzyme-free medium. Separate assay of the cells and supernatant medium indicated that about 3% of the total enzyme in the medium became adsorbed to the cells; the rest was found in the cell-free supernatant medium to account for 96% of the total (Table 1). In tests to establish whether the enzyme could be removed by washing, cells (equivalent to 39 mg [dry weight]) were incu-
TABLE 1. Adsorption of lactoperoxidase to *S. agalactiae*

<table>
<thead>
<tr>
<th>Total LP</th>
<th>LP activity (U)</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initially in medium*</td>
<td>2.690</td>
<td>100.00</td>
</tr>
<tr>
<td>Adsorbed to cells†</td>
<td>0.084</td>
<td>3.12</td>
</tr>
<tr>
<td>In supernatant medium after adsorption with bacteria</td>
<td>2.490</td>
<td>92.70</td>
</tr>
<tr>
<td>Recovered in cells plus supernatant medium</td>
<td>2.574</td>
<td>95.82</td>
</tr>
</tbody>
</table>

* Chemically defined medium with 5 μg of L-cystine per ml.
† Cells from a 3-h culture (equivalent to 47 μg [dry weight]) were suspended in 4 ml of medium containing 2.69 LP activity units, 2.86 μmol of NaSCN, and 0.5 μmol of H₂O₂. After 30 min at 24°C, the cells were recovered by centrifugation and suspended in distilled water; the final volume was 4 ml. Portions of cell suspension and supernatant medium were assayed for LP activity.

bated for 30 min in four tubes, each with 4 ml of chemically defined culture medium that contained 1.0 U of LP, 0.74 μmol of NaSCN, and 0.25 μmol of H₂O₂ per ml. The cells were then removed by centrifugation and washed either zero, one, two, or three times with 4 ml of enzyme-free medium. The cells were resuspended in distilled water to a volume of 4 ml, and portions were assayed for LP activity. Cells washed zero, one, two, or three times had 0.074, 0.097, 0.052, and 0.075 U of LP activity, respectively. Cells incubated in medium with no LP were devoid of activity. The LP adsorbed to the cells was not removed by washing, and the adsorbed enzyme remained catalytically active. Growth of cells that were allowed to adsorb LP and then placed in a second enzyme-free medium with additional SCN and H₂O₂ was inhibited for more than 20 h but less than 44 h. Exposure of *S. agalactiae* inoculum to H₂O₂ alone, followed by addition of SCN, did not inhibit growth, nor was growth inhibited if the inoculum was exposed to H₂O₂ alone, followed by addition of an LP-free filtrate of the LP-SCN-H₂O₂ reaction mixture (Table 2), thus emphasizing the need for LP contact with the bacteria for growth inhibition.

Data on the LP-catalyzed incorporation of SCN into *S. agalactiae* cellular material are shown for four cell preparations in Table 3. The molar sulphhydryl content in each reaction, as determined by assay of the cell suspension, was approximately equal to the H₂O₂ added. In one of the experiments with the frozen-thawed cells, the sulphhydryl content was in excess; in one experiment with Cetab-treated cells, it was somewhat less than the ideal ratio of 1:1 (1). Fewer than 10% of the total sulphhydryl groups in the whole cells reacted to bind SCN by an LP-catalyzed reaction, but with Cetab- and ethanol-treated cells, the nearly 1:1 relationship between SCN bound and sulphhydryls present suggested a quantitative conversion to sulphenylthiocyanate derivatives (3, 23). The sulphhydryls of the frozen-thawed cells were intermediate in their reactivity. Modification of cell membrane permeability is believed to be the reason for more sulphhydryl reactivity in the treated cells. That changes in permeability of the cells of *S. agalactiae* occurred was made evident by the loss of protein and sulphhydryl groups from the freeze-thawed, Cetab-treated, and ethanol-treated cells. In freeze-thawed cells, loss of protein was 25% and loss of sulphhydryls was 5%; in Cetab-treated cells, loss of protein was 16 to 25% and loss of sulphhydryls was 50 to 65%; and in the ethanol-extracted cells, loss of sulphhydryls was about 42%. The loss of sulphhydryl content from cells by treatment was compensated for by using more cell material to provide about 70 nmol of sulphhydryls per reaction. Most of the SCN estimated to be bound to *S. agalactiae* by an LP-catalyzed reaction was released with DTT (Table 3). In an attempt to measure sulphenylthiocyanate displacement of the cell-bound SCN with [¹⁵C]thioura, about 78% of the net cell-bound SCN was released with DTT and about 67% was displaced.

TABLE 2. Antibacterial action of LP-SCN-H₂O₂ on *S. agalactiae*

<table>
<thead>
<tr>
<th>Addition to medium</th>
<th>Subsequent addition to filtrate</th>
<th>Growth as change in optical density at:</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>9 h</td>
</tr>
<tr>
<td>LP-SCN-H₂O₂</td>
<td></td>
<td>0.572</td>
</tr>
<tr>
<td>1. H₂O₂</td>
<td>NaSCN</td>
<td>0.000</td>
</tr>
<tr>
<td>2. H₂O₂</td>
<td>LP-SCN-H₂O₂</td>
<td>0.452</td>
</tr>
<tr>
<td></td>
<td>filtrate</td>
<td>0.520</td>
</tr>
</tbody>
</table>

* The medium was the same as that described in Table 1, with a volume of 4 ml.
† 0.70 U of LP, 0.7 μmol of NaSCN, and 0.125 μmol of H₂O₂ per ml of medium.
‡ (1 and 2) 2 ml of inoculated medium containing 2 μmol of H₂O₂. (1) The inoculum (equivalent to 100 μg of cells [dry weight]) was incubated with H₂O₂ for 20 min at 24°C in 2 ml of medium, followed by addition of 2 ml of medium containing NaSCN. (2) Same as (1) but with subsequent addition of LP-free filtrate from the length of time from mixing reagents to addition of filtrate was 10 min. Amicon CF50A membrane cone filter retained the LP.
§ 2 ml of medium containing 2.8 μmol of NaSCN.
¶ Filtrate from 2 ml of medium containing 2.8 U of LP, 2.8 μmol of NaSCN, and 0.5 μmol of H₂O₂.
with thiourea. Table 3 gives the sulfhydryl content of each cell preparation and the concentration of sulfhydryl groups in each reaction mixture. A small amount of labeled SCN was bound to the cells in the absence of LP and H₂O₂. This amount seemed to be unaffected by the cell treatment, and it was not removed from the cells with DTT. An additional small amount that became bound to the cells in the presence of LP and H₂O₂ also was not removed with DTT. It seemed that these amounts are not likely to be bound through reaction with sulfhydryl groups in the bacterial proteins but perhaps are more likely to be bound to histidine, tyrosine, and tryptophan residues from which the SCN would not be released with DTT (23).

Assuming that the foregoing explanations are valid, one can recalculate the data of Table 3 to show the molar relationship of the amount of SCN incorporated to the sulfhydryl content of the cell preparations, as shown in Table 4. The difference in values of columns 2 and 3 can then represent the LP-catalyzed incorporation of SCN per mole of sulfhydryl present. Only with the Cetab- and ethanol-treated cells was there evidently a quantitative reaction of sulfhydrys with SCN in the presence of LP and H₂O₂.

**DISCUSSION**

An assayable amount of LP was adsorbed from the medium by the bacterial cells (Table 1). The enzyme was not removed by ordinary washing procedures, and the subsequent addition of SCN and H₂O₂ to the medium inhibited growth for at least 20 h. All of the components of the LP complex must be in the medium and in contact with the cells simultaneously for an antibacterial effect against *S. agalactiae*. This conclusion supports the view that the antibacterial action was not due to a toxic, diffusible substance(s), unless the substance(s) was so labile that it was degraded during the short time interval between the mixing of the three components and the adding of the enzyme-free filtrate to the inoculated medium (Table 2). Glucose transport in *S. agalactiae* by the LP complex also was inhibited only when all three components were simultaneously in contact with the cells (13).

Steele and Morrison (21) suggested that the antibacterial effect might be the result of an LP-catalyzed oxidation of cell wall or a membrane component(s), followed by a reaction with SCN or a product of SCN oxidation. Although the

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**Table 3. LP-catalyzed incorporation of SCN into *S. agalactiae* cells**

<table>
<thead>
<tr>
<th>Cell prepn</th>
<th>nmol of SCN incorporated per mg of dry cells</th>
<th>% of LP-H₂O₂-bound SCN displaced by DTT</th>
<th>Sulphydryl (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No LP or H₂O₂</td>
<td>LP + H₂O₂</td>
<td>Per mg of cells</td>
</tr>
<tr>
<td>Whole cells</td>
<td>2.04</td>
<td>9.01</td>
<td>62.5</td>
</tr>
<tr>
<td>Frozen-thawed cells (2X)</td>
<td>1.81</td>
<td>19.40</td>
<td>77.4</td>
</tr>
<tr>
<td>Cetab-treated cells</td>
<td>0.90</td>
<td>30.66</td>
<td>82.5</td>
</tr>
<tr>
<td>Ethanol-extracted cells</td>
<td>0.71</td>
<td>39.20</td>
<td>96.4</td>
</tr>
</tbody>
</table>

(a) Average values from two experiments, except for the ethanol-extracted cells. 1 to 3 mg of dry cells containing about 70 nmol of sulfhydryl groups, 50 μmol of phosphate buffer (pH 7), 1 μmol of Na₂¹⁵CN (0.25 μCi), 0.7 U of LP and H₂O₂ in a total volume of 1 ml. H₂O₂ (0.002 M) added in 10-nmol increments, 1 min apart, until 70 nmol was added. No LP or H₂O₂ was added to the controls. Incubation was for 15 min at 37°C, followed by the addition of 10 ml of cold 20% trichloroacetic acid and recovery of the cells for determination of radioactivity. DTT (12 μmol) in dry form was added immediately after incubation, and the reaction mixture was held for 2 h before adding the trichloroacetic acid.

**Table 4. LP-catalyzed incorporation of SCN into various cell preparations of *S. agalactiae* in relation to their sulfhydryl content**

<table>
<thead>
<tr>
<th>Cell prepn</th>
<th>mol of SCN incorporated per mol of sulfhydryl present</th>
<th>Net value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No LP or H₂O₂</td>
<td>LP + +</td>
</tr>
<tr>
<td></td>
<td>H₂O₂</td>
<td>H₂O₂ DTT</td>
</tr>
<tr>
<td>Whole cells</td>
<td>0.028</td>
<td>0.130</td>
</tr>
<tr>
<td>Frozen-thawed cells (2X)</td>
<td>0.024</td>
<td>0.258</td>
</tr>
<tr>
<td>Cetab-treated cells</td>
<td>0.039</td>
<td>1.350</td>
</tr>
<tr>
<td>Ethanol-extracted cells</td>
<td>0.021</td>
<td>1.139</td>
</tr>
</tbody>
</table>

(a) The data of Table 3 were recalculated to show the molar relationship of the amount of SCN that was bound to the four cell preparations to the sulfhydryl content of the cells.
oxic roles of LP and H$_2$O$_2$ were understandable, the role of SCN in the antibacterial effect was less clear. A role for SCN was suggested by reports of the LP-catalyzed incorporation of SCN into crystalline bovine serum albumin in which the protein sulfhydryls were oxidized to sulfenylthiocyanate derivatives (3; T. M. Aune and E. L. Thomas, Fed. Proc. 35:1386, 1976). Recent work on the products of the LP-catalyzed oxidation of SCN (1, 2, 8) and their role in the formation of sulfenylthiocyanate derivatives of protein sulfhydryls offered for the first time the definition of a specific role for SCN in the antibacterial effect of the LP complex.

The LP-catalyzed incorporation of SCN into the cell proteins of S. agalactiae herein reported suggested that the role of SCN in the antibacterial effect may be explained by a similar mechanism. With the four cell preparations used, the amount of SCN incorporated was apparently related to the exposure of sulfhydryls to LP-catalyzed oxidation. The LP-catalyzed incorporation of SCN into whole cells exceeded the controls by 3- to 5-fold, the frozen-thawed cells by 8- to 14-fold, and the Cetab-treated and ethanol-extracted cells by 30- to 50-fold. Most of the SCN incorporated into cellular material by LP catalysis was displaced from the combination with DTT. The amount displaced ranged from 77 to 96% with the treated cells. With whole cells, when the total SCN incorporation was only three- to fivefold greater with LP catalysis than without LP, the portion of cell-bound SCN released with DTT was about 63%. The nearly 1:1 ratio of SCN incorporated to sulfhydryl groups present in the Cetab- and ethanol-treated cells indicated a nearly quantitative exposure to sulfhydryl groups in the bacterial protein to LP-catalyzed oxidation. That ratio also suggested that the protein sulfhydryls were oxidized primarily to sulfenylthiocyanate derivatives. The release of cell-bound thiocyanate with DTT and replacement of it by thiourea also support the view that thiocyanate incorporated by LP catalysis was bound primarily as a sulfenylthiocyanate derivative of cell sulfhydryl groups. The sodium dodecyl sulfate (0.75%) in the reagents used for determining the total sulfhydryl content of the bacterial cells would be as likely to expose protein sulfhydryl groups, the free sulfhydryls, and those masked by the configuration of the protein (7) as effectively as did the Cetab and ethanol treatments. The displacement of the SCN incorporated by LP catalysis into sulfenylthiocyanate derivatives by DTT (23), the inhibition of glucose transport in S. agalactiae by the LP complex and its reversibility with DTT, and the inhibition of glucoysis and its reversibility with DTT (13) all suggested that the functional protein sulfhydryls involved in the transport of glucose and the enzymes of substrate degradation were sites where the LP complex can exert antibacterial effects. Recently, it has been shown (M. Adamson and K. Pruitt, Fed. Proc. 76:1579, 1978) that LP catalyzed the incorporation of SCN into purified hexokinase and was accompanied by enzyme inactivation. Inactivation of triosephosphate dehydrogenase in cell-free extracts from Streptococcus pyogenes by the LP complex and its reversal with cysteine and glutathione have also been shown (11). With Escherichia coli, the antibacterial effect caused by OSCN$^-$, a product of the LP-catalyzed oxidation of SCN, was due to the oxidation of protein sulfhydryls to sulfenylthiocyanate derivatives, which resulted in inhibition of bacterial respiration. The effect could be reversed with DTT or by the removal of theOSC$^-$ from the cells (23).

The hot-ethanol extraction used for the removal of glutathione from the bacterial cells evidently exposed reactive sulfhydryl groups as effectively as did the treatment with Cetab. About 1 mol of SCN per mol of sulfhydryl was incorporated, in the presence of LP and H$_2$O$_2$, by cells treated with either procedure. The increased incorporation of SCN into S. agalactiae proteins after treatment with Cetab or after extraction with 80% ethanol is believed to result from changes in membrane permeability, causing exposure of protein sulfhydryls, normally protected by the cell membrane, to the oxidative action of LP and H$_2$O$_2$. Cetab treatment of S. pyogenes caused the cell membrane to become permeable to citrulline and carbamyl phosphate (20). No observable morphological changes were noted in electron microscopic examination of S. pyogenes, nor were cell structure changes noted in S. agalactiae when the cells were examined with a scanning electron microscope ($\times$20,000 magnification) after freeze-thawing or Cetab treatment. Freeze-thawing of the bacteria several times also increased the exposure of protein sulfhydryls but less effectively than did Cetab or ethanol treatment.

The low SCN incorporation into whole cells in the presence of LP and H$_2$O$_2$ suggested that only protein sulfhydryls on the exterior of the cell membrane were accessible to oxidation. Perhaps, they are the sulfhydryls that are functional in membrane transport, and their modification may be all that is necessary for the antibacterial effect. Incorporation of SCN into the protein sulfhydryls of S. agalactiae would remove the reacted sulfhydryls from a functional role in glucose transport and glucoysis, and this could account for the antibacterial effect. The inhibition of glucoysis would be of secondary impor-
tance because without a functioning glucose transport system, the glucoytic enzymes would have no substrate to act upon. Paigen and Williams have estimated that in bacteria with a doubling time of 60 min and an internal glucose concentration of 1 mM at any one time, enough glucose would be present to sustain growth for slightly longer than 1 s (17). S. agalactiae has a doubling time of about 21 min during rapid growth; therefore, cessation of growth would be almost immediate when glucose transport was stopped. Results in recent experiments at this laboratory have shown that L-valine transport system, the inhibition of glucose transport, the inhibition of valine transport was reversible with DTT (J. W. Moran, unpublished data).

The incorporation of SCN into the sulfhydril groups of the bacterial proteins defines a specific role for the SCN of the LP complex in the growth inhibition of S. agalactiae.

ACKNOWLEDGMENTS

Grateful appreciation is expressed to Joseph E. Gallagher for the scanning electron microscope examination of S. agalactiae.

LITERATURE CITED