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Purification and Characterization of *Streptococcus mutans* Group *d* Cell Wall Polysaccharide Antigen[1]

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The *Streptococcus mutans* group *d* antigen of strain B13 has been purified and characterized with respect to chemical composition and immunochemical properties. The antigen was extracted from lyophilized cells or cell walls by using 5% trichloroacetic acid at 5 C for 16 h. The antigen could also be extracted with water or 0.01 N HCl at 100 C for 20 min. The antigen was purified by ion-exchange and gel chromatography and was found to contain 96% carbohydrate, 1.6% protein, and 0.3% phosphorus. Characterization by gas chromatography indicated that the polysaccharide was composed of galactose and glucose in a 2:1 ratio. The antigen contained two serologically active sites: one site specific for group *d* and a second site common to both group *d* and group *a* strains. Agar diffusion and immunoelectrophoresis indicated that the two sites existed on a single molecule. The immunological specificity of the group *d* polysaccharide site depended on a terminal D-galactose. The purified B13 antigen did not react with antisera specific for the glycerol teichoic acid from streptococci. Anti-*d* serum rapidly agglutinated whole cells, indicating that the antibody receptor sites of the polysaccharide antigen were at the surface of the streptococcal cell.

The ability of *Streptococcus mutans* to form plaque on the smooth surfaces of teeth and its role in the formation of dental caries are well documented (5, 8, 10, 14, 24). *S. mutants* produces extracellular glucans from sucrose by a dextran-sucrase (12, 30); these polymers are involved in the adherence of the bacteria to teeth (11). Antisera against whole cells of *S. mutans* has been shown to inhibit dextran-sucrase activity and the ability of cells to adhere to smooth surfaces (9, 20-22). This inability to adhere results from an interference in the binding of dextran-sucrase to the cell by antibodies against polysaccharide and dextran located on the cell surface (20, 21). Consequently, it is of value to extract and characterize these surface antigens and determine their possible participation in reactions which are a part of the process of adherence on the *S. mutans* cell surface.

S. mutans strains are divided into five serological groups based on the presence of antigens extracted by hot acid (3, 4). Groups *a*, *b*, *c*, and *d* parallel the four genetic groups from deoxyribonucleic acid-deoxyribonucleic acid reassociation experiments (6). The fifth group possesses the streptococcal group E antigen (4). The antigens of group *a* (19), group *b* (18), and group E (27) have been described and are known to be polysaccharides located in or on the cell wall of the streptococci.

The present report describes the extraction, purification, and characterization of the *S. mutans* group *d* antigen. This work was presented in part at the 74th annual meeting of the American Association for Microbiology (R. Linzer and H. D. Slade, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 168, 1974).

MATERIALS AND METHODS

Streptococcal strains. *S. mutans* strains HS6, OMZ61, FA1, BHT, GS5, NCTC 10449, 6715, OMZ176, KIR, and B13 were kindly provided by A. L. Coykendall, D. Edman, and I. Shklair (Naval Dental Research Institute, Great Lakes, Ill.). The cells were grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 1.8% glucose and salts (13).

Serological procedures. Anti-B13 and anti-HS6 sera were prepared with New Zealand red rabbits. A 1% suspension of whole cells in saline was sonically treated for 30 s. After sonic treatment, the suspension was made 0.1% in formaldehyde and incubated for 16 h at 5 C. The suspension was diluted 10-fold with saline to a final cell concentration of 0.1%. The rabbits received a total of nine injections over a 3-week period. The first three injections of the cell suspension were 0.5 ml each; the other six injections were 1.0 ml each. During week 4, the rabbits were bled from the

[1] Dedicated to the 60th birthday of Otto Westphal, Max Planck Institut für Immunbiologie, Freiburg-Zähringen, Germany.

ear and the titer of antibody in their serum was checked by the capillary precipitin reaction. When a 4+ reaction was achieved, the rabbits were bled from the heart.

Specific group *d* antiserum was obtained by adsorbing whole anti-B13 serum with HS6 (group *a*) cells (10 mg/ml) (19). This procedure removed an antibody which cross-reacted with *S. mutans* group *a*. The cross-reacting *a–d* antibody was recovered by incubating the HS6 cells at pH 2 for 1 h at 5 C.

Anti-polyglycerophosphate serum, specific for glycerol teichoic acid, was kindly provided by Grove Wiley, Du Pont Institute, Wilmington, Del.

The capillary precipitin test (28), whole cell agglutination (18), agar gel diffusion (23), and immunoelectrophoresis (16) procedures have been described. The quantitative precipitin assay was performed as reported previously (25).

Preparation of crude antigens. Acid extracts were prepared from strains HS6 and OMZ61 (group *a*), FA1 and BHT (group *b*), GS5 and NCTC 10449 (group *c*), and 6715, OMZ176, KIR,and B13 (group *d*). Lyophilized cells (20 mg/ml) were extracted with 0.01 N HCl at 100 C for 20 min. The cells were removed by centrifugation at 12,000 × *g* for 10 min, and the extracts were neutralized with 1 N NaOH.

Extracts from B13 cells (20 mg/ml) were prepared with (i) 0.01 N HCl at 100 C for 20 min, (ii) water at 100 C for 20 min, (iii) 5% trichloroacetic acid at 100 C for 20 min, and (iv) 5% trichloroacetic acid at 5 C for 16 h. After removal of the cells by centrifugation, the trichloroacetic acid samples were extracted twice with an equal volume of cold ether to remove the acid. All samples were neutralized with 1 N NaOH.

Antigen extracts were also obtained from B13 cell walls. Purified cell walls were prepared by breaking the cells in a Mickle shaker (26) and treating the washed walls with 2% sodium dodecyl sulfate to remove contaminating lipoprotein from the cytoplasmic membrane (18). The walls (5 mg/ml) were extracted by procedures i through iv described above.

Purification of group d antigen. Lyophilized cells (15 g) of *S. mutans* strain B13 were suspended in 300 ml of 5% trichloroacetic acid and stirred at 5 C for 20 h. The suspension was centrifuged, extracted with cold ether, and neutralized as described above. The antigen extract was concentrated in a rotating vacuum evaporator and then dialyzed against 100 volumes of 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.4) at 5 C for 16 h. The final volume was 18 ml. Part of the dialyzed extract (10 ml) was applied to a column of diethylaminoethyl (DEAE)-Sephadex A-25 (1.5 by 30 cm). The column was washed with 60 ml of the equilibrating buffer (0.01 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.4) and developed with a linear gradient (0.05 to 1.0 M NaCl) in the same buffer. The fractions showing a positive precipitin test with anti-B13 serum were combined and lyophilized. After dialysis against 0.01 M $NaHCO_3$ (pH 7.4), the antigen was purified further on a column of CM-Sephadex C-25 (1.5 by 30 cm). The column was washed with 50 ml of the equilibrating buffer (0.01 M $NaHCO_3$, pH 7.4) and developed with a linear gradient (0.05 to 1.0 M NaCl) in this buffer. The fractions showing a positive precipitin reaction with anti-B13 serum were combined, dialyzed against water, and lyophilized. The antigen was dissolved in 5 ml of water and applied to a BioGel A-0.5m agarose column (2.5 by 60 cm). The antigen was eluted from the column with 0.01 M $NaHCO_3$ (pH 7.4), dialyzed against water, and lyophilized. The final yield of antigen from 15 g of cells was 170 mg.

Lability of antigen. Purified antigen (100 μg) was incubated in 0.01 M buffer (1 ml) at 37 C for 1 h in the presence of 10 μg of each of the following enzymes: glucose oxidase (pH 6), galactose oxidase (pH 6), β-galactosidase (pH 7, 0.01 M NaCl), β-glucosidase (pH 5), cellulase (pH 5), dextranase (pH 6), lysozyme (pH 8.1), and trypsin (pH 8.1, 2 mM $CaCl_2$). The buffers were sodium acetate, pH 5; sodium phosphate, pH 6 and 7; and tris(hydroxymethyl)aminomethane-hydrochloride, pH 8.1. Portions were removed and diluted 10-fold with saline prior to measuring the serological activity of the antigen by the quantitative precipitin assay.

Partial hydrolysis of the purified antigen (200 μg) was performed in 0.04 N HCl (100 μliters). Samples were incubated for 0, 1, 3, and 5 min at 100 C, rapidly cooled to 5 C, and neutralized with 0.04 N NaOH (100 μliters). Portions (10 μliters) were diluted further, and the serological activity of the antigen was determined by the quantitative precipitin assay. A second set of samples (100 μliters) was filtered on a column of Sephadex G-25 (0.9 by 30 cm) to fractionate the sugars released during hydrolysis from the unhydrolyzed antigen. These sugars were analyzed by gas-liquid chromatography.

Analytical methods. Total reducing sugar was measured by the phenol-sulfuric acid method (7). Sugars were converted to their methyl esters and quantitated by gas-liquid chromatography with dimethylformamide-hexamethyldisilazane-trimethylchlorosilane as the silation reagent (17). Protein was determined by the method of Lowry et al. (15) and total phosphorus was determined by the method of Bartlett (1).

Materials. Sephadex G-25, DEAE-Sephadex A-25, and CM-Sephadex C-25 were the products of Pharmacia Fine Chemicals, Inc., Piscataway, N.J. BioGel A-0.5m was obtained from Biorad Laboratories, Richmond, Calif.

β-Galactosidase, β-glucosidase, and dextranase were purchased from Worthington Biochemical Corp., Freehold, N.J. Glucose oxidase and galactose oxidase were obtained from Sigma Chemical Co., St. Louis, Mo. Cellulase was the product of Nutritional Biochemicals Corp., Cleveland, Ohio. Lysozyme and trypsin were purchased from Schwarz/Mann, Orangeburg, N.Y.

RESULTS

Specificity of anti-B13 serum. Serum prepared against cells of *S. mutans* strain B13 was assayed for group *d* specificity. The capillary precipitin reaction was used with acid extracts from 10 strains of *S. mutans* (Table 1). The serum reacted strongly with four group *d* strains

and gave a weak reaction with two group *a* strains. The anti-B13 serum did not react with extracts from group *b* or *c*.

The serum was adsorbed with HS6 (group *a*) cells to remove the cross-reacting *a-d* antibody. This procedure yielded an anti-*d* serum specific for group *d* in the capillary precipitin and agar diffusion tests (Table 1, Fig. 1). Agglutination of whole B13 cells by the anti-*d* serum occurred within 20 min. The anti-*a-d* globulin was released from the HS6 cells when the cells were incubated at pH 2 for 1 h at 5 C. The released globulin cross-reacted with groups *a* and *d* extracts in capillary precipitin tests (Table 1).

Extraction of antigen. Cells of *S. mutans* B13 were extracted by various procedures (see above and Fig. 2). Incubation in 5% trichloroacetic acid at 5 C for 16 h or incubation in 0.01 N HCl at 100 C for 20 min were the most efficient means of extraction. Incubation in 5% trichloroacetic acid at 100 C for 20 min appeared to destroy the antigen. Group *d* antigen was also extracted from purified cell walls (see Fig. 2e). Incubation with pepsin, trypsin, or lysozyme did not enhance the release of antigen from walls.

TABLE 1. *Specificity of anti-B13 serum*

S. mutans antigen		Specificity of:		
		Whole anti-B13 serum	Adsorbed serum[a]	
Group	Strain		Anti-*d*	Anti-*a-d*
a	HS6	2+	–	1+
	OMZ61	2+	–	
b	FA1	–	–	
	BHT	–	–	
c	GS5	–	–	
	NCTC 10449	–	–	
d	B13	4+	3+	2+
	OMZ176	4+	3+	
	KIR	3+	2+	
	6715	3+	2+	

[a] Whole anti-B13 serum was adsorbed with HS6 cells to obtain anti-*d* serum. Anti-*a-d* globulin was released from the cells by incubation at pH 2.

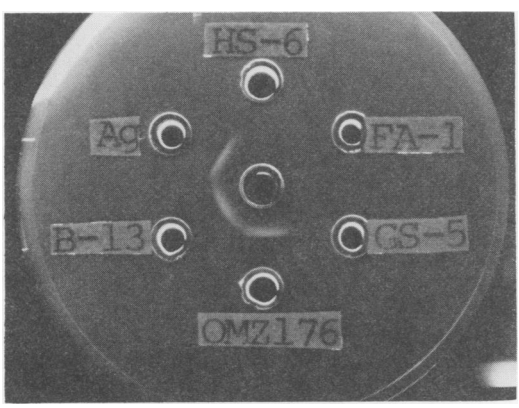

FIG. 1. *Specificity of anti-d serum on agar diffusion gel. Center well contained 40 μliters of anti-d serum. The outer wells contained 20 μliters of acid extracts from the respective S. mutans strains. Ag contained 10 μg of the purified antigen from B13.*

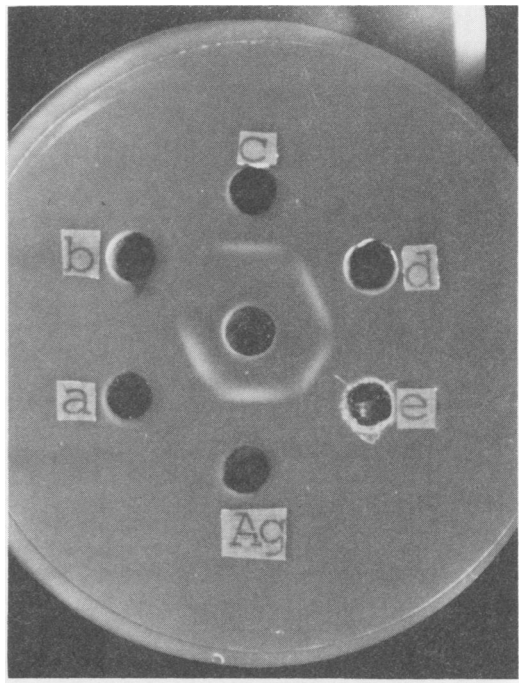

FIG. 2. *Extraction of antigen from whole cells and cell walls of B13. Center well contained 40 μliters of anti-d serum. Wells a through d contained extracts of whole B13 cells: (a) 0.01 N HCl at 100 C, (b) 5% trichloroacetic acid at 100 C, (c) 5% trichloroacetic acid at 5 C, and (d) water at 100 C. Well e contained an extract of cell walls by water at 100 C. Ag contained 10 μg of the purified antigen.*

Purification of antigen. A cold acid extract of B13 lyophilized cells was purified by column chromatography to permit further characterization of the group *d* antigen. The dialyzed extract was applied to a column of DEAE-Sephadex A-25 (Fig. 3). The fractions containing antigen (Fig. 3A) were identified by the capillary precipitin reaction. The antigen did not appear to be negatively charged because it was eluted from DEAE-Sephadex with the equilibrating buffer. The column fractions were also tested with anti-polyglycerol phosphate serum to locate glycerol teichoic acid. Precipitin-positive material was located at 230 ml (Fig. 3B). An agar diffusion experiment (Fig. 4)

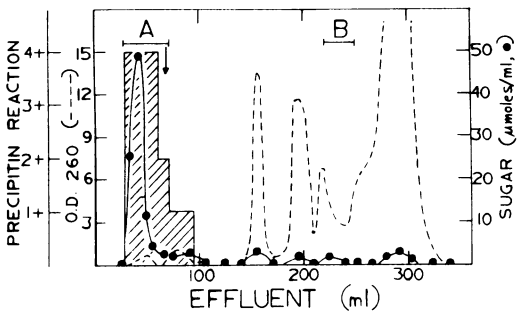

FIG. 3. *Chromatography of cold acid extract from B13 whole cells on DEAE-Sephadex. Dialyzed extract (10 ml) was applied to a column of DEAE-Sephadex (1.5 by 30 cm), and the column was rinsed with 0.01 M tris(hydroxymethyl)aminomethane-hydrocloride, pH 7.4 (equilibration buffer). Arrow, Column was developed with a linear gradient between 0.05 and 1.0 M NaCl in buffer. The precipitin reaction with anti-B13 serum was measured on a 100-fold dilution of the fractions. The B13 antigen was located in the shaded area, fraction A. The samples yielding a positive precipitin reaction with anti-PGP serum were designated fraction B.*

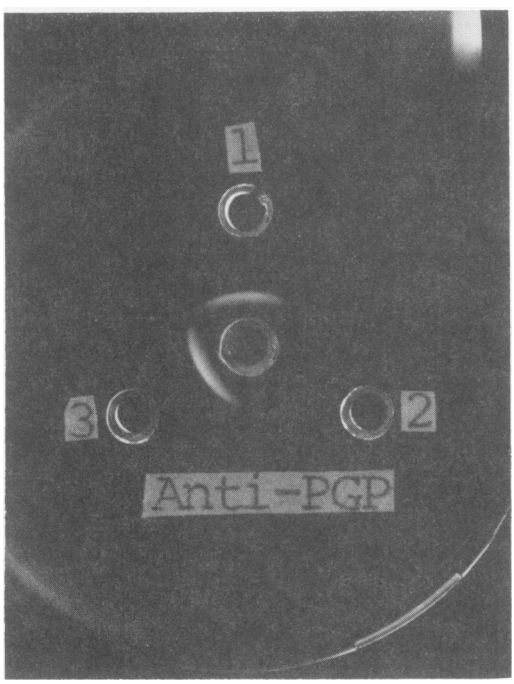

FIG. 4. *Reaction of anti-PGP serum with extract from B13. The center well contained anti-PGP serum (30 μliters). The outer wells contained: (1) cold acid extract, (2) purified B13 antigen (10 μg), and (3) fraction B from DEAE-Sephadex column (see Fig. 3).*

demonstrated that the material which reacted with anti-PGP was separated from the group *d* antigen on DEAE-Sephadex.

The antigenic material from the DEAE-Sephadex column was purified further on CM-Sephadex C-25 (Fig. 5). The antigen was eluted with the equilibrating buffer, as was the single carbohydrate peak. The fractions containing the antigen were dialyzed against water, lyophilized, and filtered on BioGel A-0.5m agarose (Fig. 6). The antigen was eluted as a single, broad carbohydrate peak.

Characterization of antigen. The chemical composition of the purified antigen is presented in Table 2. The antigen is a polysaccharide with a small amount of peptide (about 1.6%). Galactose and glucose comprise 95% of the total and are present in a 2:1 ratio. Rhamnose represents less than 1% of the dry weight. The absence of a significant quantity of phosphorus again shows the absence of teichoic acids in the preparation.

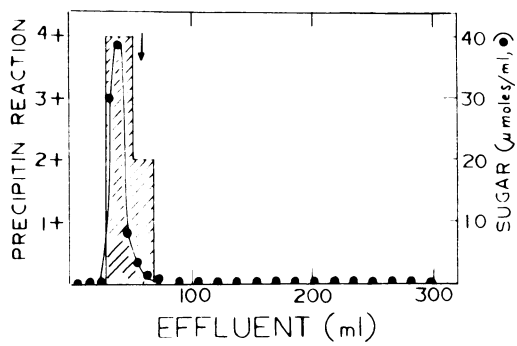

FIG. 5. *Chromatography of B13 antigen on CM-Sephadex. Antigenic material which had been partially purified on DEAE-Sephadex was applied to a CM-Sephadex column (1.5 by 30 cm). The column was rinsed with 0.01 M $NaHCO_3$ (equilibration buffer). Arrow, Column was eluted with a linear gradient between 0.05 and 1.0 M NaCl in buffer. The precipitin reaction with anti-B13 serum was measured on a 100-fold dilution of the fractions.*

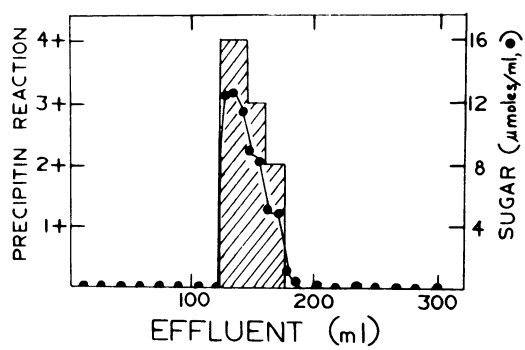

FIG. 6. *Chromatography of B13 antigen on BioGel A-0.5m. Antigenic material which had been purified on DEAE- and CM-Sephadex was applied to a BioGel A-0.5m column (2.5 by 60 cm). The column was eluted with 0.01 M $NaHCO_3$, pH 7.4. The precipitin reaction with anti-B13 serum was measured on a 100-fold dilution of the fractions.*

The purified antigen possessed both the *d* and the cross-reacting *a-d* antigenic sites. Agar diffusion and immunoelectrophoresis were used to determine whether these two sites were present in one molecule. On agar plates, the purified antigen showed one major band with whole anti-B13 serum, anti-*d* serum, and anti-HS6 serum. The latter possessed the *a-d* globulin (Fig. 7). Each of these bands demonstrated a reaction of identity with the bands produced by the other two sera.

The antigen moved toward the cathode (Fig. 8) during immunoelectrophoresis. Incubation of electrophoresed antigen with anti-B13, anti-*d*, and anti-HS6 sera resulted in the formation of single, identically positioned bands. Therefore, agar diffusion and immunoelectrophoresis data suggested that the *d* and *a-d* antigenic sites were located on the same molecule.

TABLE 2. *Chemical composition of B13 antigen*

Components	Amt (mg/100 mg)
Galactose	62
Glucose	33
Rhamnose	0.9
Phosphorus	0.3
Amino acids	1.6

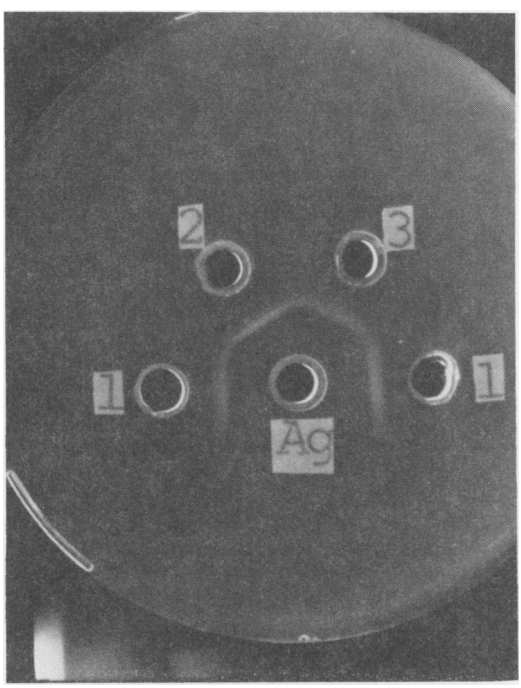

FIG. 7. *Reaction of purified B13 antigen with anti-d and anti-a-d globulins. The center well contained 10 μg of purified B13 antigen. The outer wells contained: (1) anti-B13 serum (30 μliters), (2) anti-HS6 serum (30 μliters), and (3) anti-d serum (36 μliters).*

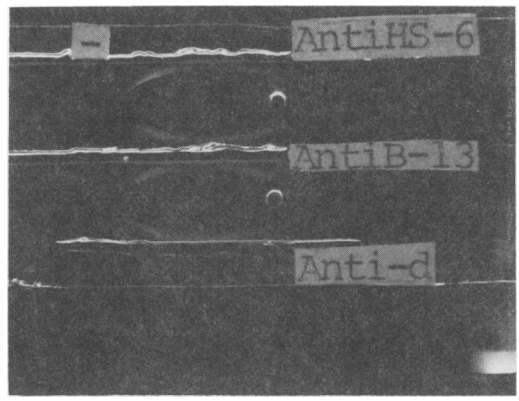

FIG. 8. *Immunoelectrophoresis of B13 antigen. Each well contained 20 μg of purified B13 antigen. The troughs contained the indicated antisera.*

A quantitative precipitin assay was performed to compare the amounts of *d* and *a-d* antibodies present in anti-B13 serum. Equivalent amounts of whole anti-B13 serum and anti-*d* serum were assayed with increasing amounts of antigen (Fig. 9). The comparison of the amounts of protein precipitated as the sera reached their equivalence points (17.0 and 11.8 μg, respectively) suggested that the antibodies specific for the *d* site comprised about 70% of the antibodies in the whole anti-B13 serum.

To characterize the specificity of the *d* site of the antigen, inhibition studies and inactivation studies were performed. Various sugars were incubated with anti-*d* serum for 1 h at 37 C prior to the quantitative precipitin assay (Table 3). The most significant effect was observed with galactose, which produced 31% inhibition. Lactose effected a 9% inhibition; glucose inhibited only 5%. Rhamnose, galactosamine, glucosamine, melibiose, cellulobiose, stachyose, and maltose inhibited less than 5%. Incubation of the antigen with galactose oxidase, glucose oxidase, β-galactosidase, β-glucosidase, cellulase, dextranase, lysozyme, or trypsin prior to the quantitative precipitin reaction did not decrease the serological activity of the antigen.

Partial hydrolysis of the antigen was performed to correlate loss of activity with release of specific sugars (Fig. 10). After 1 min at 100 C in 0.04 N HCl, there was a 30% loss in activity. Galactose was the only sugar released from the antigen during this period. Therefore, the hydrolysis data agree with the inhibition studies in suggesting that the serological specificity of the *d* antigen is dependent on a terminal galactose.

DISCUSSION

Antiserum prepared against *S. mutans* strain B13 (group *d*) demonstrated a moderate precip-

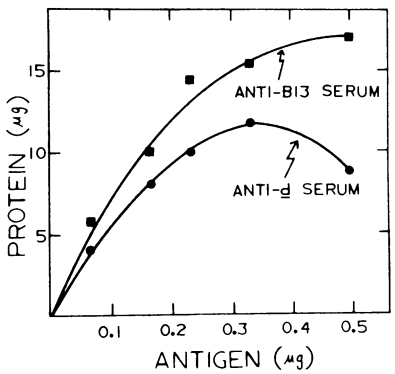

FIG. 9. *Quantitative precipitin curves with anti-B13 and anti-d sera. Equivalent amounts of anti-B13 (10 μliters) and anti-d (12 μliters) sera were reacted with increasing concentrations of group d antigen plus saline to a final volume of 50 μliters.*

TABLE 3. *Inhibition of the precipitin reaction between B13 antigen and anti-d serum*[a]

Inhibitor	Inhibition (%)
Galactose	31
Glucose	5
Rhamnose	0
Lactose	9
Melbiose	4

[a] Anti-*d* serum (12 μliters) was incubated with 500 μg of the inhibitor for 1 h at 37 C prior to the addition of the purified B13 antigen (0.25 μg).

itin reaction with two group *a* strains (Table 1). This cross-reaction was similar to that observed by Bratthall in his serological classification of *S. mutans* (3, 4). Serum specific for group *d* was obtained by adsorbing anti-B13 serum with HS6 (group *a*) cells (Table 1, Fig. 1). The use of adsorbed serum has proved essential for the classification of group *d* strains of *S. mutans*, as well as in the classification of group *a* strains.

Antigen was extracted from whole cells and purified cell walls of strain B13 by cold trichloroacetic acid, hot water, or hot dilute acid (Fig. 2). A cold acid extract of the cells was purified by DEAE-Sephadex, CM-Sephadex, and BioGel A-0.5m chromatography (Fig. 3, 5, 6). The purified antigen was a polysaccharide composed of galactose and glucose (95%) in a 2:1 ratio (Table 2).

Crude extracts of the cells contained glycerol teichoic acid (Fig. 4) as did crude extracts of group *a* (19), group *b* (2), and group *c* cells (unpublished observations). Glycerol teichoic acid was resolved from the polysaccharide antigen during DEAE-Sephadex chromatography. The teichoic acid did not contain any group-specific immunological activity (Fig. 3). Recently, it was suggested that a glycosyl-substituted glycerol teichoic acid was responsible for the specificity of the group *b* antigen (29). However, Bratthall has confirmed that the group *b* polysaccharide purified in this laboratory (18) was serologically identical to his group *b* antigen (personal communication).

The purified B13 polysaccharide antigen possessed two serologically distinct sites. One site reacted with anti-*d* serum from which the *a-d* antibodies had been absorbed with HS6 (group *a*) cells. The second site was specific for the cross-reacting anti-*a-d* globulin released by acid from the HS6 cells used to adsorb whole anti-B13 serum (Table 1). Agar diffusion and immunoelectrophoresis studies suggested that these two antigenic sites were present on one molecule (Fig. 7 and 8). These findings were similar to those from studies of the *S. mutans* group *a* antigen (19). The group *a* antigen was characterized as a polysaccharide containing two serologically distinct sites on one molecule. One site was specific for anti-*a* serum and the second site reacted with anti-*a-d* globulin prepared from whole anti-HS6 serum. The B13 polysaccharide antigen reacted with the anti-*a-d* globulin prepared from whole anti-HS6 serum but did not react with anti-*a* serum.

The specificity of the group *d* antigen appeared to be dependent on a terminal galactose. In a partial hydrolysis experiment (Fig. 10), 30% of the group *d* serological activity was lost after 1 min at 100 C. During this period, galactose

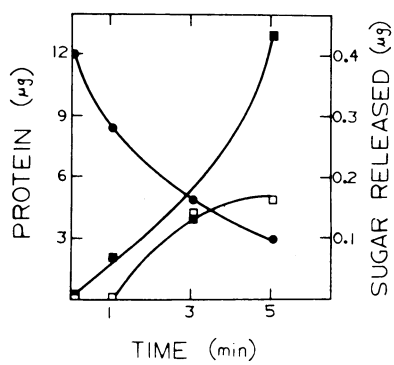

FIG. 10. *Partial hydrolysis of B13 antigen. Samples of B13 antigen (200 μg) were incubated in 0.04 N HCl at 100 C for the indicated times. After neutralization with 0.04 N NaOH, fractions (0.3 μg) of the samples were tested for serological activity by the quantitative precipitin assay with anti-d serum (12 μliters) (●). A second set of fractions (100 μg) were fractionated on Sephadex G-25, and the released mono- and oligosaccharides were characterized by gas-liquid chromatography (■, galactose; □, glucose).*

was the only sugar released from the antigen. In inhibition studies (Table 3), galactose inhibited 31% of the reaction with anti-*d* serum; glucose effected only 5% inhibition. Studies with di- and tetrasaccharides possessing terminal α-galactose showed less inhibition than free galactose. This suggested that group *d* specificity depended on a terminal β-galactose.

Bratthall has confirmed that the group *a* and *b* antigens characterized in this laboratory (18, 19) were serologically identical to the group antigens used in his laboratory for the classification of *S. mutans* (3, 4) (personal communication). These antigens were characterized as polysaccharides located in or on the cell wall (18, 19). This report has shown that the group *d* antigen is also a polysaccharide associated with the cell wall. The streptococcal group E antigen, which one group of *S. mutans* strains possess, was described as a polysaccharide located in or on the bacterial wall (27). The serological specificities of these antigens were dependent on different terminal groups: in group E, D-glucose-L-rhamnose-L-rhamnose (27); in group *a*, D-glucose-D-glucose (19); in group *b*, D-galactosamine-D-galactose (18); and in group *d*, D-galactose. Specificity of the *a-d* antigen site also appeared dependent on a D-galactose (19). The *a-d* site may differ from the *d* site in the configuration of the galactose or in the structure of the penultimate sugar.

During the course of this work, an abstract appeared describing the isolation of the group *d* antigen from *S. mutans* strain 6715 (V. J. Iacono, M. A. Taubman, D. J. Smith, and S. R. Abromson, Int. Ass. Dent. Res., p. 197, 1974). Although the composition was not detailed, the antigen was described as a carbohydrate with little or no phosphorus. These results correspond to the data presented here characterizing the group antigen from *S. mutans* strain B13.

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