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Isolation and Immunochemical Characterization of the Group-Specific Antigen of *Streptococcus mutans* 6715

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The group *d* antigen of *Streptococcus mutans* 6715 was isolated from a buffer (pH 7.3)-boiled extract of whole cells and analyzed immunochemically. Rabbits immunized in three different fashions with whole *S. mutans* 6715 each responded to the same antigenic cell surface component. This presumptive major antigen was found in culture supernatant, sonically treated supernatant, acid and buffer extracts of whole cells, and trichloroacetic acid extract of cell membranes. A crude preparation of this antigen could completely inhibit antibody-mediated cell (*S. mutans* 6715) agglutination in a spectrophotometric analysis. The antigen was purified from buffer-boiled extracts by gel filtration on columns of Sepharose 4B. The antigen did not migrate to the anode on electrophoresis nor did it contain appreciable quantities of phosphorus, glycerol, or ribitol. This suggested that the *d* antigenicity did not reside in a teichoic acid. The *d* antigen contained galactose and glucose as the sole saccharides, in a ratio of 5.9:1.0. Protein (9.5%) appeared to be a portion of the antigen, although Pronase-digested antigen retained the same electrophoretic mobility and could precipitate virtually all (98.6%) purified antibody directed to the intact antigen. The data obtained from hapten inhibition studies strongly indicated that the immunodominant region of the *d* antigen was primarily dependent upon galactose. The β-1-linkage of this galactose might also be involved. Glucose also contributed to the immunodominant region. Antibody directed to the *d* antigen may be of importance in the inhibition of adherence phenomena manifested by *S. mutans* organisms of the *d* group.

Streptococcus mutans, an etiological agent in the development of experimental dental caries (14, 52), has been characterized as a group of bacteria which, with rare exception, do not cross-react with other Lancefield-categorized groups of streptococci (5). They produce lactate from glucose (42), ferment mannitol and sorbitol (11), and synthesize extracellular polysaccharides from sucrose (18). The pathogenicity of *S. mutans* depends upon its ability to demineralize the tooth surface by the production of acids (9, 22). Adherence to hard surfaces (16, 19) appears to be a requirement for the expression of this aspect of the organism's pathogenicity. This adherence, in turn, is dependent upon the ability to synthesize dextran-like insoluble glucose polymers (glucans). Gibbons and Nygaard (16) demonstrated that restricting development of these extracellular polymers resulted in diminished plaque formation by *S. mutans*. Furthermore, strains lacking the ability to synthesize glucan were not cariogenic (8). Therefore, a primary factor in the expression of the pathogenic potential of *S. mutans* lies in its ability to participate in the formation of dental plaques through the synthesis of insoluble glucan.

Some of the cell surface components of *S. mutans* have been identified. Cariogenic streptococci have been found to lack both outer capsules of hyaluronic acid and type-specific protein antigens (M, T, and R) which are characteristic of Lancefield group A microorganisms (1). However, they possess complex cell wall polysaccharides (1). Since the Lancefield-group antigenicity of many streptococci depends upon carbohydrate cell surface components (24, 30), these structures may be important in the classification of *S. mutans*.

Most cariogenic streptococci have been classified into distinct non-Lancefield serological groups by comparative immunoelectrophoresis of acid extracts of whole cells (3). Five groups (*a* to *e*) of *S. mutans* were defined on the basis of the presence of five distinct antigens. Extracts

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of four groups (*a* to *d*) did not cross-react with any known serologic groups of streptococci, whereas extracts of group *e* were found to cross-react with Lancefield group E microorganisms. The existence of these distinct serological groups has been confirmed by deoxyribonucleic acid-deoxyribonucleic acid reassociation experiments (7). However, only the cell surface antigens which characterize the *a* and *b* groups have been isolated.

In light of the importance of the cell surface components of *S. mutans* with respect to its pathogenic potential and classification, this investigation deals with the isolation, purification, immunological properties, and chemical characterization of the antigen bearing the determinants which distinguish *S. mutans* strain 6715 as serological group *d*. (Portions of this study were presented at the 52nd General Session of the International Association for Dental Research, 21 to 24 March 1974, Atlanta, Ga.)

MATERIALS AND METHODS

Preparation of bacterial cells. *S. mutans* 6715 was routinely grown in Trypticase soy broth (BBL) and harvested as previously described (43). These cells, termed *S. mutans* 6715 (TSB), were used for preliminary antigen extraction procedures and trichloroacetic acid extraction of cell membranes. Certain of these preparations were formalin-killed (47) and used both for the adsorption of antibody from immune sera and for a spectrophotometric analysis. *S. mutans* 6715 (Fil. BHI) was grown in prefiltered (Diaflow UM-10, Amicon) brain heart infusion broth (Difco) which had been supplemented with 0.25% glucose and prepared as above. *S. mutans* 6715 (BHI) to be used for the isolation and purification of the group *d* antigen was grown in unfiltered brain heart infusion broth supplemented with 0.25% glucose and harvested in a similar fashion. The concentration of cell preparations was determined at 580 nm. *S. mutans* strain B13, a group *d* strain, was kindly provided by Harold Jordan.

Preparation of antisera. Male New Zealand white rabbits were immunized by different procedures to determine the immunogenicity of the cell surface components of *S. mutans* 6715. One rabbit (F13) was injected with 10^{10} formalin-killed *S. mutans* 6715 (TSB) in 1 ml of phosphate-buffered saline (PBS) (0.02 M phosphate, 0.15 M NaCl, pH 7.5) and 1 ml of complete Freund adjuvant (Difco) into each of the four foot pads and subcutaneously into the dorsum of the neck. This regimen was repeated 3 weeks after the first injection. The rabbit was bled once, 3 weeks after the first injection, and twice at weekly intervals after the second injection; it was exsanguinated 3 weeks after the second injection.

A second rabbit (F15) was injected intravenously (marginal ear vein) with formalin-killed *S. mutans* 6715 (TSB) in 0.5 ml of PBS: days 1, 2, and 5, 5×10^8 cells; day 6, 10^9 cells; day 8, 5×10^9 cells. Two weeks later the rabbit was injected three times: day 23, 5×10^9 cells; day 26, 2.5×10^{10} cells; day 27, 5×10^{10} cells. After 1 month, the rabbit was injected two times at weekly intervals with 10^9 cells. The rabbit was bled 1 week after each series of injections and exsanguinated 1 week after the last injection.

The third rabbit (F22) was injected with 10^{10} formalin-killed *S. mutans* 6715 (Fil. BHI) in 1 ml of PBS and 1 ml of complete Freund adjuvant into each of the foot pads, and subcutaneously on day 1 of weeks 1, 4, 7, 12, 20. Two weeks later, the rabbit was injected with 2×10^{10} cells in 2 ml of PBS into the foot pads, intramuscularly, and subcutaneously. The rabbit was bled at weeks 4, 6, 7, 9, 19, 22, and 23 and was exsanguinated 1 week after the last injection. All sera were tested in gel diffusion analyses for antibody activity. Sera that reacted similarly were pooled.

Rabbit anti-*S. mutans* 6715 serum (H.J.), obtained from rabbits immunized with heat-killed *S. mutans* cells, was generously provided by Harold Jordan.

Rabbit anti-*S. mutans* strain AHT (group *a*) sera and rabbit anti-*S. mutans* strain B13 (group *d*) sera, obtained from rabbits immunized with heat-killed whole cells (3), were the generous gifts of Douglas Bratthall. The preparation of goat antiserum directed to normal rabbit serum has been previously described, as have the goat antisera to rabbit immunoglobulin (Ig)G and IgM (44). Normal rabbit serum was obtained from rabbits prior to any immunization procedures.

Isolation of antibody directed to S. mutans cell surface components. Anti-dextran antibody was adsorbed from immune rabbit sera (F13, F15, F22) by incubation (1 h at 22 C, 16 h at 4 C) with prewashed (0.1 M acetate buffer) Sephadex G-25 (25 mg/ml of sera). After centrifugation, *S. mutans* antibody was adsorbed from the supernatant onto prewashed (0.1 M acetate buffer) formalin-killed *S. mutans* 6715 cells (10^{10} cells per ml of original sera). After incubation (1 h at 22 C, 16 h at 4 C), the centrifuged cells were washed with PBS until absorbance at 280 nm was below 0.016. After incubation of the washed cells with 0.1 M acetate (pH 3.8) for 1.5 h at 22 C, the suspension was centrifuged and the supernatant was neutralized with 1 N NaOH. The antibody-containing eluate was then concentrated by negative pressure ultrafiltration and dialyzed against PBS. When tested in gel diffusion against *S. mutans* 6715 sonic extract and culture supernatant, each eluate (F13, F15, F22) detected only the same, single antigenic component. A single precipitin band with gamma mobility was formed after each antibody preparation was reacted with goat anti-rabbit serum in immunoelectrophoresis. These preparations were IgG as confirmed with a monospecific antiserum. Both IgM and IgG were present in a pool of F22 and F15 (final bleeding) antibody and were separated by gel filtration on Sephadex G-200. The resulting IgG antibody pool will be referred to as F15-22 antibody. No bacterial cell components were detected in any antibody preparation as indicated by the lack of reaction with the respective anti-whole cell serum in gel diffusion. Anti-dextran antibody was not detected in the puri-

fied preparations. Antibody protein concentration was determined by absorbance at 280 nm in PBS using $E^{1\%}_{1cm}$ = 15 (26).

Spectrophotometric analysis. The addition of antiserum to *S. mutans* cells in the spectrophotometer resulted in an initial rise in optical density which was linearly related to the concentration of antibody (20). This phenomenon was caused by the agglutination of cells by antibody (unpublished observations). The actual analysis consisted of the addition of 100 μliters of antibody-containing solution to 1 ml of 10^8 formalin-killed *S. mutans* 6715 (TSB) (optical density at 530 nm = 0.320). After the suspensions were mixed for 1 min, the optical density at 530 nm was monitored at various times up to 45 min. This analysis could detect less than 7.6 μg of antibody contained in a 100-μliter sample.

Preparation of crude antigen (CA), acid extract, buffer extract, and trichloroacetic acid extract of cell membranes. Five milliliters of 100-fold concentrated culture supernatant from *S. mutans* 6715 (Fil. BHI) were filtered on a column of Sepharose 6B. Fractions demonstrating antigenic activity in gel diffusion (pooled F13 and F15 sera) eluted just after the void volume peak of optical density (230 nm). These fractions were pooled, concentrated, and used as the CA preparation.

An acid extract was prepared according to the procedure of Lancefield (25) as modified by Van de Rijn and Bleiweis (49). Washed *S. mutans* 6715 (Fil. BHI) (2.25×10^{11} cells per 250 ml) were suspended in 2 ml of 0.15 M NaCl, and 1 N HCl was added until a final pH of 2.5 was reached. The suspension was boiled for 10 min, cooled in an ice bath, and centrifuged, and the supernatant was neutralized with 1 N NaOH.

Antigenic extracts of *S. mutans* 6715 (Fil. BHI) (2.25×10^{11} cells per 250 ml) were also prepared by boiling (10 min, 1 h) in 0.067 M, pH 7.3 potassium phosphate buffer (PB) (39). The same procedure was followed to obtain an antigenic extract of *S. mutans* strain B13.

A trichloroacetic acid extract of cell membranes was obtained by a modification of the method of Wicken and Knox (51) as described by Van de Rijn and Bleiweis (49). Twenty-eight grams (wet weight) of *S. mutans* 6715 (TSB) was disrupted by sonic treatment. The suspensions were filtered on a sintered glass filter, washed with 40 ml of PBS, and centrifuged (27,000 × *g*). The supernatant was dialyzed against distilled water, further centrifuged (78,000 × *g*, 2 h) (Beckman L2-658), and lyophilized. The dried material (864.3 mg) was stirred with 14.4 ml of ice cold 10% trichloroacetic acid for 24 h at 4 C, and the precipitate was collected by centrifugation (20,000 × *g*). The extract was shaken with 3 volumes of ether to remove acid from the aqueous fractions which were then dialyzed against distilled water and lyophilized.

Buffer-boiled extract for purification of d antigen. The isolation of the *d* antigen for purification was performed by a modification of the methods described by Prytz and Jablon (39) and Mukasa and Slade (35). Whole cells from cultures (40 liters) of *S. mutans* 6715 (BHI) in 8 ml of PBS per liter of culture were placed in a boiling-water bath for 1 h, cooled in an ice bath, and centrifuged (16,300 × *g*). After dialysis against distilled water and lyophilization, the dried extracts (915 mg) were brought up to 100 ml (50 ml of distilled water, 50 ml of 95% ethanol) and incubated at 4 C for 16 h. The ethanol-insoluble fractions were dissolved in a total of 9 ml of distilled water and dialyzed against 100 volumes of 0.1 M $(NH_4)_2CO_3$ (pH 6.9) for 12 h at 4 C. The ethanol-soluble fractions were dialyzed exhaustively against distilled water at 4 C and lyophilized.

Radial immunodiffusion analysis. Radial immunodiffusion analysis was carried out by a modification of the method of Mancini et al. (31). Microscope slides were coated with 1.2% agarose (Seakem Brand, Bausch and Lomb) into which was added rabbit anti-*S. mutans* 6715 serum (H.J.) to a final dilution of 1 part serum to 239 parts 1.2% agarose.

Quantitative precipitin assay. Standard quantitative precipitin reactions were carried out by a modification of the method of McCarty and Lancefield (30). Immune serum or antibody (F15-22) was added to a range of concentrations of antigen or Pronase-treated *d* antigen (to be described) in PBS (final volume = 200 μliters) and incubated at 37 C for 1 h and overnight at 4 C. The mixtures were centrifuged (1,500 rpm), washed three times with PBS, and air-dried. This material was dissolved in 1 ml of 0.1 N NaOH and the optical density of the solutions was determined at 287 nm.

Quantitative precipitin inhibition assay. Preliminary assays with a wide range of concentrations (1 μmol to 100 μmol) indicated that 100 μmol of each compound demonstrated substantial inhibition. Compounds (100 μM) were incubated with 250 μg of purified antibody at 37 C for 30 min. Antigen, at a concentration (22.5 μg) which precipitated the maximal amount of antibody, was then added to the reaction mixture and incubated at 37 C for 1 h, and overnight at 4 C. The precipitates were treated as described above.

The saccharides tested in the inhibition assay were: D-(+)-galactose (Matheson, Coleman and Bell); α-methyl galactopyranoside and β-methyl galactopyranoside (Pfanstiehl); D-(−)-glucose, L-(+)-rhamnose and D-(+)-trehalose (Baker); cellobiose, α-, and β-methyl glucopyranoside (Calbiochem); D-(−)-ribose and D-(+)-maltose (Eastman); lactose (Fisher); sucrose (Mallinckrodt); D-(+)-melibiose (Nutritional Biochemicals); stachyose and β-gentiobiose (Sigma). D-(+)-galactosamine and D-(+)-glucosamine (Sigma) were also tested.

Analytical methods. Total hexose was determined by either the phenol-H_2SO_4 assay (10) using dextran as a standard or by the anthrone reaction (40) using glucose-galactose (1:6) as standards. Individual neutral sugars were identified and quantitated by means of the Technicon automated borate-complex anion exchange chromatography system (Technicon Chromatography Brochure, 1966, Technicon Chromatography Corp.). Neutral sugars for quantitation on the Technicon system were prepared as follows: 1 to 2 mg of material were hydrolyzed in 1.6 ml of 1 N HCl at 100 C for 1 h. The neutral sugar fraction was obtained

by passing the dilute hydrolysate through coupled columns of Dowex 50-X4 (Biorad), 200 to 400 mesh (H^+ form), and Dowex 1-X8 (Biorad), 200 to 400 mesh (formate form). The columns were then washed with distilled water equal to 4 to 5 column volumes. The effluent and wash from these columns containing the neutral sugars were lyophilized.

Amino sugars were assayed by the Boas (2) modification of the Elson-Morgan reaction after elution from the aforementioned Dowex 50-X4 column with 2 N HCl equal to 3 to 4 column volumes.

The presence of ribitol and glycerol in the neutral sugar fractions was determined by descending paper chromatography on Whatman no. 1 paper using the system of Fischer and Nebel (13). After 22 h, the chromatograms were stained by the Ag_2NO_3-NaOH method (48). Glucose was also quantitated by the use of a Glucostat reagent (Worthington Biochemical Corp.).

Total protein was analyzed both by the method of Lowry and co-workers (29), using bovine serum albumin as standard, and by amino acid analysis (Technicon Development Bulletin no. 124, 1968, Technicon Chromatography Corp.). For determination of amino acids, samples hydrolyzed in constant boiling HCl in sealed tubes under nitrogen for 28 h at 105 C were applied to a Technicon amino acid analyzer.

Total phosphorus was determined by the method of Lowry and co-workers (28), as modified by Mukasa and Slade (33).

Pronase treatment of the d antigen. Two microliters of a Pronase (B grade, 45,000 proteolytic P.K.U./g, Calbiochem) solution at a concentration of 0.5 mg/ml was added to antigen (40 μliters, 5.0 mg/ml), both in 0.1 M tris(hydroxymethyl)aminomethane-acetate buffer (pH 7.8) with 0.005 M calcium acetate, and incubated for 8 h at 37 C. Controls consisted of (i) the same amount of antigen and buffer (2 μliters) (buffer digest) and (ii) the same amounts of antigen and Pronase boiled for 3 min prior to incubation (boiled Pronase digest). After incubation, the solutions were placed in a boiling water bath for 3 min to inactivate any enzyme activity. Enzymatic activity of the Pronase was confirmed by following the course of proteolysis using the ninhydrin reaction with leucine as a standard (32).

RESULTS

Antigen identification. It had been reported that the group *a* antigen of *S. mutans* strain AHT and *S. mutans* strain HS6 could be obtained by several methods of extraction (35, 49). To determine whether the group *d* antigen could also be obtained by these methods, which included acid extraction (pH 2.5), 10-min and 1 h buffer extractions (pH 7.3) of whole cells, and trichloroacetic acid extraction of cell membranes (trichloroacetic acid extract), these procedures were performed on *S. mutans* 6715. The extracts were then compared with the CA isolation from culture supernatant, by immunoelectrophoretic analyses with antiserum directed to *S. mutans* (Fig. 1). This serum detected at least four components in the buffer extracts. The same predominant cathodally migrating precipitin band seen in the CA was present in each of the buffer extracts and trichloroacetic acid extract. This suggested that the major antigen in the CA was also the major antigen in each of the extracts, and that it had a net positive charge at the pH of electrophoresis (pH 8.6).

To determine whether the antigen present in the trichloroacetic acid and buffer extracts was responsible for group *d* antigenicity, the preparations were examined by immunoelectrophoresis and gel diffusion (Fig. 2) using antisera to group *d* (strain B13) as well as antisera to group *a* (strain AHT), which has been shown to cross-react with extracts of group *d* strains (4, 35, 49). Immunoelectrophoretic analysis of extracts against anti-B13 revealed a single cathodally migrating precipitin band emphasizing that the antigen in these preparations was group specific rather than strain specific. Gel diffusion also substantiated the group specificity of this antigen. However, immunoelectrophoresis and gel diffusion (Fig. 2) also showed that the antigen reacted with an anti-*a* group antiserum (AHT) indicating the additional presence of a group *a* to *d* determinant in the trichloroacetic acid and buffer extracts. This was confirmed by absorbing an anti-*d* group antiserum with AHT cells. The absorbed antiserum gave no reaction in gel diffusion with a buffer extract of AHT cells, although reaction remained with extracts of strains 6715 and B13. These results show that the trichloroacetic acid and buffer extracts of strain 6715 contained the group antigen, having both *d* and *a-d* specificities.

A spectrophotometric analysis was used to further confirm that the antigen, which appeared to be the *d* antigen, was the major antigen of *S. mutans* 6715. If all agglutinating antibody in the immune serum was directed to the group *d* antigen, then this antigen should remove all such antibody. A quantitative precipitin reaction was performed using uniform concentrations of CA. Supernatants from reactions in antibody excess, equivalence, and moderate antigen excess were chosen for analysis of residual antibody activity using the spectrophotometric technique described above (Fig. 3). CA at a concentration of 0.045 optical density units removed all detectable antibody activity from the immune serum. Complete removal was indicated by the lack of rise in optical density when equivalence supernatant was reacted with

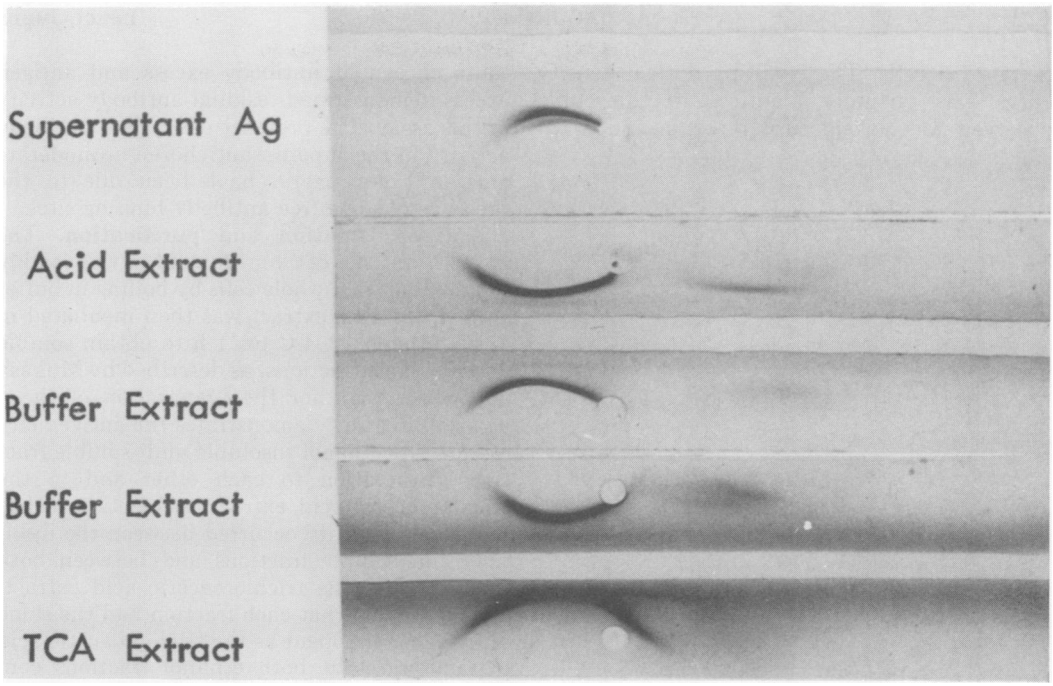

FIG. 1. *Immunoelectrophoretic analyses demonstrating the presence of the same major antigenic component in the CA and in several antigenic extracts of S. mutans 6715. The cathode is to the left. The troughs contained the same rabbit immune serum directed to whole S. mutans 6715 (pooled F13 and F15). From top to bottom, the wells contained CA (supernatant Ag), acid extract, 10-min buffer extract, 1-h buffer extract, and the trichloroacetic acid extract.*

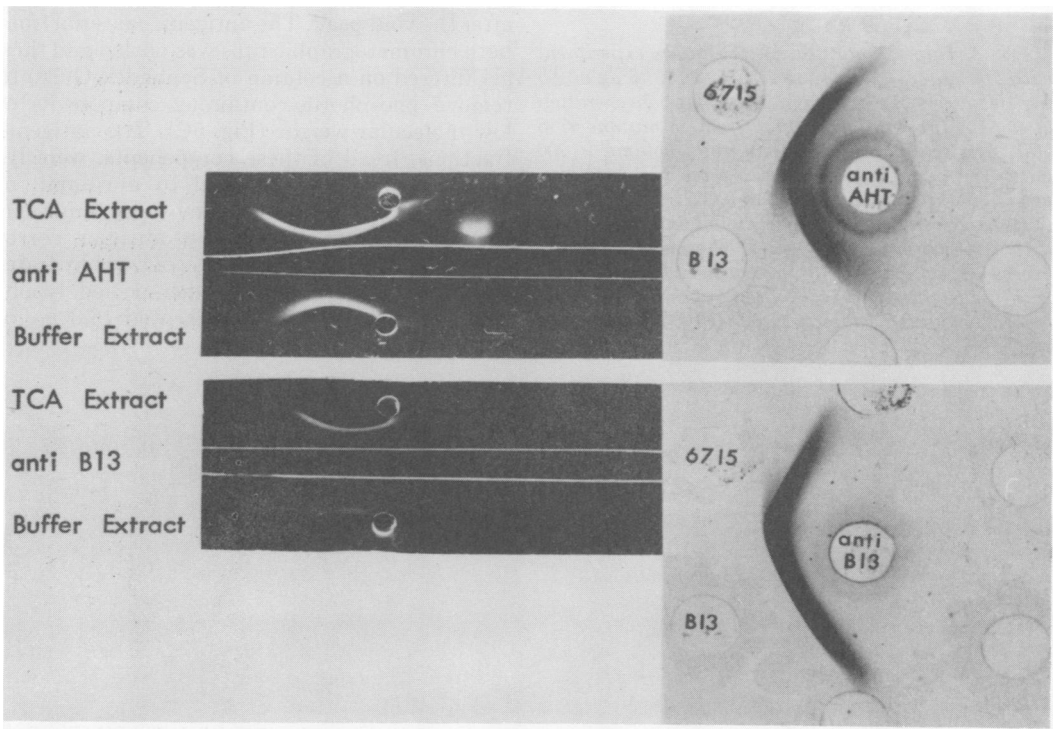

FIG. 2. *Immunoelectrophoretic and gel diffusion analyses demonstrating the presence of the group d antigen in antigenic extracts of S. mutans 6715. Left: in the immunoelectrophoretic patterns, the wells contained extracts of S. mutans 6715, as indicated. The upper and lower troughs contained anti-AHT and anti-B13 sera, respectively. The cathode is to the left. Right: in the gel diffusion patterns, the peripheral wells contained buffer extracts of the S. mutans strains indicated. The central wells contained anti-B13 or anti-AHT sera.*

S. mutans cells. The resulting optical density curve was virtually identical to the curve observed for normal rabbit serum. Supernatants chosen at antibody excess and antigen excess demonstrated residual antibody activity in this assay. The presence of residual antibody activity in the supernatant chosen at moderate antigen excess could have been due to the presence of some free antibody binding sites.

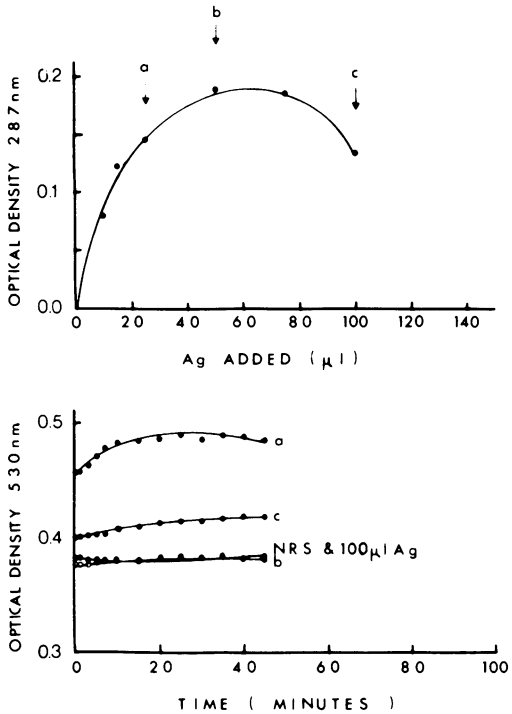

FIG. 3. *Upper: quantitative precipitin experiment with 100 μliters of immune serum (F15, 46 μg of Ab per 100 μliters of serum) and increasing concentration of the CA preparation isolated from S. mutans 6715 (Fil. BHI) culture supernatant (total volume = 200 μliters). CA concentration is expressed in microliters, where 50 μliters is equivalent to 0.045 optical density units at 280 nm. Lower: spectrophotometric analysis with supernatants (100 μliters) chosen at antibody excess (a), equivalence (b), and antigen excess (c) and with the supernatant (100 μliters) obtained after the reaction of normal rabbit serum and 100 μliters of CA.*

Antigen isolation and purification. The group *d* antigen of *S. mutans* 6715 was readily extractable from whole cells by boiling in buffer (Fig. 1, 2). This extract was then incubated in 47.5% ethanol at 4 C for 1 h to obtain soluble and insoluble fractions, as described by Mukasa and Slade (35), for the preparation of the *a* antigen. Figure 4 demonstrates the antigenicity of both the ethanol-insoluble and -soluble fractions in relation to each other and to the trichloroacetic acid extract. A reaction of immunologic identity occurred between the insoluble and soluble fractions and between both fractions and the trichloroacetic acid extract. This indicated that each fraction had the same antigenic component as the trichloroacetic acid extract and that both ethanol fractions contained the same antigen. Therefore, the ethanol-insoluble and -soluble fractions were treated identically and were individually filtered on columns of Sepharose 6B (Fig. 5A and B). In both cases, radial immunodiffusion analyses indicated that the antigenicity eluted just after the void peak. The antigenic material from both chromatographic runs was pooled and then gel filtered on a column of Sephadex G-200 to remove phosphorus-containing components of low molecular weight (Fig. 5C). The antigenic fractions, freed of these components, were lyophilized and then applied to a column of Sepharose 4B (Fig. 5D) to remove high-molecular-weight impurities. The antigen corresponded to the elution position of carbohydrate-containing material which, when pooled, lyophilized, and desiccated, represented 16.8 mg of

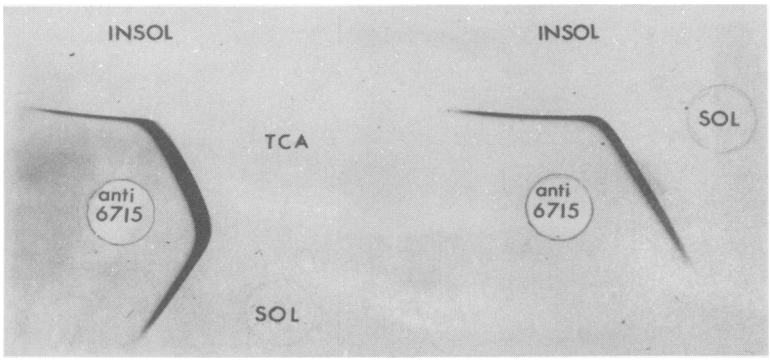

FIG. 4. *Gel diffusion analysis demonstrating the presence of the same antigenic component in the ethanol-insoluble (INSOL) and -soluble (SOL) fractions and the trichloroacetic acid extract. The center well in each pattern contained the same anti-S. mutans 6715 serum (H.J.).*

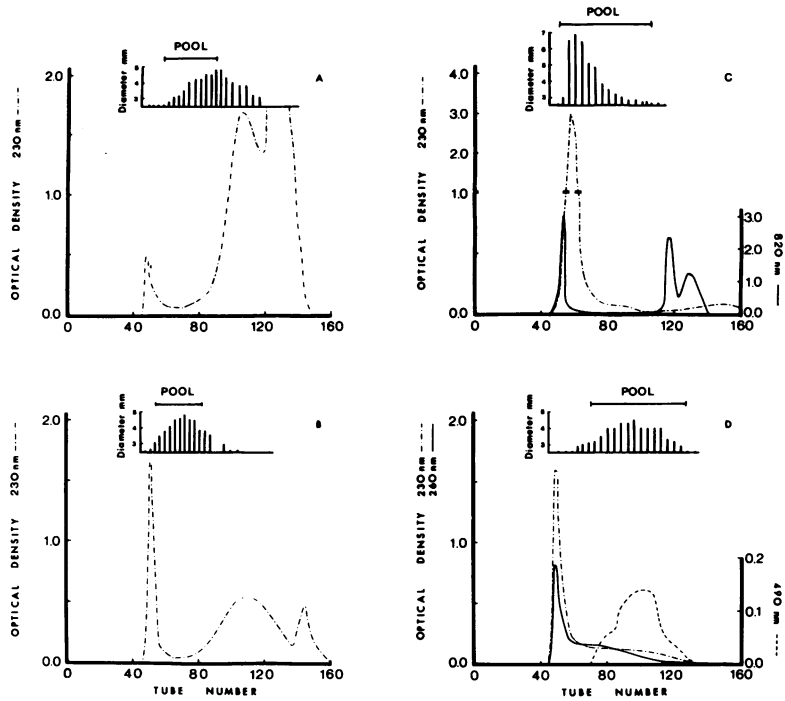

FIG. 5. *(A) Gel filtration of a 47.5% ethanol-soluble fraction of the 1-h buffer extract of S. mutans 6715 (BHI). The sample (70 mg) was applied to a column (2.5 by 90 cm) of 6% agarose and eluted with 0.1 M $(NH_4)_2CO_3$ (pH 6.9) at 4 C. Fractions (3.4 ml) were collected at a rate of 15.6 ml/h. The relative amount of antigen in each fraction, as determined by radial immunodiffusion with anti-S. mutans 6715 serum (H.J.), is indicated by the diameter (mm) of the precipitin ring for each fraction. Tubes 58 to 90 were pooled, dialyzed exhaustively against distilled water and lyophilized (5.6 mg). (B) Gel filtration of a 47.5% ethanol-insoluble fraction of the 1-h buffer extract of S. mutans 6715 (BHI). The sample (129 mg) was applied to a column (2.5 by 90 cm) of 6% agarose and eluted with 0.1 M $(NH_4)_2CO_3$ (pH 6.9) at 4 C. Fractions (3.4 ml) were collected at a rate of 20 ml/h and the relative amounts of antigen in each fraction tested is indicated. Tubes 54 to 82 were pooled, dialyzed, and lyophilized (8 mg). (C) Gel filtration of the total combined 47.5% ethanol-soluble and -insoluble fractions after gel filtration on agarose. The sample (43 mg) was applied to a column (2.5 by 90 cm) of Sephadex G-200 and eluted with 0.1 M $(NH_4)_2CO_3$ (pH 6.9) at 4 C. Fractions (3.4 ml) were collected at a rate of 16.8 ml/h and the relative amount of antigen in each fraction tested is indicated. Tubes 51 to 106 were pooled, dialyzed, and lyophilized (37 mg). (D) Gel filtration of the d antigen on Sepharose 4B. The sample (37 mg) was applied to a column (2.5 by 90 cm) of 4% agarose and eluted with 0.1 M $(NH_4)_2CO_3$ (pH 6.9) at 4 C. Fractions (3 ml) were collected at a rate of 16.8 ml/h and the relative amount of antigen in each fraction tested is indicated. The presence of carbohydrate in 100 μliters of fraction is indicated by absorbance at 490 nm. Tubes 70 to 127 were pooled, dialyzed, and lyophilized (16.8 mg).*

the purified group *d* antigen. This material contained less than 0.5% phosphorus. The antigen was tested for immunochemical purity and compared with the whole buffer extract in immunoelectrophoretic analysis (Fig. 6A). A single cathodally migrating precipitin band was formed with the *d* antigen as compared to the multiple bands formed with the buffer extract. To determine whether the *d* antigenicity was dependent upon carbohydrate, the antigen was treated with Pronase and tested in gel diffusion analysis (Fig. 6B). The release of peptide was confirmed by following the course of proteolysis using the ninhydrin reaction. A reaction of immunological identity occurred between the nontreated antigen and all digests. These results suggested that the antigenicity did not reside in a peptide moiety but was probably dependent upon carbohydrate. However, at this time we are unable to state whether peptide and carbohydrate were covalently linked.

Chemical characterization of the d antigen. The results of the chemical analyses of the *d* antigen are shown in Table 1. As expected, the antigen contained carbohydrate (70.9%) and a small amount of phosphorus (0.4%). Galactose and glucose, present in a ratio of 5.9:1.0, accounted for virtually all of the carbohydrate. Glucosamine was present in a trace amount. The predominant amino acids present were

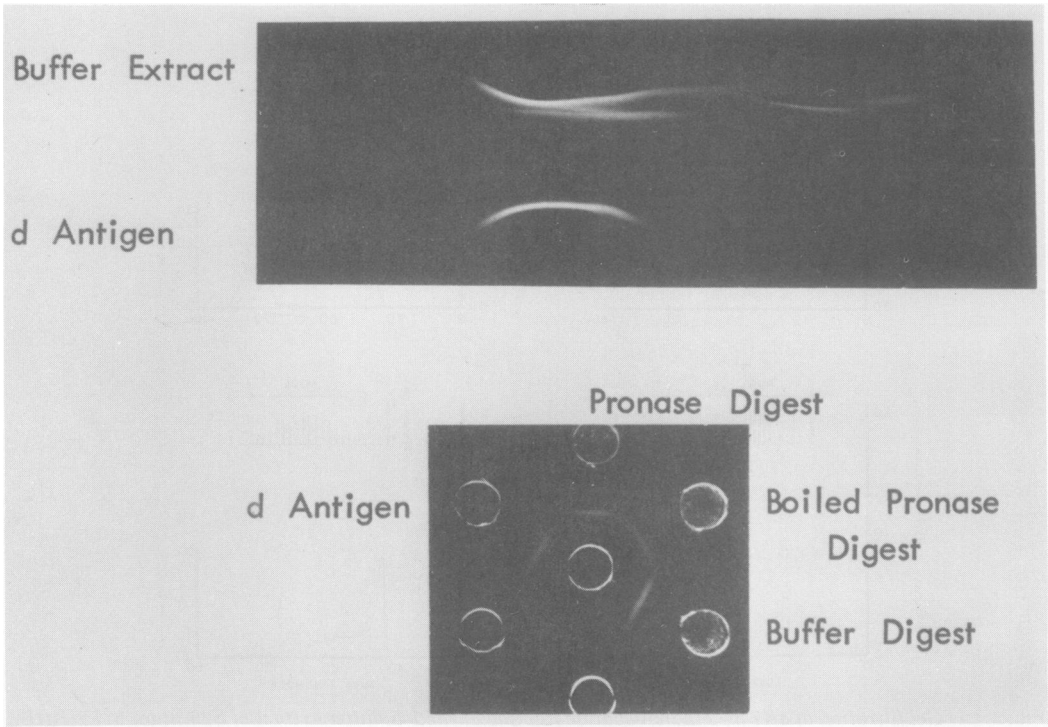

FIG. 6. *Upper: immunoelectrophoretic analysis demonstrating the purity of the d antigen. The upper well contained whole 1-h buffer extract. The lower well contained the d antigen and the trough contained antiserum (F 22) directed to S. mutans. The cathode was to the left. Lower: gel diffusion analysis of the pronase digest of the d antigen and the nontreated antigen. The peripheral wells contained the materials indicated. The center well contained antiserum (F 22) directed to S. mutans.*

glutamate (1.8%) and aspartate (1.3%). Ribitol and glycerol were not detected. In light of the absence of ribitol and glycerol and the small amount of phosphorus, these findings suggest the absence of teichoic acid. Chemical analysis of concentrated buffer eluted from all the columns described did not demonstrate any galactose or glucose.

Quantitative precipitin inhibition assay. The equivalence point for hapten inhibition experiments was determined in a quantitative precipitin reaction between the *d* antigen and purified antibody (F15-22). Equivalence was attained with 22.5 μg of intact antigen (Fig. 7). After treatment with Pronase, the antigen precipitated virtually all antibody (98.6%), further confirming that antibody was directed to the carbohydrate portion of the *d* antigen.

Table 2 contains the results of several quantitative precipitin inhibition assays performed with 250 μg of antibody, 22.5 μg of antigen, and 100 μmol of various mono- and disaccharides and amino sugars. Of the monosaccharides tested, galactose gave the greatest inhibition (34.1%). The β-methyl derivative of galactose and melibiose (galactose-α-1,6-glucose) were the most effective inhibitors (52.7 and 56.5%, respectively). Stachyose {[*O*-α-D-galacpyranosyl-(1,6)]$_2$-α-D-glucopyranoside-(1,2)-D-fructose} was not as effective (21.7%). Glucose (25.6%), and its α- and β-methyl derivatives (23.2 and 20.5%), gave virtually identical inhibition. Of the glucose disaccharides tested, cellobiose (glucose-β-1,4-glucose) and maltose (glucose-α-1,4-glucose) gave the greatest inhibition (30.2 and 33.3%, respectively). Other carbohydrates were also tested, but little or no inhibition was noted. Inhibition also occurred with galactosamine (34.6%) and glucosamine (25.2%). This was remarkably similar to inhibition by the respective parent compounds of galactose (34.1%) and glucose (25.6%). These results suggested that the immunodominant region of the *d* antigen was primarily galactose, and that the β-1-linkage of this galactose might be involved. Glucose, in either its alpha or beta configuration, was also a part of the immunodominant region.

TABLE 1. *Chemical composition of the d antigen*[a]

Compound	Percentage of Composition by Weight
Carbohydrate	
Galactose	60.6
Glucose	10.3
Ribose	ND
Glucosamine	Trace
Total	70.9
Amino acids[b]	
Glutamic acid	1.8
Aspartic acid	1.3
Alanine	1.0
Lysine	0.7
Threonine	0.6
Glycine	0.6
Leucine	0.6
Valine	0.6
Serine	0.4
Proline	0.4
Isoleucine	0.4
Tyrosine	0.4
Arginine	0.3
Phenylalanine	0.3
Histidine	0.1
Total	9.5
Glycerol	ND
Ribitol	ND
Phosphorus	0.4

[a] Recovery of the *d* antigen was 80.8%. ND, Not detectable.

[b] Amino acids not listed were absent. Analyzer could detect as little as 5 nmol.

DISCUSSION

The group *d* antigen of *S. mutans* 6715 was isolated, purified, and chemically characterized. The present results strongly indicate that this is the major antigenic component of *S. mutans* 6715. All sera from rabbits immunized by different procedures contained antibody mainly directed to this antigen. Anti-group *d* antibody could be eluted from cells incubated with these immune sera. All agglutinating antibody activity in immune sera was inhibited by a crude preparation of this antigen (CA). Although some antibody preparations contained anti-glucosyltransferase activity, virtually all antibody could be precipitated with the purified *d* antigen (Fig. 7). The presence of such activity is not surprising since glucosyltransferase has been demonstrated on the surface of these cells. Small amounts of anti-glucosyltransferase antibody would not alter the interpretation of our immunochemical findings.

The nature of the cell surface antigens which characterize each of the five groups of *S. mutans* have been recently investigated. Van de Rijn and Bleiweis (49) suggested that a membrane-associated glycerol teichoic acid with a β-galactoside, probably also containing glucose, was the major antigen of the group *a* strain *S. mutans* AHT. Vaught and Bleiweis (50) also reported the existence of a cell wall glycerol teichoic acid of *S. mutans* BHT, a group *b* strain. Hapten inhibition studies revealed that the antigenicity resided in a β-galactoside component of this antigen. However, Mukasa and

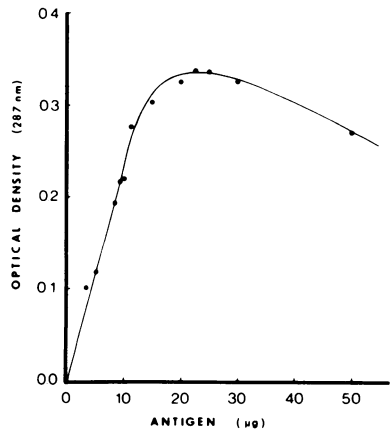

FIG. 7. *Quantitative precipitin curve for the reaction of purified d antigen and antibody. F15-22 antibody (250 μg) was added to increasing concentrations of the d antigen (final volume = 200 μliters) and incubated at 37 C for 1 h and overnight at 4 C. The precipitates were dissolved in 1 ml of 0.1 N NaOH and measured spectrophotometrically.*

TABLE 2. *Quantitative precipitation-inhibition assay*

Inhibitor (100 μmol)	Inhibition (%)
Galactose	34.1
α-Methyl galactopyranoside	27.1
β-Methyl galactopyranoside	52.7
Galactosamine	34.6
Melibiose	56.5
Lactose	45.0
Maltose	33.3
Cellobiose	30.2
Glucose	25.6
α-Methyl glucopyranoside	23.2
β-Methyl glucopyranoside	20.5
Glucosamine	25.2
Stachyose	21.7
Rhamnose	6.9
Ribose	5.9
Sucrose	4.8
Trehalose	4.8
Gentiobiose	0.0

Slade (35) characterized the group *a* antigen of *S. mutans* HS6 as a cell wall polysaccharide containing glucose, galactose, glucosamine, and galactosamine (33). Similarly, these investigators characterized a polysaccharide and mucoprotein as the group antigen of *S. mutans* FA1 (group *b*). The antigenicity resided in D-galactose and D-galactosamine. Burgess and Edwards (6) also isolated a carbohydrate-based antigen rather than a teichoic acid from *S. mutans* FA1. Our studies of the group *d* antigen agree with the evidence from group *a* and *b* which suggests that these group-specific antigens of *S. mutans* are primarily carbohydrate rather than teichoic acid. The antigen of *S. mutans* 6715 did not migrate to the anode in immunoelectrophoresis, nor did it contain significant amounts of phosphorus, glycerol, or ribitol, all characteristics of teichoic acid. It may be suggested that the isolation procedures disrupted the esterified carbohydrate component of a teichoic acid, such that only a portion of the group antigen was analyzed. However, a trichloroacetic acid extract was obtained from *S. mutans* 6715 by the classical method for extraction of teichoic acids (23), in which the ribitol and polyglycerol phosphate remain relatively intact. This trichloroacetic acid extract had the same antigenic component (*d* antigen) as both the ethanol-soluble and -insoluble fractions of the buffer-boiled extract (Fig. 4). Chemical analyses of the trichloroacetic acid extract also indicated the absence of ribitol and glycerol (21).

Despite several purification procedures, amino acids (9.5%) were always present as part of the *d* antigen (Table 1). The group *b* antigen isolated from *S. mutans* FA1 was found to be composed of carbohydrate and peptide material which could be separated by electrophoresis (34). However, the polysaccharide and peptide components of the group *d* antigen migrated as a single component to the cathode. Pronase-treated antigen had the same migration characteristics as the nontreated antigen. Others (35) have found that the peptide component could not be separated from the group *a* antigen of *S. mutans* HS6 despite treatment with several proteolytic enzymes. This is of interest since antibody directed to the group *d* antigen cross-reacts with the group *a* antigen. As suggested for the *a* antigen, *d* antigen amino acids may be a remnant of a component, at or near the surface, to which the *d* antigen is linked. The chemical analysis of the *d* antigen showed only a trace of glucosamine and indicated the lack of *N*-acetylmuramic acid. Although glucosamine did inhibit precipitation with the *d* antigen (Table 2), this may have been due to structural similarity to the glucose found in this antigen. This evidence suggested that the cell wall peptidoglycan is probably not the component responsible for the *d* antigen linkage.

Carbohydrate analyses and hapten inhibition studies of the *d* antigen strongly suggest that a β-galactoside constitutes a major portion of the immunodominant region of this antigen (Table 1, 2). Glucose also appears to contribute to the antigenicity. Appreciable inhibition with melibiose (Table 2) seems to indicate that a portion of the antigenic determinant probably contains a 1-6 linkage. Galactose seems to be important for the immunochemical specificity of other types of streptococci. For example, group R streptococci possess specificity attributable to α-galactose (41), whereas in group F streptococci immunological specificity is dependent upon β-galactose (52). Similarly, the β-linkage of galactose appears to be important in studies of the group *a* antigen of *S. mutans* (49). It is quite clear that a galactoside is also a part of the immunodominant region of the *a-d* antigenic site of the group *a* antigen of *S. mutans* (35). The *a-d* site was found to cross-react with extracts of *S. mutans* B13, a group *d* strain. The *d* antigen isolated from *S. mutans* 6715 also cross-reacts with extracts of strain B13, as well as strain AHT, a group *a* strain (Fig. 2). However, the antigenicity of the *a-d* site resided in galactosamine, as well as a galactoside (35). No measurable amount of galactosamine could be detected in the *d* antigen of *S. mutans* 6715. This suggests that the cross-reactivity of antibody directed to the *d* antigen with the *a-d* site could be due to the galactose component alone and that the presence of galactosamine is possibly only a characteristic of the *a* group of *S. mutans*. The group *d* antigen was not completely precipitated in 47.5% ethanol (Fig. 4), although this procedure has been used previously to prepare the *S. mutans* group *a* antigen (35). It may be possible to effectively purify the *d* antigen using higher concentrations of ethanol.

The combination of extraction procedures, including sonic treatment, which were utilized to obtain the *d* antigen suggested a rather superficial location of this material on the bacterial cell. However, an identical antigen could be extracted from presumptive cell membranes using the classical trichloroacetic acid extraction method. Although the *d* antigen might be associated with the cell membrane, the data are insufficient to rule out the possibility that this apparant association was an artifact of isolation, representing newly synthe-

sized material from ribosomes or resulting from cell wall material contaminating the 78,000 × *g* fraction.

Adherence is important for the pathogenic potential of *S. mutans*. Antibody has been shown to interfere with plaque formation (15) and adherence phenomena exhibited by *S. mutans* in vitro (38). Although this antibody-mediated inhibition has been attributed to effects on the glucosyltransferase enzymes (12), effects on other cell surface structures or products of *S. mutans* have been described. Several recent reports (36, 37) have presented evidence which suggests that the *a-d* site may play a role in the adherence of *S. mutans* to the tooth surface. Purified antibody directed to the *a-d* site completely blocked adherence of *S. mutans* HS6 to smooth glass surfaces (36). This antibody was found to inhibit the binding to the cell surface, but not the enzymatic activity of glucosyltransferase enzymes. Therefore, it was suggested that the *a-d* site was a receptor, or more probably was in close physical approximation to the actual cell receptor site for these glucosyltransferases (37). Similarly, in light of the relatedness of the *a-d* and *d* antigens, antibody directed to the *d* antigen may also inhibit binding of the enzyme to its receptor.

In our laboratory immunization studies have been conducted with *S. mutans* 6715 as an immunogen (47, also see reviews 45, 46). We have been able to demonstrate in vivo effects of the presence of antibody on disease caused by these organisms. As we have shown in this manuscript and previously in rabbits and in rats, much of the antibody synthesized after immunization with *S. mutans* 6715 is directed to the *d* antigen (43). Therefore, this antigen could be of importance in initiating a protective immune response.

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ADDENDUM

Following submission of this manuscript for publication, a report appeared describing the purification and characterization of the group antigen of *S. mutans* B13, a group *d* strain (26). The chemical composition and immunochemical properties of the *d* antigen from strain B13 generally confirmed the results presented in this manuscript for the group *d* antigen of *S. mutans* 6715.

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