Cytoplasmic Proteins of *Streptococcus mutans* (Serotype c) and Their Interaction with Fluoride

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The protein profile of the cytoplasmic proteins of *Streptococcus mutans* GS-5 was determined by two-dimensional gel electrophoresis. Use of this recently developed, high-resolution analytical tool showed in excess of 140 cytoplasmic proteins. The profile consisted of mostly acidic components with pl values between 3.70 and 5.30 and relative molecular weights mainly in the 13,000 to 90,000 range. With sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the proteins were resolved into 40 to 45 components. The binding of fluoride by the proteins reached a maximum value in 15 min, and it was linear with exogenous $F^-$ doses of up to 60 to 80 ppm per mg of protein (60 to 80 μg/mL). The proteins bound 22 to 138 times more $F^-$ than from assay mixtures containing 1 mM CaCl2 than from assay mixtures containing such ions as HgCl2, ZnCl2, CuCl2, MgCl2, MnCl2, or SnCl2. When NaF, SnF2, NH4F, CsF, (CH3)4NF, and Na3PO4F were used as sources of $F^-$ (adjusted to 10 ppm of $F^-$ in all cases), the proteins bound 2.1, 1.8, 1.6, 1.4, and 0.3 ppm of $F^-$ per mg of protein, respectively. Initial fractionation of the plasma proteins by preparative column isoelectric focusing indicated that proteins with pl values of 4.1 to 4.5 as well as those with pl values of 5.0 to 5.3 bound twice as much $F^-$ as did the proteins outside these pl values.

Elevated content of fluoride in the environment has been associated with a diminution in the number of dental caries (5, 6, 10). Furthermore, the addition of fluoride to communal water supplies and industrial products such as dentifrices significantly reduces tooth decay (1, 18, 21).

During the past decade, extensive experimentation has been conducted on the immunology and physiology of *Streptococcus mutans*, which is thought to be involved with dental caries (12). No reports could be found in the literature concerning the biology of cytoplasmic proteins of *S. mutans* GS-5. Studies on the cytoplasmic proteins of *S. mutans* are of interest because of (i) the paucity of data in this area, (ii) the possible interaction of various anticaries agents such as fluoride (22, 36) with proteins of *S. mutans*, (iii) the biotyping of *S. mutans* in studies dealing with assessments of the cytoplasmic protein complement during various phases of growth, (iv) experiments on genetic polymorphism in *S. mutans*, and (v) investigations pertaining to the mechanism of drug resistance.

High resolution of proteins at the nanogram level can be achieved by two-dimensional electrophoresis (24, 29, 34). The aim of this study is to examine the number of cytoplasmic proteins of *S. mutans* GS-5 that can be resolved electrophoretically, to establish a relative pl and molecular weight map of these proteins, and to determine the amounts of fluoride bound by these cytoplasmic proteins under various experimental conditions.

**MATERIALS AND METHODS**

**Preparation of cytoplasmic proteins.** Unless stated otherwise, *S. mutans* GS-5 grown for 48 to 72 h in the synthetic medium developed by Terleckyj et al. (31) was used. These cells were in the stationary phase of the growth cycle. The cells were harvested by centrifugation and washed twice with deionized, double-distilled water. Chemicals of the highest purity available (Sigma Chemical Co., St. Louis, Mo. and Fisher Scientific Co., Itasca, Ill.) were used to prepare the culture medium, and only new disposable tubes, flasks, and laboratory utensils constructed from polycarbonate or borosilicate glass were employed.

The cells were suspended in deionized, double-distilled water and disrupted by shaking at 1 to 8°C with 0.1- to 0.3-mm-diameter glass beads in a Bead-Beater (Biospec Products, Bartlesville, Okla.) or a Mickle cell disintegrator (Brinkmann Instruments, Inc., Des Plaines, Ill.). The cell chamber of the Bead-Beater was immersed in an ice slurry. The entire assembly was kept in a walk-in cold room maintained at 4°C. The Bead-Beater was turned on and off at 1-min intervals to avoid temperature increases in the cell chamber. Cell breakage was achieved after a total period of 30 min of shaking. The broken-cell suspensions were centrifuged at 2,000 × g for 20 min to remove intact cells and glass beads. The supernatant fluid was then centrifuged at 105,000 × g for 1 h at 4°C in an International Equipment refrigerated ultracentrifuge model B-25. The resulting pellet was discarded, whereas the supernatant fluid from the above ultracentrifugation was subjected to three ammonium sulfate fractionations (30). The precipitates resulting from the 35, 65, and 95% saturations with ammonium sulfate were dissolved and dialyzed against deionized, double-distilled water. The dialyzed proteins were either kept at −70°C or lyophilized. The addition of trichloroacetic acid at a final concentration of 10% to the supernatant of the 95% (NH4)2SO4 fraction indicated that the cytoplasmic proteins had been precipitated. The ammonium sulfate fractionation scheme was used to minimize contamination of the cytoplasmic proteins with polysaccharides, nucleic acid components, and other cellular moieties.

**Analytical procedures.** Protein was assayed by the method of Lowry et al. (23) with bovine serum albumin as a standard. The albumin was dried over phosphorus pentoxide to remove any moisture before weighing and construction of the standard curve. RNA was determined by the orcinol reaction (19). Absorbances were measured at 660 nm within 10 min of completion of the reaction. Yeast RNA was used as a standard (14). Rhamnose was determined by the method of Dische and Shettles (11).

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ATPase assay. ATPase activity was measured at 37°C by the liberation of Pi in 1 ml of reaction mixture containing 100 mM KCl, 4.0 mM MgCl₂, 2.5 mM ATP (vanadate free), and 50 mM Tris-acetate buffer (pH 6.5). The reaction was started by the addition of 100 to 1,000 μg of cytoplasmic proteins, and after 30 min at 37°C, it was terminated by the addition of 0.5 ml of 1.5 M perchloric acid. The tubes were centrifuged at 2,000 × g for 15 min at 4°C. The controls included the reaction mixture in which the cytoplasmic proteins were substituted by S. mutans GS-5 cytoplasmic membrane ATPase (30) and the addition of an ATPase sample after perchloric acid treatment; all control samples and reactants were exposed to conditions similar to those of the test samples. The P_i released was assayed in the supernatant by the method of Chen et al. (9). Values were obtained from a standard curve, after absorbance readings at 820 nm. Enzyme activity (1 U) was expressed as the amount liberating 1 μmol of P_i in a 30-min period; specific activity was expressed as units per milligram of protein.

Electron microscopy. The proteins were dissolved in deionized, double-distilled water, precipitated with 10% cold trichloroacetic acid, and concentrated in a pellet by centrifugation for 1 h at 105,000 × g. The protein pellet was prefixed for 1 h with 4% glutaraldehyde in Millonig phosphate buffer (27), rinsed twice with the buffer, and postfixed for 1 h with 1% osmium tetroxide. Then, the protein pellet was dehydrated in a graded series of acetone, infiltrated, and embedded in Epon. Thin sections were cut on an LKB III Ultratome, collected on uncoated grids, and examined with a Hitachi HV-11B electron microscope, operated at 75 kV.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis analysis of proteins was performed as described by Anderson and Anderson (4).

All protein samples subjected to SDS-polyacrylamide gel electrophoresis were mixed with SDS treatment buffer (0.01 M Tris-hydrochloride [pH 7.0], 2% [wt/vol] SDS, 5% [vol/vol] 2-mercaptoethanol) in sealed 1.5-ml polyethylene microcentrifuged tubes and heated at 100°C for 2 min (32). Samples were layered into the gel pockets and overlaid with SDS-polyacrylamide gel electrophoresis electrode buffer (0.025 M Tris-hydrochloride, 0.192 M glycine, 0.1% [wt/vol] SDS, pH 8.3). Electrophoresis was performed at a constant current of 30 mA for 5.5 h. Gels were stained with 0.2% (wt/vol) Coomassie brilliant blue R-250 in 50% ethanol-5% acetic acid; destaining was performed in a series of ethanol-acetic acid solutions (2). The following proteins (Pharmacia Fine Chemicals, Piscataway, N.J.) were subjected to electrophoresis as described above for SDS-polyacrylamide gel electrophoresis and served as relative molecular weight (Mr) standards: aldolase, 158,000; transferrin, 90,000; γ-globulin (heavy chain), 50,000; ovalbumin, 45,000; chymotrypsinogen, 25,000; and RNase A, 13,700.

Two-dimensional gel electrophoresis. Proteins were separated by two-dimensional gel electrophoresis based on the method described by O'Farrell (29) and modified by Anderson and Anderson (2). Because we were interested in the complete profile of the cytoplasmic proteins of S. mutans, equal amounts of the 35, 65, and 95% ammonium sulfate fractions were combined before electrophoresis.

All samples subjected to isoelectric focusing in two-dimensional gel electrophoresis analysis were mixed with 2% (wt/vol) SDS-5% (vol/vol) 2-mercaptoethanol-20% (vol/vol) glycerol and heated to 100°C for 2 min. After cooling, urea was added to saturate (760 mg of urea to 1 ml of sample volume). Electrode solutions for isoelectric focusing consisted of 20 mM NaOH (cathode) and 10 mM H₃PO₄ (anode). The samples were focused for 18 h at 260 V. After isoelectric focusing, the gels were extruded into SDS-equilibration buffer (0.125 M Tris-hydrochloride [pH 6.8], 2% [wt/vol] SDS, 5% [vol/vol] 2-mercaptoethanol, 10% [vol/vol] glycerol) for 15 min at 37°C and used immediately or frozen at -70°C. For pH gradient determination, one unequilibrated focused gel was sliced into 5-mm sections and placed into tubes containing 1 ml of degassed, distilled water, and the pH was determined as previously described for isoelectric focusing of the equilibrated first-dimension isoelectric focusing gel was performed as described above for one-dimensional SDS-polyacrylamide gel electrophoresis with the following modification: the isoelectric focusing gel was placed on top of the stacking gel in SDS-polyacrylamide gel electrophoresis and sealed into place with 1% agarose in SDS-polyacrylamide gel electrophoresis electrode buffer. Electrophoresis and staining were performed as described above for SDS-polyacrylamide gel electrophoresis.

Assay of fluoride binding. Experiments on binding were done in 15-ml polystyrene disposable conical centrifuge tubes, and unless otherwise stated, the incubation mixture was composed of 1 ml of diluting gel containing protein—ca. 10 ppm of F– in a total volume of 1.2 ml. The tubes were capped and incubated at 37°C for 15 min, and then 1.2 ml of 95% ethanol was added to precipitate the protein. The mixture was allowed to stand at 4°C for 30 to 60 min and then was centrifuged at full speed on a Clay-Adams clinical centrifuge for 10 min. The pellet was washed twice with 2 ml of ethanol and finally treated for 30 min at 4°C with 1.2 ml of 0.5 N HClO₄ in stoppered tubes. The mixture was centrifuged for 10 min, and the supernatant was adjusted to pH 5 to pH 6 with an equivalent volume of 0.5 N KOH and finally centrifuged for 10 min. Total fluoride was assayed with fluoride-specific electrodes (Orion Research Inc., Cambridge, Mass.) by mixing equal volumes of the extract and total-ionic-strength activity buffer containing 1,2-cyclohexylenedinitrilo tetraacetic acid. Each experiment was performed in triplicate, and control experiments conducted in fluoride-free medium were used to estimate endogenous fluoride levels. The total fluoride reported in this paper represents HClO₄-extracted fluoride (22 to 24°C) from washed protein pellet and is called tightly bound fluoride.

RESULTS

Release of cytoplasmic proteins. The preparation of sufficient quantities of cytoplasmic proteins from S. mutans GS-5 requires an apparatus which is capable of disrupting the cells in an efficient and rapid manner. Unfortunately, the cells of S. mutans GS-5 are not readily ruptured by physical means. Recently, a new device known as the Bead-Beater was marketed for cell disintegration. The Bead-Beater released in 30 min of cell breakage four to five times more protein from S. mutans GS-5 cells than the protein released from cells subjected to breakage in the Mickle disintegrator.

Assays for cell wall and membrane contaminant. Clean cytoplasmic protein preparations should not contain such typical cell wall streptiococcal components as rhamnose (33) or such cell membrane enzymes as ATPase (28) and should appear homogeneous in electron microscope examinations. To this end, 2 to 8 mg of cytoplasmic proteins was examined for cell wall and cell membrane contamination by the procedures described above. The results of these studies are shown in Table 1. The rhamnose content of the cytoplasmic proteins ranged between 0.00 and 1.43% and yielded a mean
value of 0.68%. None of the five randomly selected preparations of cytoplasmic proteins showed any detectable ATPase activity. The finding of 11 to 23% RNA (with a mean value of 18.0%) in the cytoplasmic protein preparations suggests that ribonucleoproteins have not been excluded from these preparations. Electron microscope examinations of the cytoplasmic proteins of *S. mutans* GS-5 did not reveal any cell wall or cell membrane fragments in such preparations.

**Direct SDS-polyacrylamide gel electrophoresis.** Cytoplasmic proteins precipitated with 35, 65, and 95% ammonium sulfate and prepared from stationary-phase cells were subjected to electrophoresis at concentrations of 100 to 200 µg in SDS-polyacrylamide gel slabs (10% acrylamide–2.5% N,N′-methylenebisacrylamide). Under the experimental conditions, the cytoplasmic proteins precipitated with the 35, 65, and 95% ammonium sulfate were resolved into 49 to 52, 55 to 60, and 38 to 41 Coomassie blue-stained bands, respectively (Fig. 1). The profiles of the cytoplasmic proteins contained bands which had *Mr* values usually in the 13,700 to 138,000 range. Several well-defined bands were found between the gel application slot and the 158,000 marker as well as below the 13,700 marker in the profiles of proteins precipitated with the 35 and 65% ammonium sulfate fractions but not with the 95% ammonium sulfate fraction. Variation of the amount of protein beyond the 100 to 200 µg loaded on the SDS gel did not increase the resolution of the electropherogram. Similarly, combination of equal amounts

![FIG. 1. SDS-polyacrylamide gel electrophoresis analysis of *S. mutans* GS-5 cytoplasmic proteins. Lanes 1 through 3, 35% fraction; lanes 4 through 6, 65% fraction; lanes 7 and 8, 95% fraction.](image)

of the 35, 65, and 95% ammonium sulfate protein fractions did not result in any apparent alterations of the protein electrophoretic profile.

**Two-dimensional gel electrophoresis.** To delineate further the protein species encountered in the cytoplasmic proteins of *S. mutans* GS-5, two-dimensional gel electrophoresis was employed. The protein components in a given sample are cataloged by their apparent pi and *Mr*. Cytoplasmic proteins isolated from stationary-phase cells were solubilized in 2% SDS–5% 2-mercaptoethanol–20% glycerol and heated to 100°C for 2 min; the protein-to-SDS ratio was 1:1.9 by weight. After cooling, urea was added to saturation. The sample was subjected to isoelectric focusing in the presence of 4% acrylamide–6% N,N′-methylenebisacrylamide–2% Ampholine ampholytes (pH 3.5 to pH 10 and pH 4 to pH 7)–9.2 M urea–2% Nonidet P-40. This optimum mixture of ampholytes produced a linear, reproducible pH gradient from 3.3 to 7.7. The most suitable duration for focusing was determined to be between 4,030 V·h and 4,680 V·h. Prolonged times and excessive voltages produced distorted regions in the pH gradient. During isoelectric focusing, the SDS gradually migrated out of the sample and produced a white zone at the extreme acidic end of the gel. Before two-dimensional gel electrophoresis, the focused gel was equilibrated for 5 min at 37°C in 10% glycerol–5% 2-mercaptoethanol–2.1% SDS made up in 0.125 M Tris-hydrochloride, pH 6.8. This treatment improved the speed and entry of the focused components into the second dimension without a concomitant loss of any detectable protein components.

When the cytoplasmic proteins from stationary-phase cells of *S. mutans* were precipitated with 35, 65, and 95% ammonium sulfate, combined in equal portions (200 µg of each fraction), and then subjected to electrophoresis, more than 140 Coomassie blue-staining spots could be discerned in the electropherogram (Fig. 2). The protein spots shown in this typical electrophoretic profile were superimposable in repetitive experiments when similar protein isolation procedures and electrophoretic conditions were followed. The

![FIG. 2. Two-dimensional analysis of *S. mutans* GS-5 cytoplasmic proteins.](image)

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**TABLE 1.** Rhamnose, RNA, and ATPase content in cytoplasmic proteins of *S. mutans* GS-5

<table>
<thead>
<tr>
<th>Prepn</th>
<th>Rhamnose (%)</th>
<th>RNA (%)</th>
<th>ATPaseb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.43</td>
<td>11.3</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>0.88</td>
<td>23.1</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>0.43</td>
<td>15.7</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>0.00</td>
<td>22.0</td>
<td>ND</td>
</tr>
</tbody>
</table>

a The control value for membrane ATPase activity was 0.5 to 0.8 µM P, per 30 min per mg of membrane protein. ND, Not detected.
protein profile consisted of acidic components with pI values between 3.70 and 5.30. Over 80% of these proteins had pI values of 4.18 to 5.3 and Mr values in the 13,000 to 90,000 range. The 10% uniform polyacrylamide gel provided an excellent distribution of protein spots which included a relative range of molecular weight separation extending from 158,000 to 13,700. A component in this system was defined as an area represented by a discernible, stainable spot. The spots appeared to differ in quantity as judged by the intensity of staining. Components present in greater proportions gave the characteristic teardrop shape as noted by O’Farrell (29); the components present in lesser amounts appeared as greyish spots. Protein loads beyond the 140-μg level overshadowed many less intense spots and did not improve the electrophorogram shown in Fig. 2.

To examine the possibility that some proteolysis may have occurred during cellular fractionation, the serine type inhibitor phenylmethylsulfonyl fluoride (0.1 mM) was added in several cell fractionation procedures. Cytoplasmic proteins prepared in the presence and absence of this inhibitor and then subjected to electrophoresis in gel did not show any detectable changes in the electrophoretic patterns. Furthermore, incubation of protein samples at 37°C for 1 h before electrophoresis did not increase the number of Coomassie blue-staining spots on the electrophorograms, suggesting that minimal, if any, proteolytic degradation occurred during the processing of the cytoplasmic proteins.

TLIEF. The inability of the two-dimensional gel electrophores to demonstrate basic cytoplasmic proteins raised the possibility that such proteins could be demonstrated under nondenaturing conditions. Thus, thin-layer isoelectric focusing (TLIEF) was performed (19). The data indicated that TLIEF failed to demonstrate the presence of any basic proteins under nondenaturing conditions at the same protein load. Furthermore, it was thought that if fluoride ions bind to cytoplasmic proteins of S. mutans, such binding could alter the electropherogram and result in the appearance of certain protein bands in the neutral or basic area of the gel. Therefore, 140 μg of the combined cytoplasmic proteins precipitated with 35 to 95% ammonium sulfate were incubated with 0, 10, 50, or 100 ppm of fluoride for 15 min at 37°C and then subjected to TLIEF. Additions of 10 to 100 ppm of fluoride ions to the cytoplasmic proteins of S. mutans GS-5 failed to demonstrate the presence of any basic proteins in the assay mixture. Interestingly, as the fluoride concentration increased, several bands at the approximate pI values of 4.3, 5.0, and 6.0 became prominent.

Studies on fluoride binding. When one conducts binding assays, it is essential that such determinations be performed with systems that have reached equilibrium and become saturated. Time course experiments involved incubation of 1.2 mg of cytoplasmic proteins in a medium containing 50 ppm of F− for various time intervals, followed by precipitation of proteins with 10% HgCl2 in 1.2 ml of ethanol, centrifugation, washing with 95% ethanol, and F− estimation. Figure 3 indicated that the binding of F− by the cytoplasmic proteins reached a maximum value within 15 min. All subsequent F−-binding assays were allowed to proceed for 15 min. The effect of F− concentration on the binding of this anion to cytoplasmic proteins was studied with assay mixtures containing various levels of F−. It was found that the binding of F− by the cytoplasmic proteins of S. mutans GS-5 was linear with exogenous F− doses up to ca. 60 ppm (Fig. 4).

Effect of divalent ions on the binding of F− to cytoplasmic proteins. Previous work in this laboratory (25) shows that intact cells of S. mutans accumulated 20 to 30 times more F− from assay systems that contained 0.1 to 1 mM CaCl2 than other ions that are found in the oral cavity, or that are of interest in the demineralization and remineralization of teeth. To determine the influence of divalent ions on the binding of F− to cytoplasmic proteins of S. mutans GS-5, deionized, double-distilled water containing 10 ppm of F− was supplemented with 1 mM CaCl2, HgCl2, ZnCl2, CuCl2, MgCl2, MnCl2, or SnCl2 and added to the usual F−-binding assay mixtures. The results in Table 2 indicated that the cytoplasmic proteins bound 22 to 138 times more F− from assay systems containing 1 mM CaCl2 than from test mixtures that were charged with other divalent ions. The high concentration of F− extracted from the ZnCl2 as well as CaCl2 controls is most likely due to higher endogenous levels of F− in CaCl2 and ZnCl2 than in the other salts tested.

The stimulatory action of calcium on the binding of F− to cytoplasmic proteins was not likely due to CaF2 precipitation because, as determined by optical density assays, CaF2 formation required at least 2 mM CaCl2 and 50 ppm of F− in the reaction mixture. CaF2 precipitation with 1 mM CaCl2 was not observed even with 100 ppm of F− in the reaction mixtures.

Effect of the source of F− on its binding by the cytoplasmic proteins. Fluoride is added to toothpaste in the form of various salts. Thus, NaF, SnF2, NH4F, CsF, (CH3)4NF, and Na2O3F were selected as sources of F− and added, in all
instances, at a concentration of 10 ppm to the assay mixtures that had been supplemented with 1 mM CaCl₂. The results in Table 3 indicated that NaF and SnF₂ were better F⁻ sources for the binding of this anion to cytoplasmic proteins than were NH₄F, CsF, or (CH₃)₂NF, whereas Na₃PO₄F resulted in markedly less F⁻ binding than all the other salts tested.

**Binding of fluoride by cytoplasmic proteins of varying solubility, Mᵣ, and pl.** The data shown so far on the binding of F⁻ by the cytoplasmic proteins of *S. mutans* have been obtained with a mixture of cytoplasmic proteins containing equal concentrations of the 35, 65, and 95% (NH₄)₂SO₄ fractions. When the three (NH₄)₂SO₄ fractions were tested separately for F⁻ binding, the 35 and 65% (NH₄)₂SO₄ fractions bound similar levels of F⁻, whereas the 95% fraction bound about half the level bound by the other two fractions.

Filtration of the cytoplasmic protein F⁻ binders through Sephadex G-50, G-75, G-100, and G-150 media was not helpful for the fractionation of F⁻ binders. Even Sephadex G-100 (Mᵣ, 1 × 10⁶ to 8 × 10⁶), which was expected to separate the enolase that F⁻ has been shown to interact with (8), yielded a protein fraction that bound F⁻ levels equal to proteins of different Mᵣ values.

Because studies with TLIEF indicated that the addition of F⁻ to cytoplasmic proteins caused several Coomassie blue-stained bands to become prominent at the pl around 4.3, 5.0, and 5.3, the cytoplasmic proteins were fractionated with preparative isoelectric focusing (20), and fractions with various pl values were collected and tested for F⁻ binding. The results in Table 4 indicated that proteins with pl values of 4.1 to 4.5 as well as those with pl values of 5.0 to 5.3 bound twice as much F⁻ as did the others outside these pl values.

**DISCUSSION**

If this electrophoretic study of the cytoplasmic proteins is to reflect accurately the protein profile of the cytoplasm of *S. mutans* GS-5, then any contaminating cellular components which may contribute proteinaceous material to the assay macromolecules must be removed or at least minimized. Electron microscopy of the isolated cytoplasmic proteins showed that they were devoid of any cell wall or cell membrane contaminants. However, as cytological examinations alone cannot demonstrate homogeneity of a sample, several key chemical analyses were conducted. The data indicated that the cytoplasmic proteins contained 0.68% rhamnose and 18% RNA and did not show any detectable ATPase activity. Because rhamnose and ATPase constitute common and acceptable cell wall and cytoplasmic membrane markers, it may be concluded that cell wall or cell membrane-derived proteins contributed very little, if at all, to the protein profile of the cytoplasm of *S. mutans* GS-5. Finally, the presence of RNA in the cytoplasmic proteins of *S. mutans* GS-5 indicates the difficulty of eliminating RNA with the methodology employed.

The data obtained from the SDS gel electrophoresis must be judged in the light of the following theoretical assumptions and deficiencies: disruption of all but covalent interactions occurs within and between the chains; SDS is constantly bound per unit polypeptide chain length; and all conformational differences between polypeptides are destroyed (32).

The two-dimensional gel electrophoresis of the cytoplasmic proteins of *S. mutans* GS-5 represents the only investigation thus far reported, and it may be valuable as a reference analytical assay for cataloging and identifying individual cytoplasmic proteins of *S. mutans* GS-5. At the same time it provides primary information on the apparent pl and Mᵣ of each protein, information which is useful for designing isolation schemes.

With appropriate staining techniques, two-dimensional gel electrophoresis can easily resolve over 10,000 proteins. The Coomassie blue stain can detect about 0.01 μg of protein (29, 34). The amount of protein applied (140 μg) was relatively large for this system, and it would appear to be sufficient for the resolution of the cytoplasmic proteins of *S. mutans* GS-5.

The two-dimensional electrophoresis indicated that the cytoplasmic proteins consisted of acidic components with pl values of 3.70 to 5.30. However, the possibility exists that the cytoplasmic proteins contained either basic (pl 10 to 13) or acidic (pl < 3.5) components that were beyond the workable pH gradient range and commercial availability of ampholytes for this system. The absence of any detectable basic components in the electropherograms of TLIEF in which the pH gradient extended to 11.0 argues against this possibility.

### Table 2. Effect of divalent cations on fluoride binding by the cytoplasmic proteins of *S. mutans* GS-5

<table>
<thead>
<tr>
<th>Cation</th>
<th>F⁻ extracted from assay mixture (ppm)</th>
<th>F⁻ extracted from control (ppm)</th>
<th>F⁻ bound (ppm ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>0.057</td>
<td>0.021</td>
<td>0.036 ± 0.002</td>
</tr>
<tr>
<td>Hg⁺</td>
<td>0.025</td>
<td>0.012</td>
<td>0.013 ± 0.002</td>
</tr>
<tr>
<td>Zn⁺</td>
<td>0.035</td>
<td>0.018</td>
<td>0.017 ± 0.002</td>
</tr>
<tr>
<td>Cu⁺</td>
<td>0.056</td>
<td>0.012</td>
<td>0.024 ± 0.002</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0.080</td>
<td>0.024</td>
<td>0.056 ± 0.003</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>0.065</td>
<td>0.004</td>
<td>0.061 ± 0.003</td>
</tr>
<tr>
<td>Sn²⁺</td>
<td>0.226</td>
<td>0.145</td>
<td>0.081 ± 0.003</td>
</tr>
<tr>
<td>Ca⁺⁺</td>
<td>2.46</td>
<td>0.660</td>
<td>1.80 ± 0.10</td>
</tr>
</tbody>
</table>

* The incubation mixture contained 10 ppm of F⁻, 1 mM of the cation under study, and 1 mg of cytoplasmic protein per ml and was incubated at 37°C for 15 min. Controls were run simultaneously without protein.

### Table 3. Effect of different sources of fluoride on its binding by cytoplasmic proteins of *S. mutans* GS-5

<table>
<thead>
<tr>
<th>Source</th>
<th>F⁻ extracted (ppm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaF</td>
<td>2.08 ± 0.16</td>
</tr>
<tr>
<td>SnF₂</td>
<td>1.81 ± 0.15</td>
</tr>
<tr>
<td>NH₄F</td>
<td>1.63 ± 0.12</td>
</tr>
<tr>
<td>CsF</td>
<td>1.62 ± 0.15</td>
</tr>
<tr>
<td>(CH₃)₂NF</td>
<td>1.37 ± 0.12</td>
</tr>
<tr>
<td>Na₃PO₄F</td>
<td>0.33 ± 0.02</td>
</tr>
</tbody>
</table>

* The concentration of each salt in the incubation mixture was such that it yielded a final concentration in each case of 10 ppm of F⁻. The incubation mixtures (1.2 ml) also contained 1 mM CaCl₂ and 1 mg of cytoplasmic proteins per ml and were incubated at 37°C for 15 min.

### Table 4. Binding of fluoride by cytoplasmic proteins of *S. mutans* of varying pl value

<table>
<thead>
<tr>
<th>pl value of protein fraction</th>
<th>F⁻ extracted (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.7-4.1</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>4.1-4.5</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>4.6-5.0</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>5.0-5.3</td>
<td>1.9 ± 0.3</td>
</tr>
</tbody>
</table>

The results of the incubation mixtures indicated that the one which contained 37°C for 15 min. Controls were run simultaneously without protein.
The preparation method used in two-dimensional gel electrophoresis is a denaturing one, which therefore has the advantage of producing protein subunits most of which are protein gene products. These gene products appear as spots in the electrophoretic profile and are defined by their apparent pI and Mr. The probability of two unrelated protein components occupying exactly the same position on a two-dimensional gel is negligible (26). Because isoelectric focusing is considered the best method currently available for the detection of protein charge variability (2), the conditions conducive to protein heterogeneity must be stated. Some protein gene products are post-translationally modified by phosphorylation, deamination, acetylation, carbamylation, or other chemical modifications and additions of charged carbohydrate moieties (29). Chemical alteration of proteins by deamination or carbamylation will influence the overall protein charge and result in the formation of many Coomasie blue-stained spots situated in horizontal rows of similar Mr values in the electrophoretic protein profile (13). This phenomenon was not observed in the two-dimensional electrophoretic experiments of this investigation because urea was always used fresh to prevent isocyanate formation which could lead to protein carbamylation.

The identification of the fluoride-binding cellular components of the cariogenic organism S. mutans constitutes a basic step toward the understanding of the action of fluoride on the physiology of this important oral bacterium. It has been stated that it is imperative to determine the levels of F⁻ bound by cariogenic bacteria as well as the site in the cell where such binding occurs (7).

From 1 to 2 ppm of F⁻ can be bound by the cytoplasmic proteins under suitable experimental conditions. The binding of F⁻ by the cytoplasmic proteins of S. mutans GS-5 indicated that these macromolecules constitute respectable F⁻ depots for possible exchange of F⁻ between S. mutans proteins and those of other oral bacteria, such as Streptococcus sanguis (15, 16, 17, 35). It is also feasible that F⁻ bound to cytoplasmic proteins may participate in the remineralization and solubility of tooth enamel and thus render it less susceptible to caries.

Time course studies on the binding of fluoride by the cytoplasmic proteins of S. mutans revealed that the fluoride binding proceeded slowly and reached equilibrium within 15 min. This finding suggests that if S. mutans is to accumulate levels of fluoride significant enough to hinder its cariogenic potential, prolonged release of fluoride from antacids preparations is likely to be effective. In this regard, intraoral fluoride-releasing devices containing saturating levels of fluoride or repeated mouth rinses with fluoride are apt to saturate the target fluoride binders in S. mutans.

Enhanced fluoride binding to cytoplasmic proteins of S. mutans occurred in assay systems containing such divalent metals as calcium or tin. Similarly, the attachment of fluoride to cytoplasmic proteins of S. mutans was increased when NaF or SnF₂ was employed as source of F⁻ in the assay systems. These findings raise the question of whether F⁻ incorporation in toothpastes in the form of NaF or SnF₂ and calcium (1 mM) may be preferable to Na₃PO₄F, NH₄F, CsF, or other organic sources of F⁻.

Finally, it is noteworthy that although many cytoplasmic proteins of S. mutans were capable of binding fluoride, those having pI values of 4.1 to 4.5 and 5.0 to 5.3 bound twice as much fluoride as did the ones outside this range. Isolation and characterization of the germane target fluoride cytoplasmic proteins may provide useful information for the interpretation of the cariogenic action of fluoride.

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LITERATURE CITED


