Glucose Transport in *Streptococcus agalactiae* and Its Inhibition by Lactoperoxidase-Thiocyanate-Hydrogen Peroxide

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Transport of 2-deoxyglucose or glucose in *Streptococcus agalactiae* was strongly inhibited if the cells were first exposed to a combination of lactoperoxidase-thiocyanate-hydrogen peroxide (LP-complex). The inhibition was completely reversible with diithiothreitol. N-ethylmaleimide and p-chloromercuribenzoate inhibited sugar transport, and the inhibition was also reversible with diithiothreitol. Sodium fluoride also inhibited sugar transport. Glucolysis was completely inhibited, and diithiothreitol completely reversed the inhibition. Phosphoenolpyruvate-dependent phosphotransferase activity in *S. agalactiae* was not strongly inhibited by the LP-complex. Interference of the entry of glucose into cells of *S. agalactiae* by the LP-complex could well account for its growth inhibitory properties with this organism. The inhibition of glucose transport by the LP-complex and its reversibility with diithiothreitol suggest the modification of functional sulfhydryl groups in the cell membrane as a cause of transport inhibition.

The inhibition of the growth of streptococci in raw milk has been known since the observations made by Jones and Simms (8). Portmann and Auclair (22) established that lactoperoxidase (LP) was a component of the naturally occurring bacterial growth-inhibitory complex. Reiter et al. (24) showed that thiocyanate, present in milk, in addition to LP and hydrogen peroxide (H₂O₂), was necessary for growth inhibition of streptococci. Although only organisms that form H₂O₂ in their metabolism will be inhibited in their growth, the inhibition can be extended to a variety of organisms by the addition of exogenous H₂O₂ (11).

Various workers have attempted to show that the growth inhibition of streptococci was caused by some short-lived intermediate formed during the LP-catalyzed oxidation of thiocyanate by H₂O₂. Oxidation products such as cyanide (5, 29), intermediates such as sulfur dicyanide (19), and cyanosulfurous and cyanosulfuric acids (14) have been proposed as growth inhibitors. Steele and Morrison (30) demonstrated the binding of LP to *Streptococcus cremoris* 972 cells. They were unable to show that a dialyzable product resulting from thiocyanate oxidation was responsible for the inhibition of growth. Jago and Morrison (7) studied the antistreptococcal activity of LP in milk and whey with *S. cremoris* 972 and stated that growth inhibition was probably the result of the oxidation of a specific hydrogen donor group on the cell membrane. Rothstein (26), in discussing the action of mercurials, reasoned that the major site of action of heavy metals on cells and tissues would likely be the cell membrane and that the most important reaction sites in the cell membrane are enzymes and other components involved in transport. Therefore, the mechanism of toxicity would likely be due to interference with the movement of substrates and ions across the cell membrane.

The purpose of this study was to determine whether the growth-inhibitory action of LP, thiocyanate, and H₂O₂ was due to an alteration of the properties of the bacterial cell membrane, resulting in the interference with glucose transport into the cells of *S. agalactiae*.

**MATERIALS AND METHODS**

**Cultures and growth conditions.** *S. agalactiae* 50 (this is the same strain referred to as 660 by R. W. Brown, J. Dairy Sci. 50:1572-1584, 1967), a pigmented strain reported on earlier (17), was used. The stock culture was maintained by bimonthly transfer in a liquid medium composed of 1.0% yeast extract (Difco), 0.5% Casitone (Difco), 0.5% glucose, and 0.1 M potassium phosphate, pH 7.0. Phosphate buffer was sterilized separately and added to the cooled medium. Cells for use in the transport experiments were grown in the chemically defined me-
Phosphate, transport. The culture was washed to a concentration of 15 mmol/ml, containing 0.05 M phosphate, pH 7. The buffer was sterilized separately. Cells were harvested by centrifugation, washed once with 0.075 M KCl, and resuspended in 0.05 M phosphate, pH 7, containing 0.001 M MgSO$_4$, so each milliliter contained about 200 mg of cells (wet weight). The suspension was stored at −27°C for at least 24 h. The suspension was then thawed at room temperature and washed three times with 4 ml portions of 0.05 M phosphate -0.001 M MgSO$_4$, pH 7. This was found to be necessary to reduce the endogenous phosphotransferase activity so a good response to PEP could be obtained. This washed cell preparation retained good phosphotransferase activity for up to 5 weeks when stored at −27°C. Reactions were run in centrifuge tubes (16 by 100 mm) containing 0.5 μmol of [U-$^{14}$C]2-deoxyglucose (0.06 μCi/μmol), 0.75 μmol of PEP, cell preparation (0.5 mg of cell protein), and 0.05 M phosphate buffer containing 0.001 M MgSO$_4$ and other reagents to equal 0.5 ml of total volume. When the LP-complex was present, 0.6 U of LP, 0.40 μmol of NaSCN, and 0.07 μmol of H$_2$O$_2$ per ml were added. The reaction mixtures were incubated in a water bath for 30 min at 37°C and then placed in an ice bath; 0.5 ml of 0.6 M trichloroacetic acid was added, and the extraction was continued for 40 min with occasional mixing. The reaction tubes were then centrifuged for 5 min at 10,000 × g at 4°C, the supernatant liquid was decanted into screw-capped tubes (13 by 100 mm) and each was extracted three times with ethyl ether to remove the trichloroacetic acid. The volume of each extract was measured, and 0.5 ml was added to 4.5 ml of 0.03 M BaBr$_2$ in 80% ethanol and held at room temperature for 10 to 15 min while a white precipitate containing the phosphorylated derivatives of 2-deoxyglucose formed. The precipitates were collected on 0.45-μm membrane filter disks (Millipore Corp.), and each was washed with 5 ml portions of 80% ethanol for a total of 45 ml of wash solution. The filter disks were dried in scintillation vials for at least 1 h at 37°C; the vials were filled with Cab-O-Sil (Cabot Corp.) and 15 ml of XDC scintillation fluid (3) and counted in a Packard Tri-Carb model 3380 liquid scintillation counter.

Cell protein determination. Cell protein was determined on 5-ml portions of the washed frozen-thawed cell suspension after digestion with 0.01 ml of 1 N NaOH for 10 min at 100°C, followed by neutralization with 0.01 ml of 1 N HCl, by the method of Lowry et al. (13).

Lactic acid and O$_2$ uptake measurements. Oxygen uptake was measured manometrically, and lac-
tic acid analysis was done by the Barker and Summerson procedure as described by Umbreit et al. (31).

**LP preparation.** Purified LP was prepared from fresh skim milk by the method of Morrison and Hultquist (18). The 412/280-nm adsorption of the 0.5 M sodium acetate eluate from the second resin column was 0.86. This eluate was distributed in 1-ml amounts into small screw-capped vials and stored at -60°C. Each milliliter of eluate contained about 250 U of LP activity as measured by the procedure of Oram and Reiter (20). The procedure was modified by reducing the amount of H₂O₂ from 1.0 to 0.40 μmol to obtain maximum color development during the assay. The reaction was carried out in a volume of 4.5 ml containing 2.0 ml of 0.2 M sodium acetate (pH 5.7), enzyme sample plus distilled water to 4.32 ml, 0.1 ml of 0.1% O-dianisidine in 95% ethanol, and 0.08 ml of freshly prepared 0.005 M H₂O₂. The reaction was allowed to proceed for 10 min at 25°C and was stopped by adding 0.5 ml of 4 N HCl. One unit of activity was defined as that amount of LP that gave an optical density of 1.0 when measured in a Klett-Summerson colorimeter with a no. 42 filter.

Thiocyanate was added as the sodium salt. A solution containing 2 mg/ml was filter-sterilized and added to the reaction mixture to give a final concentration of 0.40 μmol/ml. The hydrogen peroxide concentration was 0.07 μmol/ml and was added from a freshly made solution.

**RESULTS**

**Inhibition of sugar transport in intact cells.**

A cell suspension of *S. agalactiae* that had an active respiration with glucose as a substrate showed no respiration with 2-deoxyglucose (Fig. 1). In transport experiments with intact cells, radioactivity values in samples taken at 60 to 90 s after glucose addition were nearly identical to those with the non-metabolizable 2-deoxyglucose. Table 1 shows the results of both transport experiments with the U-¹³C-labeled sugars and the effect on transport in intact cells of pretreatment of the cells with various reagents and combinations of reagents.

Cells that were incubated from the LP-complex for 30 min and then separated from the LP-complex and used for measurement of sugar transport showed a strong inhibition of the entrance of labeled carbon from either of the two sugars. Exposure of the cells to single or dual combinations of the components of the LP-complex had negligible effects on the transport of either of the sugars. Addition of 1 mM DTT to the treated cells immediately before the addition of the substrate resulted in complete reactivation of sugar transport (Fig. 2). If the cell suspension was incubated for 15 min with 0.2 mM pCMB before adding the sugar, the transport of 2-deoxyglucose was completely inhibited. The addition of 1 mM DTT before the addition of the sugar resulted in an almost complete reversal of the inhibition. If the cells were incubated with 5 mM NEM for 15 min before adding the sugar, the transport of 2-deoxyglucose was inhibited 70%, and addition of 5 mM DTT before the addition of the sugar caused an almost complete reversal of the inhibition of transport. The addition of 0.1 mM pCMB and 2.5 mM NEM inhibited the trans-
port of 2-deoxyglucose 65 and 13%, respectively. NaF at a 0.01 M concentration strongly inhibited the transport of 2-deoxyglucose and glucose (Table 2). The presence of a 0.1 or 1.0 mM concentration of 2-deoxyglucose with the cells during exposure to the LP-complex did not prevent the inactivation of transport. Preincubation of the cells for 30 min with 1 and 5 mM KCN did not affect the transport of glucose.

Sugar transport system in S. agalactiae. The inhibition of glucose transport by NaF in intact cells suggested the inhibition of enolase and consequent PEP formation, which would suggest the involvement of a PEP-dependent phosphotransferase system in glucose transport (10). Table 3 shows that S. agalactiae does possess an active PEP-dependent phosphotransferase. The second half of Table 3 shows data to emphasize the importance of washing the frozen-thawed cells to reduce the endogenous phosphotransferase activity so PEP dependence of the reaction was evident. The same cell preparation was used, the only difference being that unwashed cells were used in one case and in the other the cells were washed three successive times with 0.05 M phosphate-0.001 M MgSO4, pH 7, before use. The amount of 2-deoxyglucose-6-P found was directly related to the amount of cell protein present, up to a concentration of 1.2 mg/ml of reaction mixture (Fig. 3).

Table 4 shows data of experiments in which the phosphotransferase assay with frozen-thawed cells was carried out in the presence of the LP-complex. In contrast to the strong inhibition on the transport of glucose in intact cells, the LP-complex caused a small inhibition of phosphotransferase activity (25 to 30%), and the inhibition was only partially reversible with DTT.

Table 5 shows the effect of pCMB and NEM on phosphotransferase activity. Each of the sulfhydryl inhibitors caused nearly complete inhibition of phosphotransferase activity in the concentrations used, and in combination with DTT the inhibition with NEM was about 70% reversible and with pCMB about 84% reversible.

Inhibition of gluolysis in S. agalactiae by preexposure of the cells to the LP-complex. Interference with PEP generation during gluolysis by preexposure of the bacterial cells to the LP-complex might inhibit the functioning of the phosphotransferase system and the transport of glucose into the cells. Table 6 shows the effect of the LP-complex on the formation of lactic acid by S. agalactiae. Gluolysis was inhibited 80 to 90% when the cells were preincubated with the LP-complex for 30 min and then removed and placed in a glucose-buffer solution. The addition of PEP did not reconvert gluolysis, but DTT completely restored gluolyl activity to the bacterial cells.
FIG. 3. Effect of cell protein concentration on phosphorylation of 2-deoxyglucose with frozen-thawed cells of S. agalactiae. The procedure was the same as that used for data in Table 3, except for varying the cell protein concentration.

**TABLE 3. Phosphotransferase activity in S. agalactiae**

<table>
<thead>
<tr>
<th>Assay conditions</th>
<th>nmol of 2-deoxyglucose phosphorylated/mg of cell protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>3× washed, frozen-thawed cells</td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>57.00</td>
</tr>
<tr>
<td>No PEP</td>
<td>10.65</td>
</tr>
<tr>
<td>Heated cells</td>
<td>1.59</td>
</tr>
<tr>
<td>Unwashed, frozen-thawed cells</td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>42.95</td>
</tr>
<tr>
<td>No PEP</td>
<td>53.80</td>
</tr>
<tr>
<td>Heated cells</td>
<td>5.79</td>
</tr>
</tbody>
</table>

* Per 1-ml reaction mixture: 1 mg of cell protein; 1.5 μmol of [U-14C]2-deoxyglucose (0.06 μCi/μmol) per ml; 1.5 μmol of PEP. Heated cells, 100°C, 10 min. Reaction was at 35°C for 30 min.

**DISCUSSION**

Our objective was to obtain an explanation of the growth inhibition of *S. agalactiae* by the LP-complex. Preincubation of cells with the three components resulted in the complete inhibition of 2-deoxyglucose or glucose transport, which could be reversed by DTT. Thus, growth inhibition may well be caused by the inhibition of transport activity.

This inhibition is probably not mediated by cyanide resulting from thiocyanate oxidation (5) since, if complete oxidation of 0.74 mM and lower concentrations of thiocyanate used in growth media to cyanide did occur, the concentration of cyanide resulting would not be high enough to cause the inhibition of growth that has been observed. Cyanide concentrations of 1.0 mM added to the medium did not inhibit growth, whereas 1.5 mM caused partial growth inhibition of *S. agalactiae*. Preincubation of the cells with 1 and 5 mM KCN did not affect the transport of glucose. The necessity of thiocyanate would support the view suggested by Steele and Morrison (30), that there may be an interaction between an oxidation product in the cell wall or membrane and thiocyanate, or an intermediate product of thiocyanate oxidation, to cause the inhibition of growth. Peroxidation of membrane lipids by LP-iodide-H₂O₂ (4) and the modification of sulfhydryl groups of proteins by the LP-complex to form sulfenyl thiocyanate (T. M. Aune and E. L. Thomas,
TABLE 6. Effect on glucolysis in S. agalactiae of pretreatment of cells with the LP-complex

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>μmol of lactic acid formed in</th>
<th>Expt 1</th>
<th>Expt 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.388</td>
<td>0.300</td>
<td></td>
</tr>
<tr>
<td>LP-KSCN-H₂O₂</td>
<td>0.086</td>
<td>0.035</td>
<td></td>
</tr>
<tr>
<td>LP-KSCN-H₂O₂ + PEP</td>
<td>0.099</td>
<td>0.046</td>
<td></td>
</tr>
<tr>
<td>LP-KSCN-H₂O₂ + DTT</td>
<td>0.402</td>
<td>0.501</td>
<td></td>
</tr>
</tbody>
</table>

*Freshly harvested cells from a 3-h culture in synthetic medium with 6 μg of L-cystine per ml. Cells were washed twice with 0.05 M phosphate, pH 7, and incubated for 30 min at 36°C in a mixture of: LP, 0.8 U/ml; KSCN, 0.4 μmol/ml; and H₂O₂, 0.06 μmol/ml. Cells were recovered by centrifugation, resuspended in 0.05 M phosphate buffer (pH 7) with 0.325 μmol of glucose, and incubated at 36°C for 30 min. Cells were removed by centrifugation, and lactic acid analyses were made on the supernatant liquor. The control was handled in an identical manner, except there was no exposure of cells to the LP-complex. PEP (1.5 mM) and DTT (1 mM) were added to the cell suspension 5 min before adding glucose. There was 1 mg (dry weight) of cells in a 2.0-ml volume for each incubation.*

Fed. Proc. Abstr. 67th Annu. Meet., 1976, Abstr. 1386, p. 1630 offer possible explanations for the changes in membrane permeability that may be caused by the LP-complex. The involvement of sulfhydryls is of special interest since the inhibition of transport by exposure of S. agalactiae cells to the LP-complex was reversible with DTT. The reversibility of the inhibition of glucose transport with DTT, whether the inhibition is caused by the LP-complex (Fig. 2), NEM, or pCMB (Table 2), strongly suggests that the inhibition is the result of an oxidative modification of functional sulfhydryl groups in the cell membrane, possibly changing the conformation of a membrane protein(s) so it cannot function in the transport process. Full restoration of transport activity with DTT indicated a regeneration of sulfhydryl groups that function in the transport process (1, 2, 9).

There is no exact information on the location of, or which specific sulfhydryl groups may be involved in, the glucose transport inhibition by the LP-complex, so the site(s) of inhibition cannot be clearly defined. Enzymes of the phosphotransferase system exist in S. agalactiae 50 and are presumed to be a pathway of sugar transport. The role of a phosphotransferase system in the transport of sugars in microorganisms and its particular significance as a mechanism of transport for organisms that are primarily fermentative have been discussed (24, 26). Roseman (25) believes that most sugars penetrate the bacterial membrane by group translocation, mediated by a sugar-specific enzyme, which is located in the cell membrane. PEP-dependent phosphotransferase activity has been reported earlier in several other streptococci, including S. lactis (15) and streptococci of the oral cavity, S. mutans, S. sanguis, and S. salivarius (27). The results with S. agalactiae were similar to those reported by Schachtele and Mayo (27) for the oral streptococci. If the assay for phosphotransferase activity was carried out in the presence of the LP-complex, the formation of sugar phosphate was not strongly inhibited and the inhibition was only partially reversible with DTT. Therefore, sugar transport inhibition in intact cells by the LP-complex cannot be accounted for solely by its effect on enzymes of the phosphotransferase system. If the assay was carried out in the presence of 0.2 mM pCMB or 5.0 mM NEM, concentrations that inhibited sugar transport in intact cells, the phosphotransferase reaction was completely inhibited and the addition of DTT restored the activity to nearly its original value. Others have also demonstrated the inhibition of sugar transport in intact cells (6) and the inhibition of the enzymes of the phosphotransferase system (23, 28) with sulfhydryl inhibitors.

Oram and Reiter (20) reported hexokinase activity and glucolysis to be inhibited by the LP-complex in S. cremoris 972. The inhibition was reversible, and they suggested that this effect could account for the growth inhibition of this organism by the LP-complex. Glyceraldehyde-3-phosphate dehydrogenase in cell-free extracts of S. pyogenes was also inhibited by the LP-complex, and the inhibition was reversible with cysteine or glutathione (16). In view of more recent knowledge of glucose transport in fermentative bacteria (23, 25) and specifically in the case of S. agalactiae, which may use the phosphotransferase system for glucose transport, inhibition of glycolysis would deprive the cells of the necessary PEP for transport. Gluclolysis was completely inhibited in intact cells of S. agalactiae if the cells were preexposed to the LP-complex. Glycolysis was restored completely with DTT. Evidently in the intact cells a modification of sulfhydryl function on the cell membrane made the cells impermeable to glucose because DTT restored both the transport and glycolytic processes. The reactions were run in the presence of air. The higher lactic acid values found with the addition of DTT presumably were due to the maintenance of a redox potential that encouraged fermentation instead of oxidation. Under
aerobic conditions, *S. agalactiae* will convert as much as 60 to 65% of the glucose carbon to acetic acid, CO$_2$, and acetoin (17).

Since glucolysis in intact cells is inhibited, either the glucoytic enzymes are accessible to the LP-complex or there are other sulfhydryl proteins on the cell exterior, perhaps non-enzyme proteins, that become oxidatively modified and cause impairment of membrane permeability. Glucoytic enzymes are primarily cytoplasmic, and one would not expect them to be accessible to the inhibitor. Since LP or DTT would not be expected to enter the cell, the inhibition reaction and its reversal likely occur on the cell surface, but since the fate of possible reactive intermediate products (perhaps permeable) is not known, one cannot eliminate the possibility of reaction with a cytoplasmic component, perhaps in a multistep process. The turnover of glucose within growing cells of *S. agalactiae* is very rapid in relation to the size of the substrate pool, and the effect of blocking the entry of a continuous supply of glucose into the cell by the LP-complex would become evident by an almost immediate cessation of growth (21).

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**LITERATURE CITED**


