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Disulfide Reduction and Sulfhydryl Uptake by *Streptococcus mutans*

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Incubation of *Streptococcus mutans* cells with certain disulfide compounds resulted in accumulation of reduced sulfhydryl compounds in the extracellular medium or in both the medium and the cells. Oxidized lipoic acid and lipoamide competed for reduction. At high concentrations, these compounds were reduced at rates comparable to that of glucose metabolism, and all of the increase in sulfhydryls was in the medium. Cystamine did not compete with these compounds for reduction but was also reduced at high rates and low apparent affinity, and all of the cysteamine produced from cystamine accumulated in the medium. In contrast, glutathione disulfide (GSSG) and L-cystine were reduced slowly but with high apparent affinity, and 60 to 80% of the increase in sulfhydryls was intracellular. NADH-dependent lipoic acid or lipoamide reductase activity was present in the particulate (wall-plus-membrane) fraction, whereas NADPH-dependent GSSG reductase activity was present in the soluble (cytoplasmic) fraction. Two transport systems for disulfide and sulfhydryl compounds were distinguished. GSSG, L-cystine, and reduced glutathione competed for uptake. L-Cysteine was taken up by a separate system that also accepted L-penicillamine and D-cysteine as substrates. Uptake of glutathione or L-cysteine, or the uptake and reduction of GSSG or L-cystine, resulted in up to a 10-fold increase in cell sulfhydryl content that raised intracellular concentrations to between 30 and 40 mM. These reductase and transport systems enable *S. mutans* cells to create a reducing environment in both the extracellular medium and the cytoplasm.

Cell sulfhydryl content consists of two major pools, the protein sulfhydryls (cysteine residues) and low-molecular-weight-sulfhydryl compounds in the cytoplasm. Protein sulfhydryls are essential to enzyme activity and to other functions of many proteins (18). Sulfhydryl compounds have specific functions and also provide a reducing environment that protects protein sulfhydryls against oxidation and other forms of chemical modification (11, 14, 18).

In mammalian cells, reduced glutathione (GSH) is the major sulfhydryl compound. GSH participates in enzyme-catalyzed reduction of peroxides (11) and detoxification of other electrophilic agents (14) and also acts as a nonspecific reducing agent. Less is known about the role of sulfhydryl compounds in microorganisms, although all cells may contain substantial concentrations of GSH, cysteine, or related compounds (9, 10, 19, 26, 27; R. C. Fahey and G. L. Newton, *in* A. Holmgren, A. Larsson, B. Mannervik, and S. Orrenius, ed., *Functions of Glutathione-Biochemical, Physiological, and Toxicological Aspects*, in press).

Protection afforded by such compounds may be especially important to bacteria classified as *Streptococcus mutans*. As representatives of the lactic acid streptococci, these oral pathogens (12) are facultative anaerobes that depend primarily on glycolysis for their energy metabolism (5), but take up O_2 at rates comparable to those of aerobic organisms (1, 7, 24, 31). A portion of the O_2 taken up by streptococci is converted to potentially toxic electrophilic agents, including O_2^- and H_2O_2 (1, 3, 6, 7, 24, 31). In addition, release of H_2O_2 from catalase-negative lactic acid bacteria results in peroxidase-mediated production of antimicrobial oxidizing agents in exocrine secretions (13, 30) and phagocytic leukocytes (15, 17, 21).

Incubation of *S. mutans* cells with glucose and certain disulfide or sulfhydryl compounds results in a dramatic increase in cell sulfhydryl content and increased resistance to at least one oxidizing agent (32). To characterize this phenomenon, we studied the reduction of disulfides and transport of disulfide and sulfhydryl compounds. These bacteria were found to have reductase and transport activities that can create pools of sulfhydryls in both the cytoplasm and the extracellular medium.

MATERIALS AND METHODS

Materials. All chemicals were of reagent grade. Sodium dodecyl sulfate (labeled "specially pure") was from BDH, Poole, England. [^{35}S]GSH (48.5 Ci/mmol) and [glycine-2-^{3}H]GSH (2.2 Ci/mmol) were from New England Nuclear Corp., Boston, Mass.). L-[3,3′-^{3}H]cystine (0.88 Ci/mmol) was from Amersham Corp., Arlington Heights, Il. To obtain an exchange of label between glutathione disulfide (GSSG) and either [^{3}H]GSH or [^{35}S]GSH, or between L-cysteine and [^{3}H]cystine, we diluted the labeled compounds with a 200-fold molar excess of the unlabeled compound and incubated them for 1 h at 37°C. Reduced or oxidized lipoic acid (DL-6,8-thioctic acid) or lipoamide (DL-6,8-thioctic acid amide) was added as a 0.2 M solution in dimethyl sulfoxide. Other compounds were added as solutions in 0.1 M sodium chloride–1 mM magnesium sulfate–0.05 M potassium phosphate, adjusted to pH 7. The presence of dimethyl sulfoxide did not influence results with any of the compounds tested.

Bacteria. As described previously (31, 32), *S. mutans* strains of serotypes *a* (AHT, HS-6), *b* (FA-1, BHT), *c* (GS-5, Ingbritt), *d* (OMZ-176, B-13), *e* (LM-7), *f* (OMZ-175), and *g* (6715-15) were grown under aerobic or anaerobic conditions in filter-sterilized Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.), harvested by centrifugation, and washed and resuspended in the NaCl-$MgSO_4$-KPO_4 medium. Similar results were obtained with all strains. All results shown below were obtained with GS-5 cells harvested in the stationary phase (24 h) from aerobic cultures.

Incubation conditions. Incubations were at a cell density corresponding to 2.3 mg (dry weight) per ml in a 2-ml total volume of the NaCl-$MgSO_4$-KPO_4 medium with 10 mM

glucose under N_2 at 37°C. Incubation mixtures with radiolabeled sulfhydryl compounds also contained 5 mM dithiothreitol.

Uptake of radiolabeled compounds. After incubation, 0.2-ml portions of the incubation mixtures were cooled to 4°C to prevent further uptake. Cells were collected on 0.45-μm filters (Millipore Corp., Bedford, Mass.) and washed with cold 0.1 M NaCl–1 mM $MgSO_4$. The filters were dissolved in scintillation fluid (2), and radioactivity was determined with a liquid scintillation spectrometer.

Sulfhydryl content of the medium and cells. After incubation, the medium and cells were separated by centrifugation at 20,000 × *g* for 15 min at 4°C. The cells were suspended and washed by centrifugation in 6 ml of cold 0.1 M NaCl–1 mM $MgSO_4$. The sulfhydryl content was determined by reacting portions of the medium and the washed, detergent-solubilized cells with an excess of the disulfide compound 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2) and by determining the yield of 5-thio-2-nitrobenzoic acid (Nbs) from the absorbance at 412 nm, assuming a molar extinction coefficient of 13,600 (8). Portions of the medium were diluted with 10 mM EDTA–0.1 M Tris-hydrochloride (pH 7) and incubated for 5 min at 37°C with 0.3 mM Nbs_2. The cells were suspended in a solution containing 2 ml of water, 20 μl of 1 M potassium EDTA, 1 ml Tris-hydrochloride (pH 8), 0.1 ml of 3 mM Nbs_2, and 0.5 ml of 20% (wt/vol) sodium dodecyl sulfate, added successively (29, 32). The mixture was incubated for 1 h at 37°C under N_2, cooled, and centrifuged, and the absorbance of the supernatant was measured.

Reductase activities. Washed cells were disrupted, as described previously (31), by homogenization in 1 mM EDTA–20 mM Tris-hydrochloride (pH 8) with 0.1-mm glass beads. The homogenate was centrifuged at 4°C for 30 min at 20,000 × *g* to obtain a clarified supernatant (soluble fraction) and a particulate fraction. The soluble fraction was dialyzed against 0.1 M phosphate (pH 7). The particulate fraction was washed twice by centrifugation and suspended in 20 mM Tris-hydrochloride (pH 7). Protein was determined by the method of Lowry et al. (20), with bovine serum albumin as the standard. The soluble and particulate fractions represented 73 and 27%, respectively, of the protein recovered, which was 11% of the dry weight.

Portions of the fractions were incubated at 37°C under N_2 with disulfide compounds, NADH or NADPH, and 0.3 mM Nbs_2 in 0.02 M Tris-hydrochloride (pH 7). Incubations were stopped by dilution with 12.5 volumes of 0.4% (wt/vol) sodium dodecyl sulfate in 0.1 M Tris-hydrochloride (pH 7). Mixtures containing particulate fractions were clarified by centrifugation. The yield of sulfhydryls was calculated from the absorbance at 412 nm.

RESULTS

Disulfide reduction. When cells were incubated with certain disulfide compounds (RSSR), those compounds were reduced to the corresponding sulfhydryl compounds (RSH) as indicated by increased sulfhydryl content of the medium or of both the medium and the cells. The total amount of reduction was calculated as the sum of the medium SH and the increase in cell SH. Reduction was linear with time up to at least 1 h, and rates were proportional to the number of cells per milliliter. Rates of reduction were calculated as the rate of appearance of RSH, which would be two times the rate of disappearance of RSSR.

Reduction could also be measured by incubating *S. mutans* cells with the RSSR compound and excess Nbs_2 (R′SSR′). During the incubation, Nbs_2 acted as a trap for RSH.

$$RSH + R'SSR' \rightarrow RSSR' + R'SH$$

The amount of reduction was calculated as the sum of Nbs (R′SH) in the medium and the increase in cell SH.

Reduction required glucose and was blocked by inhibitors of glucose metabolism (KF or EDTA at 20 mM) or at 4°C. Similar results were obtained under aerobic or anaerobic conditions, although anaerobic conditions prevented the loss of RSH compounds by autoxidation or reactions with O_2 metabolites released by the cells.

Oxidized lipoic acid and lipoamide were reduced at similar rates (Table 1). The rate of lipoic acid reduction was up to five times higher when reduction was measured by incubating *S. mutans* cells in the presence of Nbs_2. This observation may indicate that the product (reduced lipoic acid) inhibited reduction or that reduction was reversible, so that Nbs_2 increased the net rate of reduction by trapping the product.

The rate of glucose metabolism under these conditions was about 150 μM/min (31). Therefore, a large fraction of the reducing equivalents obtained from glucose metabolism was diverted into reduction of lipoic acid or lipoamide. Reducing equivalents for RSSR reduction or other reductive processes (7) may be obtained at the expense of reduction of pyruvate to lactate.

Cystamine, GSSG, and L-cystine were also reduced. The highest apparent affinities (lowest K_m values) were obtained with GSSG and cystine. Rates of reduction were much lower than those obtained with lipoic acid and lipoamide, and the addition of Nbs_2 resulted in less than a twofold increase in rates. The presence of Nbs_2 did not alter the K_m for any compound, indicating that Nbs_2 was not a competitive substrate for reduction.

Although a number of compounds were reduced, reduction was selective. RSSR compounds that were not reduced included Nbs_2, pantethine, the L-cystine analogs D-cystine, D- or L-penicillamine disulfide, and L-homocystine, and the GSSG analogs L-cystinyl-bis-glycine and coenzyme A-SG (the mixed disulfide of coenzyme A and GSH). This specificity indicated that reduction was not due to release of a reducing agent into the medium. In particular, reduction of only the L-isomer of cystine indicated stereospecific binding to the active site of a transport system or reductase enzyme.

TABLE 1. Disulfide reduction[a]

Compound	Nbs_2	V_{max} (μM/min)	K_m (mM)
Lipoic acid	+	150–250	1
	−	30–40	
Lipoamide	+	40–80	1
	−	30–40	
Cystamine	+	20	5
	−	11	
GSSG	+	1.8	0.02
	−	1.1	
L-Cystine	+	0.8	0.02
	−	0.5	

[a] Rates of reduction were calculated from the sum of the increases in sulfhydryl content of the medium and cells during incubation with (+) or without (−) 0.6 mM Nbs_2.

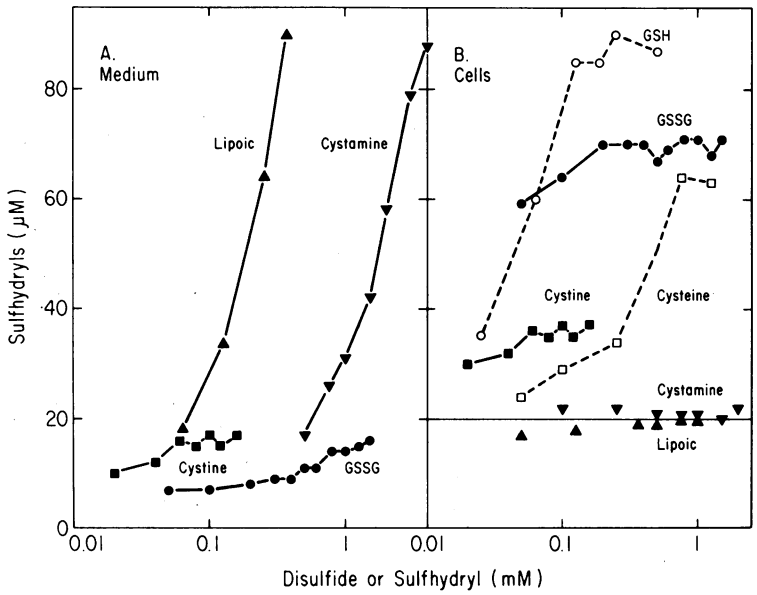

FIG. 1. Distribution of sulfhydryls between the medium and cells. (A) Sulfhydryl content of the medium was measured after a 15-min incubation with oxidized lipoic acid (▲) or cystamine (▼) or after a 1-h incubation with L-cystine (■) or GSSG (●). (B) Sulfhydryl content of the washed-cell suspensions was measured after 1 h of incubation with GSH (○), GSSG (●), L-cysteine (□), L-cystine (■), cystamine (▼), or oxidized lipoic acid (▲). When cells were incubated without added disulfide or sulfhydryl compounds, the mean sulfhydryl content of the cell suspension was 20 ± 4 μM.

Figure 1 shows the distribution of the increase in SH between the medium and the cells. With oxidized lipoic acid or cystamine, all of the SH increase was in the medium. In other experiments, similar results were obtained with oxidized lipoamide. Similarly, incubation with reduced lipoic acid or cysteamine (the reduced form of cystamine) did not result in an increase in cell SH. Therefore, reduction of lipoic acid, lipoamide, and cystamine yielded RSH compounds that were either not taken up or not accumulated in the cells against a concentration gradient.

In contrast, with GSSG or cystine, about 60 to 80% of the SH increase was intracellular. Figure 1 also shows the increase in cell SH obtained by incubating *S. mutans* cells with GSH or L-cysteine. This increase was greater than that obtained with GSSG or cystine. Therefore, reduction of GSSG and cystine yielded RSH compounds that could be transported into the cells and which were accumulated against a concentration gradient.

Competition for reduction. To determine whether all the RSSR compounds were reduced at the same site or whether several reductase activities were involved, competition for reduction was studied. For example, cystine was reduced at a much slower rate than cystamine, so that cystine would act as a competitive inhibitor of cystamine reduction if both were reduced by the same enzyme. However, no evidence was obtained for competitive inhibition between any of the RSSR compounds.

These results were not conclusive in that reduction of one RSSR compound could result in nonenzymatic reduction of a second compound. For example, reduction of cystamine to cysteamine could result in reduction of cystine by cysteamine, which would eliminate the inhibitor.

$$\text{cystamine} + 2\,e^- + 2\,H^+ \xrightarrow{\text{reductase}} 2 \text{ cysteamine}$$

$$2 \text{ cysteamine} + \text{cystine} \xrightarrow{\text{nonenzymatic}} \text{cystamine} + 2 \text{ cysteine}$$

To overcome this problem, cells were incubated with two RSSR compounds to determine whether the rates of reduction were additive. If cystamine and cystine were reduced by the same enzyme, the rate of reduction obtained with saturating concentrations of both compounds would be no faster than with cystamine alone. If the compounds were reduced by two different enzymes, the rates would be additive.

Table 2 shows the rate obtained with each compound alone and in combination with a second compound. Also shown are predicted rates, which were calculated assuming that the rates were additive. Incubations were performed with excess Nbs_2 as a trap for RSH to diminish the nonenzymatic reduction described above. Nbs had little or no ability to reduce the RSSR compounds.

Oxidized lipoic acid and lipoamide competed for reduction, but lipoamide did not compete with cystamine. Cystamine also did not appear to compete with either GSSG or cystine, although the large difference in rates made it difficult to determine whether rates were additive. GSSG and cystine did compete. These results suggested the presence of as many as three reductase activities, one that was specific for oxidized lipoic acid or lipoamide, one that was specific for GSSG or cystine, and perhaps another one that was specific for cystamine. Alternatively, the three classes of substrates may have been taken up by three separate disulfide transport systems and then reduced by one or more reductase enzymes.

Relation of reduction and transport. GSSG and cystine competed for uptake (Fig. 2). GSSG inhibited the uptake of label from [^{3}H]cystine, and cystine inhibited the uptake of label from [^{3}H]GSSG. Inhibition was competitive, and the K_i values were similar to the apparent K_m values for reduction (Table 1). If GSSG and cystine were taken up by separate, concentrative transport systems and then were reduced at a common intracellular site, competition for reduction would

TABLE 2. Competition for reduction[a]

Expt	Compound	Rate of reduction (μM/min)	Predicted rate (μM/min)	% of predicted reduction
A	Lipoic	210		
	Lipoamide	60		
	Lipoic + lipoamide	170	270	63
B	Lipoamide	43		
	Cystamine	23		
	Lipoamide + cystamine	67	66	102
C	Cystamine	9.7		
	GSSG	1.3		
	Cystine	0.5		
	Cystamine + GSSG	11.4	11.0	104
	Cystamine + cystine	9.9	10.2	97
D	GSSG	0.73		
	Cystine	0.47		
	GSSG + cystine	0.73	1.20	61

[a] Cells were incubated with 1.2 mM Nbs_2 and: (A) 5 mM oxidized lipoic acid or lipoamide or both; (B) 5 mM oxidized lipoamide or 10 mM cystamine or both; (C) 5 mM cystamine, 5 mM GSSG, 0.15 mM cystine, or the indicated combinations; (D) 5 mM GSSG or 0.13 mM cystine or both. Predicted rates were calculated as the sum of rates obtained with each compound alone.

be observed, but there would be no competition for uptake of label. The results suggested that GSSG and cystine were taken up by the same disulfide transport system.

Table 3 shows apparent V_{max} and K_m values for uptake of label from radiolabeled GSSG and cystine and for the increase in cell SH obtained with these compounds. The rates of uptake and the rates of SH increase were similar. The apparent K_m values for uptake were similar to those obtained for reduction (Table 1). These observations suggest that GSSG and cystine were taken up slowly and then reduced rapidly, so that the apparent V_{max} and K_m values were characteristic of the rate-limiting disulfide transport process. An alternative interpretation is that the RSSR compounds cannot be accumulated against a concentration gradient, so that uptake of label from RSSR depends on reduction of RSSR to RSH. If RSSR cannot be accumulated, then competition between GSSG and cystine could be due to competition for transport or reduction or both.

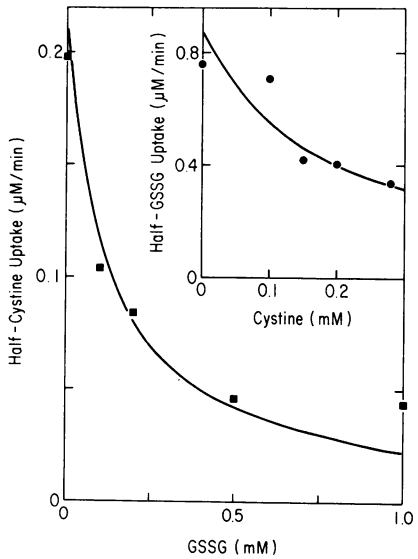

FIG. 2. Competition for disulfide uptake. Cells were incubated with 0.1 mM L-[^{3}H]cystine (■) and the indicated concentrations of GSSG or with 0.15 mM [^{3}H]GSSG (●) and the indicated concentrations of L-cystine. Uptake was calculated assuming that two intracellular sulfhydryls were obtained per disulfide taken up. Curves fitted to the data were calculated assuming competitive inhibition with K_m and K_i = 20 μM for both compounds, V_{max} = 1 μM/min for GSSG, and V_{max} = 0.26 μM/min for cystine.

Sulfhydryl uptake. Evidence for uptake of RSH compounds was provided by the increase in cell SH, the corresponding SH decrease in the medium, and the uptake of label from radiolabeled forms of the RSH compounds. Uptake was linear with time, proportional to the number of cells per milliliter, dependent on glucose, and blocked by KF or

TABLE 3. Sulfhydryl uptake[a]

Compound	Measurement	V_{max} (μM/min)	K_m (mM)
GSSG	Δ cell SH	1	0.02
	Half-[^{35}S]GSSG uptake	1	0.04
	Half-[^{3}H]GSSG uptake	1	0.06
L-Cystine	Δ cell SH	0.2	0.02
	Half-[^{3}H]cystine uptake	0.2	0.02
GSH	Δ cell SH	2	0.1
	^{35}S uptake	2	0.2
	^{3}H uptake	2	0.2
L-Cysteine	Δ cell SH	1.5	0.3
	^{3}H uptake	1.7	0.4
D-Cysteine	Δ cell SH	1.8	4
L-Penicillamine	Δ cell SH	0.4	1

[a] Rates of uptake were calculated from the increase in cell sulfhydryl content (Δ cell SH) or from the uptake of label. Uptake of labeled GSSG or cystine was calculated assuming that 2 mol of intracellular sulfhydryls were obtained per mol of disulfide taken up.

EDTA or at 4°C. Uptake of RSH compounds resulted in up to a 10-fold increase in cell SH content.

Table 3 also compares uptake of RSH compounds. GSH was the most rapidly transported. Similar uptake was obtained with GSH labeled in the cysteinyl and glycine residues. Unlabeled L-cysteine, glycine, or L-cysteinyl-glycine did not inhibit uptake of label from either of the labeled forms of GSH. Therefore, GSH was taken up as the intact tripeptide (γ-glutamyl-cysteinyl-glycine) rather than first being hydrolyzed to dipeptides or amino acids.

Uptake of label from [^{35}S]GSH or [^{3}H]GSH was not inhibited by L-alanine, cysteamine, or any of the RSH compounds shown in Table 3. Also, uptake was not inhibited by γ-glutamyl-alanine, seryl-glycine, or β-aspartyl-glycine. Therefore, GSH uptake was specific and was not due to a transport system with broad specificity for amino acids, RSH compounds, or peptides.

Both L- and D-cysteine were taken up, although L-cysteine was transported with higher apparent affinity (Table 3). This lack of absolute stereospecificity was in contrast to that observed for reduction of L-cystine. Similarly, L-penicillamine was taken up, although L-penicillamine disulfide was not reduced, indicating that the specificities of transport and reduction were different.

L-Penicillamine, L-alanine, and D-cysteine competed with L-cysteine for transport, inhibiting the rate of uptake of label from L-[^{3}H]cysteine (Fig. 3). In contrast, cysteamine was not taken up and did not inhibit cysteine uptake. Although GSH was taken up, it did not compete with L-cysteine. Therefore, RSH uptake was due to two transport systems, one specific for GSH and one accepting a number of competing substrates with decreasing apparent affinities: L-cysteine > L-penicillamine > L-alanine > D-cysteine.

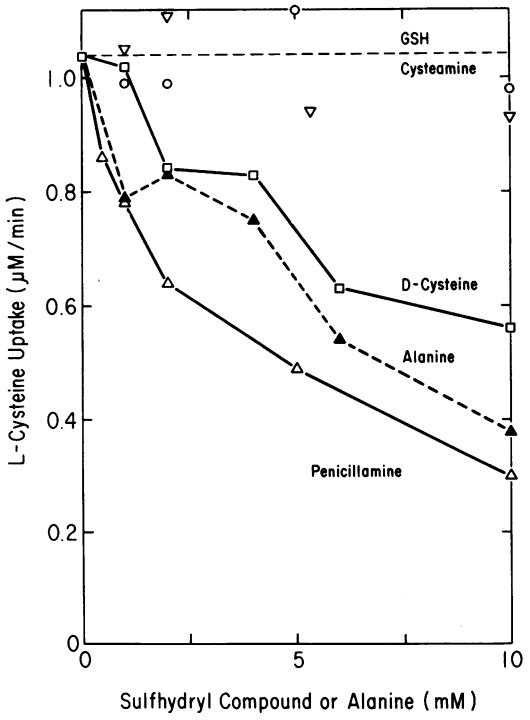

FIG. 3. Competition for sulfhydryl uptake. Cells were incubated with 0.2 mM L-[^{3}H]cysteine and the indicated concentrations of GSH (○), cysteamine (▽), D-cysteine (□), L-alanine (▲), and L-penicillamine (△).

TABLE 4. Competition for uptake[a]

Compound	Rate of increase in cell sulfhydryls (μM/min)	Predicted rate (μM/min)	% of predicted uptake
GSH	1.5		
GSSG	0.8		
GSH + GSSG	1.5	2.3	65
Cysteine	1.1		
Cystine	0.2		
Cysteine + cystine	1.3	1.3	100

[a] Cells were incubated with 3 mM GSH or 0.3 mM GSSG or both, or with 5 mM L-cysteine or 0.3 mM L-cystine or both. Predicted rates were calculated as the sum of rates obtained with each compound alone.

GSH also competed with GSSG for uptake (Table 4). When cells were incubated with saturating concentrations of both GSSG and GSH, the rate of increase in cell SH was less than the sum of the rates with each compound alone. In contrast, cysteine and cystine did not compete. These results, together with those in Table 2 and Fig. 2, suggest that one transport system serves for the uptake of GSSG, cystine, and GSH and that a separate system takes up cysteine.

In other experiments, no increase in cell SH was obtained on incubation with the RSH compounds L-cysteinyl-glycine, L-homocysteine, D-penicillamine, dithiothreitol, 2-mercaptoethanol, and coenzyme A or dephospho-coenzyme A. Glucose metabolism was not inhibited or stimulated by any of the RSH or RSSR compounds tested. Therefore, differences in rates and the inability to reduce or take up certain compounds were not due to differences in the supply of reducing equivalents or energy for concentrative uptake.

To compare rates of reduction and transport, the uptake rates shown above were expressed as the amount of SH per milliliter of cell suspension, rather than the amount per milliliter of intracellular volume. Assuming a 1.55-μl intracellular volume per amount of cells yielding 1 mg (dry weight) (16), rates of RSH uptake into the intracellular volume would be 280 times the rates shown. Before incubation with RSH or RSSR compounds, the cell suspensions contained 16 to 22 μM SH, corresponding to an intracellular concentration of 4 to 6 mM. The highest SH content of the washed-cell suspension after incubation with GSH or L-cysteine was 100 to 150 μM, or 30 to 40 mM intracellular SH.

Reductase activities. Disulfide reductase activities were detected in fractions from disrupted cells. NADH-dependent activity specific for oxidized lipoic acid or lipoamide was obtained in the particulate fraction. The ratio of specific activities of the soluble and particulate fractions was 0.7. In contrast, NADPH-dependent GSSG reductase activity was obtained primarily in the soluble fraction. The ratio of specific activities was 6.2. NADPH did not serve for reduction of lipoic acid or lipoamide, and NADH did not serve for GSSG. No reductase activity was detected with cystamine or cystine as the substrate.

Table 5 shows that the maximum rates of reduction of lipoic acid, lipoamide, and GSSG were similar, despite the large difference in rates observed with intact cells (Table 1). There was a 100-fold increase in measurable rates of GSSG reduction upon disruption of the cells, a 4-fold increase with lipoamide as the substrate, and no increase with lipoic acid. The K_m for GSSG was about 75 times that obtained with intact cells, whereas the K_m values for lipoic acid and

TABLE 5. Reductase activities[a]

Disulfide compound	V_{max} (μmol/min per mg)	K_m (mM)	Reductant	K_m (mM)
Lipoic acid	1.4	0.5	NADH	0.2
Lipoamide	1.1	0.7	NADH	0.2
GSSG	1.5	1.5	NADPH	0.35

[a] Rates of reduction of lipoic acid or lipoamide were measured per milligram of protein in the particulate fraction, and rates of reduction of GSSG were measured per milligram of protein in the soluble fraction.

lipoamide were unchanged. The increased rate of GSSG reduction and the higher K_m were consistent with other results indicating that the maximum rate and apparent affinity obtained with intact cells are characteristic of GSSG uptake rather than GSSG reduction.

DISCUSSION

The results suggest that the RSH content of *S. mutans* may be determined by the RSH and RSSR contents of the growth medium rather than by the biosynthetic capabilities of the bacteria. In the oral environment, RSH and RSSR compounds would be provided by food, saliva, and lysis of epithelial cells.

Of the compounds studied, either GSSG and L-cystine or GSH and L-cysteine are the two most likely to be encountered in significant concentrations by *S. mutans* under biologically relevant conditions. Although rates of reduction of GSSG and L-cystine were lower than those of several other compounds, the apparent affinities were higher. Also, GSH and L-cysteine were taken up faster than other RSH compounds. Lipoic acid and lipoamide were rapidly reduced at high concentrations but would be present in only small amounts in vivo. These compounds are required for pyruvate dehydrogenase activity in mammalian hosts for these bacteria and possibly also in the bacteria (4). Cystamine is an intermediate in host taurine biosynthesis (23). D-Penicillamine is a product of degradation of certain antibiotics (22), but only L-penicillamine was taken up.

Two or more reductase activities and at least two transport systems contributed to disulfide reduction and sulfhydryl uptake. Oxidized lipoic acid and lipoamide were reduced by the same enzyme, and the reduced products were released into the extracellular medium. Cystamine may have been reduced by a different enzyme, and the product (cysteamine) was released into the medium. GSSG and L-cystine competed for uptake, although it is not clear whether this competition was due to competition for transport or reduction. After uptake, GSSG and cystine were reduced, resulting in accumulation of GSH and cysteine within the cells. GSH also appeared to compete with GSSG for uptake, whereas L-cysteine was taken up by a separate transport system that also took up L-penicillamine and D-cysteine.

NADH-dependent lipoic acid and lipoamide reductase activity was present in the particulate fraction, suggesting that these lipophilic compounds were reduced on or in the cell membrane. NADPH-dependent GSSG-reductase activity was present in the soluble fraction, consistent with intracellular reduction. Transport and reduction of GSSG occurred at sites with different properties, so that transport and reduction were not concomitant steps in a single process. However, both processes required a metabolizable carbohydrate substrate to provide reducing equivalents and energy for concentrative uptake.

The high rate and low apparent affinity of cystamine reduction and the absence of cysteamine accumulation in the cells suggest that cystamine was reduced at the membrane surface. A transmembrane reductase enzyme or membrane-soluble cofactor such as lipoic acid would be required to transfer reducing equivalents across the membrane. However, it is also possible that cystamine was taken up rapidly and reduced intracellularly and that cysteamine was then rapidly transported to the medium.

The enzymes responsible for reduction of cystamine and cystine have not been identified. Inability to detect reduction in subcellular fractions could indicate that the enzymes were inactivated, that an essential cofactor or activating agent was absent, or that a compound other than NADH or NADPH was the electron donor for enzyme-catalyzed reduction. Alternatively, these compounds may have been reduced nonenzymatically in reactions with endogenous GSH or reduced lipoic acid or lipoamide. For example, the rapid reduction of intracellular GSSG could provide an indirect mechanism for reduction of cystine.

$$\text{cystine} + 2\ \text{GSH} \xrightarrow{\text{nonenzymatic}} 2\ \text{cysteine} + \text{GSSG}$$

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{reductase}} 2\ \text{GSH} + \text{NADP}^+$$

$$\text{net: cystine} + \text{NADPH} + \text{H}^+ \longrightarrow 2\ \text{cysteine} + \text{NADP}^+$$

Due to reduction at the membrane or to efflux of intracellular RSH, reduction of RSSR compounds resulted in the release of RSH compounds to the medium. The released RSH could reduce other compounds that did not interact directly with the reductases. For example, Nbs_2 was reduced in the medium when a suitable RSSR compound was added. This indirect mechanism of reduction would enable the cells to reduce many compounds that are not substrates for the reductases or transport systems. Similarly, oxidizing agents or other electrophiles (A) might be reduced in the extracellular medium at the expense of intracellular NAD(P)H, with RSSR acting as a cofactor.

$$\text{RSSR} + \text{NAD(P)H} + \text{H}^+ \xrightarrow{\text{reductase}} 2\ \text{RSH} + \text{NAD(P)}^+$$

$$2\ \text{RSH} + \text{A} \xrightarrow{\text{nonenzymatic}} \text{RSSR} + \text{AH}_2$$

$$\text{net: NAD(P)H} + \text{H}^+ + \text{A} \longrightarrow \text{NAD(P)}^+ + \text{AH}_2$$

Oral lactic-acid bacteria including streptococci and lactobacilli have been reported to create a reducing environment (5), which has been described as low oxidation-reduction potential or E_h (5, 25, 28). This environment may exclude aerobic (O_2-requiring) microorganisms, foster the growth of obligate anaerobes, and protect the bacteria against attack by oxidizing agents and other electrophiles. The accumulation of sulfhydryl compounds in the medium and the depletion of O_2 by NADH-dependent reduction (31) may be the principal mechanisms by which *S. mutans* creates a reducing environment in the extracellular medium.

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