INFECTION AND IMMUNITY, May 1980, p. 486-493
0019-9567/80/05-0486/08$02.00/0

Vol. 28, No. 2

Protein Antigens of *Streptococcus mutans*: Purification and Properties of a Double Antigen and Its Protease-Resistant Component

MICHAEL W. RUSSELL,†* LESLEY A. BERGMEIER, E. D. ZANDERS, AND T. LEHNER

Department of Oral Immunology and Microbiology, Guy's Hospital, London SE1 9RT, England

A surface protein antigen of *Streptococcus mutans* having two sets of antigenic determinants (antigens I and II) was purified by column chromatography from culture supernatants of *S. mutans* serotype *c*. The protease-resistant component, antigen II, was purified from pronase-digested antigen I/II. The antigens were analyzed chemically and immunologically, and their physicochemical properties were investigated. Antigen I/II consisted of more than 80% protein, and its peptide chain molecular weight was estimated to be 185,000. Antigen II consisted of approximately 60% protein, with a peptide chain molecular weight of 48,000. Antisera to antigens I/II and II were raised in rabbits and used to investigate the presence of the antigens in cells of other streptococci. This indicated that not only serotype *c* but also serotypes *e* and *f* possessed antigen I and II determinants, whereas serotypes *a*, *d*, and *g* possessed a determinant related to antigen I but not one related to antigen II.

An understanding of the antigenic structure of *Streptococcus mutans* is necessary for the development of an effective vaccine against dental caries. The serotype antigens corresponding to serotypes *a* through *g* (3, 20) have been described (17) and are probably related to the carbohydrates which have been found in the cell walls (10). Lipoteichoic acid, which is widespread among gram-positive bacteria, has also been described (13, 19), and its antigenic specificity resides largely in the polyglycerophosphate chain which is common to all glycerol teichoic acids (14). However, the value of these antigens in vaccines against dental caries remains to be established, and there is evidence that antibodies against them are not related to immunity to dental caries in monkeys (21).

Precipitating antibodies to various antigens of *S. mutans* serotype *c* have been observed in sera of monkeys immunized against caries (16). Examinations of culture supernatants and cell extracts of *S. mutans* serotype *c* have revealed the presence of at least four prominent antigens, designated I through IV, in addition to the serotype polysaccharide and lipoteichoic acid antigens (22). Antigens I/II, II, and III have been partially purified and used to investigate antibody responses in monkeys immunized with whole cells and cell walls of *S. mutans* (M. W. Russell, S. J. Challacombe, and T. Lehner, Immunology, in press). It appeared that precipitating antibodies to antigens I/II and II were related to protection against dental caries.

As described in the initial identification of these antigens (22), antigen II was a subcomponent of antigen I and could be derived from it by treatment with proteolytic enzymes. As antigen I was originally defined as being destroyed by proteolytic treatment, the substance or complex which contains both protease-sensitive antigen I and protease-resistant antigen II is now defined as antigen I/II.

This paper describes the purification of antigens I/II and II and their characterization by physicochemical properties and chemical analysis.

MATERIALS AND METHODS

Organisms. *S. mutans* Ingbritt was originally supplied by B. Krasse, and the Guy's strain was isolated from the dental plaque of a patient at Guy's Hospital. Both strains were identified as belonging to serotype *c*. The other *S. mutans* strains and other organisms used have been described previously (20a, 22).

For the purification of antigens, *S. mutans* Ingbritt or Guy's strain was grown in 5-liter flasks of a semidefined medium based on a low-salt acid hydrolysate of casein (Acidicase peptone 2; BBL Microbiology Systems) supplemented with selected vitamins, amino acids, glucose, and salts (2). A tryptone-yeast extract-Casamino Acids dialysate medium was also used (22). The medium was inoculated with an overnight culture in Todd-Hewitt broth and incubated at 37°C for 60 to 65 h. The culture supernatant was separated by centrifugation in a continuous flow rotor at 22,000 × *g*.

† Present address: Department of Microbiology, University of Alabama in Birmingham, Birmingham, AL 35294.

Extraction and purification of antigen I/II. The culture supernatant was concentrated to about 200 ml by dialysis against solid polyethylene glycol (Carbowax 20M), followed by dialysis against water and lyophilization. In an improvement which reduced contamination with smaller polyethylene glycol polymers and cell wall polysaccharide, the concentrate was made 3 M with respect to ammonium sulfate, and the precipitate was redissolved and lyophilized.

The dried culture supernatant was dissolved in about 50 ml of 0.01 M tris (hydroxymethyl)-aminomethane hydrochloride (pH 7.4) and chromatographed on a diethylaminoethyl cellulose (Whatman DE 52) column (30 by 1.5 cm). After the starting buffer was eluted at a flow rate of 25 ml/h, a 500-ml gradient of 0.1 to 0.3 M sodium chloride in starting buffer was applied. The eluate was monitored at 280 nm, and fractions were collected at 15-min intervals. Antigens were detected in fractions by fused rocket immunoelectrophoresis (IEP) against polyspecific antiserum to *S. mutans* serotype *c* (22), and the partially purified antigen I/II peak was collected, dialyzed against water, and lyophilized.

The partially purified antigen I/II was dissolved in up to 5 ml of 0.1 M tris(hydroxymethyl)aminomethane hydrochloride–0.5 M NaCl (pH 7.4) and chromatographed on Sepharose 6B in a column (90 by 2.5 cm) which was eluted at 20 ml/h with the same buffer. The eluate was monitored at 280 nm, and 15-min fractions were collected. Antigens were again detected by fused rocket IEP, and the antigen I/II peak was dialyzed against water and lyophilized.

Purification of antigen II. Approximately 100 mg of partially purified antigen I/II was dissolved in 5 ml of 0.1 M tris(hydroxymethyl)aminomethane hydrochloride (pH 7.4) and treated with 1 mg of pronase at 37°C for 16 h. The digest was chromatographed on Bio-Gel P150 in a column (90 by 2.5 cm) which was eluted at 10 ml/h with 0.1 M tris(hydroxymethyl)-aminomethane hydrochloride, pH 7.4. Fractions, which were monitored at 280 nm, were collected at 30-min intervals, and antigen II was located by fused rocket IEP. The antigen peak was dialyzed against water and lyophilized.

Antisera. Antisera were raised in New Zealand white rabbits. Polyspecific antisera to *S. mutans* serotype *c* were prepared against unfractionated culture supernatant antigens as described previously (22). Antisera to purified antigens I/II and II were raised by using 1-mg doses of antigen in Freund complete adjuvant given intramuscularly, followed 3 weeks later by a similar dose in Freund incomplete adjuvant injected subcutaneously. Blood was taken at least 3 weeks later.

Serological methods. Immunodiffusion, IEP, and single radial immunodiffusion in agarose gels have been described previously (20a, 22). The indirect immunofluorescence procedure in which rabbit antisera and fluoresceinated anti-rabbit immunoglobulin are used (Wellcome) has been described previously (20a).

Estimation of molecular weight. Antigens I/II and II were chromatographed on columns (approximately 90 by 2.5 cm) of Ultrogel AcA22 and AcA34, respectively, which were prepared and eluted with 0.1 M tris(hydroxymethyl)aminomethane hydrochloride–tris 0.5 M NaCl (pH 7.4) at flow rates of 10 and 20 ml/h, respectively. The columns were calibrated by eluting dextran blue (Pharmacia), thyroglobulin (molecular weight, 670,000), ferritin (apoferritin molecular weight, 443,000), immunoglobulin G (150,000), serum albumin (67,000), ovalbumin (45,000), and chymotrypsinogen (25,000). V_e/V_0 was plotted against the logarithm of molecular weight (1) by least-squares regression.

Estimation of isoelectric point. Two-dimensional immunoisoelectric focusing was performed in a polyacrylamide gel (24).

PAGE. Polyacrylamide gel electrophoresis (PAGE) under native conditions was performed in tube gels of polyacrylamide as described previously (22).

PAGE in 0.1% sodium dodecyl sulfate (SDS) was performed in a vertical slab apparatus (26), using the Laemmli buffer system (15) and 7.5% polyacrylamide gels. Molecular weights were estimated by co-electrophoresis of antigens with marker proteins labeled by ^{14}C methylation (Radiochemical Centre, Amersham, England). After the gels were stained with 0.1% Coomassie brilliant blue in 40% trichloroacetic acid, they were destained in 10% acetic acid and dried. Marker proteins were located by autoradiography for 48 h with Kodak X-Omat H X-ray film.

For detection of antigens in SDS-PAGE, an immuno-overlay system with modifications (25) was used. Unstained gels were overlaid with 0.6% agarose containing 10% antiserum and incubated at room temperature for 16 h. The agarose layer was then floated off and dried on a glass plate. After the overlay was washed extensively in 5% sodium chloride, it was stained as described above for immunodiffusion plates.

Proteolytic digestion of antigens. Antigen I/II which had been partially purified on diethylaminoethyl cellulose (150 μg) was digested for 16 h at 37°C with 1.5 μg of trypsin, 1.5 μg of chymotrypsin, or 1.5 μg of *Staphylococcus aureus* V8 protease (Miles Laboratories Ltd.) (all at pH 8.0) or with 1.5 μg of papain at pH 7.0 with 0.01 M cysteine or 1.5 μg of pepsin at pH 4.0, 5.0, 5.5, or 6.0. Reactions were terminated, and the digests were analyzed by SDS-PAGE in 10% acrylamide gels.

Analytical methods. Protein was estimated by the method of Lowry et al. (18), using bovine serum albumin as a standard. Neutral carbohydrate was estimated by the phenol-sulfuric acid method (8), using glucose as a standard. Amino sugars were estimated by using 3-methyl-2-benzothiazolinone hydrazine hydrochloride (Sigma Chemical Co.), with glucosamine as a standard (27). Phosphorus was assayed by the method of Chen et al. (5). Amino acid analysis was performed on 200 μg of antigen hydrolyzed in 6 N HCl in the presence of thioglycolic acid under nitrogen at 105°C for 16 h, using an LKB amino acid analyser.

RESULTS

Purification of antigen I/II. A total of 15 liters of culture supernatant of *S. mutans* Ingbritt was concentrated and precipitated with 3 M ammonium sulfate and lyophilized to give 2.6 g of dried material, which was chromatographed on diethylaminoethyl cellulose (Fig. 1). Antigen

I/II was eluted in a peak of ultraviolet-absorbing material at the start of the gradient and was largely separated from antigen III by this process. The yield of lyophilized partially purified antigen I/II was 90 mg.

Chromatography of partially purified antigen I/II (90 mg) on Sepharose 6B is shown in Fig. 2. Antigen activity was located in a small isolated and nearly symmetrical peak, which on lyophilization yielded 4.6 mg of substance. Figure 3 shows an electrophoretic analysis of antigen I/II. PAGE indicated a single band of Coomassie brilliant blue-positive material of low mobility. However, in SDS-PAGE antigen I/II resolved into three bands, designated A, B, and C, but all three contained antigenic material, as revealed by overlaying the electrophoretic gel with agarose containing antiserum to antigen I/II. The same technique with antiserum to antigen II showed that bands A and B were positive but that band C did not react. Therefore, it was

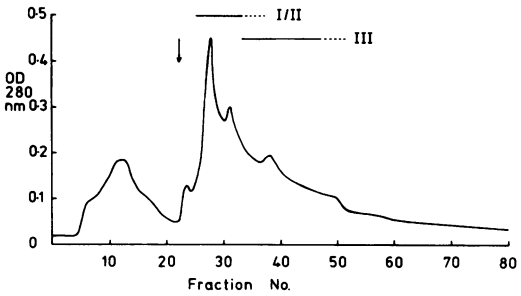

FIG. 1. *Diethylaminoethyl cellulose chromatography of 2.6 g of culture supernatant antigens of S. mutans. The arrow indicates the start of a sodium chloride gradient. The bars indicate the distributions of antigens I/II and III detected in fused rocket IEP. OD, Optical density.*

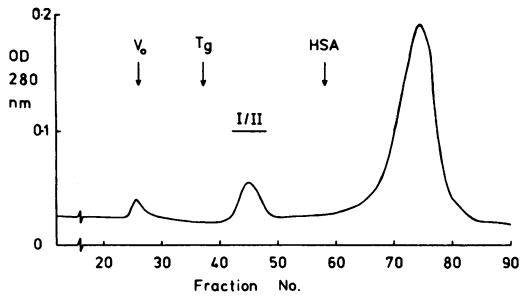

FIG. 2. *Sepharose 6B chromatography of 90 mg of partially purified antigen I/II. The horizontal bar indicates the distribution of antigen I/II detected in fused rocket IEP. V_o, Void volume (blue dextran); Tg, peak elution position of thyroglobulin (molecular weight, 670,000); HSA, peak elution position of human serum albumin (molecular weight, 67,000). OD, Optical density.*

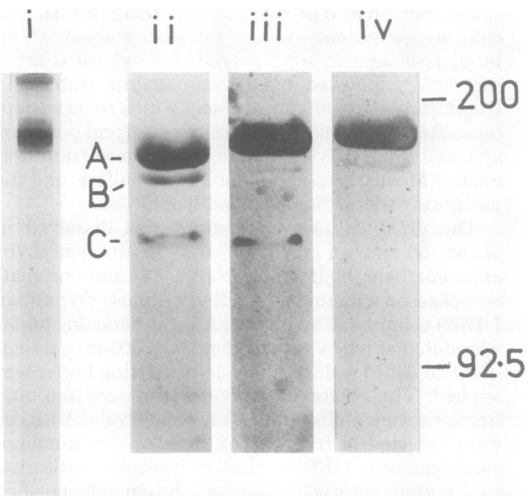

FIG. 3. *Electrophoretic analysis of antigen I/II. Lane i, PAGE, Coomassie brilliant blue stained; lane ii, SDS-PAGE, Coomassie brilliant blue stained; lane iii, SDS-PAGE, immuno-overlay with antigen I/II antiserum; lane iv, SDS-PAGE, immuno-overlay with antigen II antiserum. Markers were myosin (molecular weight, 200 × 10^3) and phophorylase b (molecular weight, 92.5 × 10^3).*

concluded that band C contained antigen I, whereas bands A and B contained antigen II and probably also antigen I.

Different preparations of antigen I/II revealed some differences in the band pattern on SDS-PAGE. Band A was always present as the major component, whereas bands B and C were variable, less intense, and sometimes absent altogether. It seemed likely that band A represented intact antigen I/II, whereas bands B and C were possibly partial breakdown products.

Purification of antigen II. Figure 4 shows chromatography of pronase-digested partially purified antigen I/II. Antigen II was eluted in a peak of ultraviolet-absorbing material soon after the void volume peak and with an elution volume similar to that of serum albumin. The yield of lyophilized antigen II was about 2.5 mg/100 mg of partially purified antigen I/II.

PAGE of antigen II revealed a single Coomassie brilliant blue-staining band, which was not further resolved on SDS-PAGE (Fig. 5). An immuno-overlay containing antiserum to antigen II showed that this protein band contained antigen II determinants.

Origin of antigens I/II and II. To confirm that antigens I/II and II were derived from *S. mutans* and not from components of the culture media, the following experiment was carried out. Batches of the semidefined medium (2), dialysate medium (22), and Todd-Hewitt broth (Oxoid) were treated with 3 M ammonium sulfate

for 16 h at 4°C. The resulting precipitates were redissolved and dialyzed against saline to achieve a 100-fold concentration and then examined by single radial immunodiffusion against antisera to antigens I/II and II. In no case was a precipitin reaction observed, whereas when the same treatment was applied to culture supernatants of *S. mutans* serotype *c* grown in these media, precipitin rings due to antigens I/II and II were readily observed.

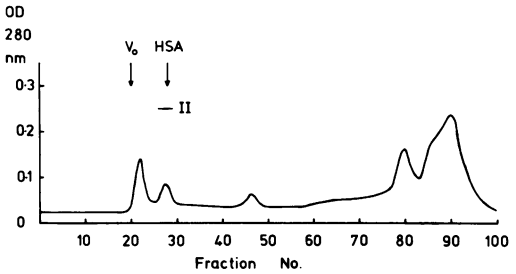

FIG. 4. *Bio-Gel P-150 chromatography of antigen II (pronase digest of 100 mg of partially purified antigen I/II). The horizontal bar indicates the distribution of antigen II detected by fused rocket IEP. V_0, Void volume (blue dextran); HSA, peak elution position of human serum albumin (molecular weight, 67,000). OD, Optical density.*

FIG. 5. *Electrophoretic analysis of antigen II. Lane i, PAGE, Coomassie brilliant blue stained; lane ii, SDS-PAGE, Coomassie brilliant blue stained. Markers were bovine serum albumin (molecular weight, 68 × 10^3), catalase (molecular weight, 57.5 × 10^3), and ovalbumin (molecular weight, 45 × 10^3).*

Serological comparison of antigens I/II and II. The reactions of antigens I/II and II against antisera in immunodiffusion and IEP are shown in Fig. 6. In immunodiffusion antiserum to antigen I/II reacted with both antigen II and antigen I/II; the latter gave a spur which indicated the presence of the additional antigenic moiety (antigen I) in antigen I/II. Antiserum to antigen II, however, reacted only with the antigen II component within antigen I/II and consequently showed apparent identity between this and pure antigen II. In IEP, antigen I/II remained close to the sample well, and antigen II migrated toward the anode. Antisera to antigens I/II and II and to unfractionated supernatant antigens formed only single arcs with each antigen, not only indicating apparent purity, but also suggesting that antigen I/II is a single substance possessing both antigenic specificities.

Enzyme digestion of antigens I/II and II. Antigen I/II was originally found to be partially susceptible to digestion by pronase and trypsin (22), and use has been made of this treatment to purify antigen II from partially purified antigen I/II. Antigen II was similarly produced when purified antigen I/II was treated with pronase or trypsin. Treatment of partially purified antigen I/II with other proteolytic enzymes was attempted, especially to discover whether free antigen I could be generated without further degradation.

Figure 7 shows the SDS-PAGE analysis of digests of partially purified antigen I/II obtained with proteases of different substrate specificities. Although undigested antigen I/II contained proteins in addition to band A, Fig. 7 shows that all of the enzymes used produced a protein band equivalent to the antigen II band formed by pronase or trypsin and that pepsin at pH 4 seemed to be the most efficient enzyme for this purpose. However, no evidence for the release of free antigen I (band C) was obtained, even when pepsin was used at suboptimal pH valves (5.0, 5.5, 6.0). Interestingly, pepsin at pH 5.0 appeared to produce the least amount of the antigen II band, together with two additional bands of lower molecular weight. Chymotrypsin also produced bands of lower molecular weight than antigen II.

Physicochemical properties. Molecular weights were estimated by gel filtrations on calibrated Ultrogel columns. Antigen I/II eluted from Ultrogel AcA22 with a V_e/V_0 value of 1.79 ± 0.035 (mean ± standard error of the mean), which corresponded to a molecular weight of approximately 350,000 (range, 260,000 to 440,000). Antigen II consistently eluted from

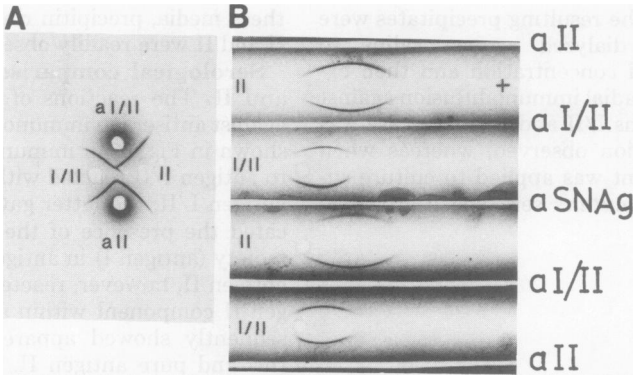

FIG. 6. *Immunological analysis of antigens I/II and II. (A) Immunodiffusion. (B) IEP. I/II antigen I/II; II, antigen II; aI/II, antiserum to antigen I/II; aII, antiserum to antigen II; aSNAg, antiserum to unfractionated culture supernatant antigens.*

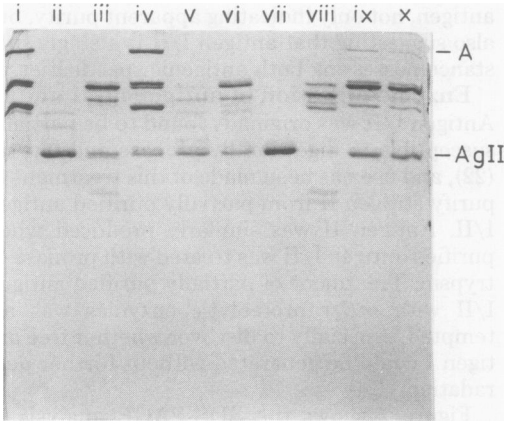

FIG. 7. *SDS-PAGE of enzymic digests of partially purified antigen I/II. Lane i, Control; lane ii, trypsin; lane iii, chymotrypsin; lane iv, V8 protease; lane v, pronase; lane vi, papain; lane vii, pepsin, pH 4.0; lane viii, pepsin, pH 5.0; lane ix, pepsin, pH 5.5; lane x, pepsin, pH 6.0. The arrows indicate the positions of antigen I/II (band A) and antigen II (band Ag II).*

Ultrogel AcA34 at a V_e/V_0 value of 1.88, which corresponded to a molecular weight of 69,000. On SDS-PAGE, the molecular weight of antigen I/II band A was estimated to be 185,000, and the molecular weights of bands B and C were estimated to be 170,000 and 150,000, respectively. The molecular weight of antigen II was estimated to be 48,000.

Isoelectric focusing revealed no microheterogeneity of either antigen I/II or II. The isoelectric points of the focused bands were as follows: antigen I/II, pH 4.7 to 4.9; antigen II, pH 4.0 to 4.1.

Antigens I/II and II possessed similar ultraviolet absorption spectra, which were typical of proteins with broad absorption peaks at 270 to 280 nm and high absorption below 230 nm. $E_{280}^{1\,cm}$ values for 1-mg/ml solutions were as follows: for antigen I/II, 1.12; for antigen II, 0.65. The E_{280}/E_{260} ratio for antigen I/II was 1.2, and that for antigen II was 1.1.

TABLE 1. *Composition of antigens I/II and II*

Type of compound	Composition (%)	
	Antigen I/II	Antigen II
Protein	82	58
Carbohydrate	1.7	12
Amino sugar	2.5	5.8
Phosphorus	0.06	0.3

Chemical composition. Table 1 shows the chemical analyses of antigens I/II and II. Antigen I/II was composed predominantly of protein (82%), with minor amounts of carbohydrates, amino sugars, and phosphorus. Antigen II was assayed at 58% protein and 12% carbohydrate.

Amino acid analysis of acid-hydrolyzed antigens I/II and II revealed the presence of most common amino acids, although they contained only low or undetectable amounts of methionine and cysteine, respectively. Tryptophan was not detected either, but this would not have survived the hydrolysis procedure. Alanine, glutamate (from glutamine), lysine, and threonine, which have been reported as the constituent amino acids of peptidoglycan from *S. mutans* (12), were not present in appreciably greater quantities than other amino acids.

Distribution of antigens I/II and II among other streptococci. Indirect immunofluorescence was used to examine the occurrence of antigens I/II and II in different serotypes of *S. mutans* and other oral streptococci (Table 2). *S. mutans* strains belonging to serotypes *c*, *e*, and *f* reacted with antisera to both antigen I/II and antigen II, whereas strains of serotypes *a*, *d*, and *g* reacted with antiserum to antigen I/II (generally less strongly than serotypes *c*, *e*, and *f*) but did not react with antiserum to antigen

TABLE 2. *Immunofluorescent reactions of streptococci with antisera to antigens I/II and II*

Organism	Serotype	Titer with:[a] Antiserum to antigen I/II	Titer with:[a] Antiserum to antigen II
S. mutans			
Guy's	*c*	5	4
Ingbritt	*c*	5	3
SS41	*c*	5	4
SR25	*c*	5	4
NCTC10449	*c*	5	4
LM 7	*e*	5	4
T93	*e*	5	4
OMZ175	*f*	4	2
AHT	*a*	2	0
OMZ61	*a*	4	0
OMZ176	*d*	4	0
6715	*g*	4	0
FA.1	*b*	0	0
Rat	*b*	0	0
S. sanguis OMZ 9		2	0
S. salivarius HHT		2	0
Streptococcus CHT		1	0

[a] Log_2 highest dilution of antiserum (starting at 1:10) showing strong fluorescence. All reactions with normal rabbit serum were negative.

II. Serotype *b* strains did not react with either antiserum. The single strains of *Streptococcus sanguis* and *Streptococcus salivarius* examined, as well as *Streptococcus* CHT, also reacted weakly with antiserum to antigen I/II, but not with antiserum to antigen II. No organism demonstrated fluorescence if treatment with the specific antiserum was omitted or was replaced by normal rabbit serum. Antigen I/II antiserum usually demonstrated clear cell surface "ring" fluorescence, but with antigen II antiserum the fluorescence was less clearly demarcated.

DISCUSSION

Two antigenic substances, antigens I/II and II, were purified from *S. mutans* and characterized, and antisera were raised against them in rabbits. These antisera confirmed that antigen I/II possesses two determinants or two sets of determinants, one of which is identical to antigen II. The other set, corresponding to antigen I, is readily destroyed by proteolytic enzymes, to which antigen II is resistant. A substance which reacts with both antisera may be antigen I/II or II, but the presence of the extra antigen I determinant(s) in antigen I/II can be demonstrated, for example by the spur reaction in Ouchterlony immunodiffusion (Fig. 6). Furthermore, antigen I/II migrates more slowly than antigen II in IEP.

Antigen I/II behaved as a single entity during purification, which failed to resolve it into separate antigens I and II, and also on PAGE in the presence of SDS after reduction by 2-mercaptoethanol. Thus antigens I and II appear to be determinants present on a single molecule which consists largely of protein. However, the molecular weight of this substance on gel filtration showed some variability in the range of 260,000 to 440,000, which was approximately double that found on SDS-PAGE. This could imply that the antigen prepared by gel filtration was dimeric, but as no cysteine was recovered on amino acid analysis and the molecule showed the same mobility in SDS-PAGE without mercaptoethanol (unpublished data), the existence of disulfide bridges appears unlikely. Alternatively, the antigen might be associated with variable amounts of other substances, such as cell wall polymers, which were removed by treatment with SDS. However, gross chemical analysis revealed the presence of only minor amounts of carbohydrates, amino sugars, and phosphorus. There was no evidence for the association of carbohydrate with antigen I/II band A on SDS-PAGE since specific staining with periodic acid-Schiff reagent or with periodic acid-dansyl hydrazine failed to give a positive reaction. Therefore, gross contamination with cell wall polymers, such as polysaccharide, peptidoglycan, or teichoic acid, seems unlikely. Furthermore, large quantities of peptidoglycan would be reflected in a preponderance of those amino acids (alanine, glutamate, lysine, and threonine) reported in *S. mutans* peptidoglycans (12), and there was no serological cross-reaction of antigen I/II with cell wall (serotype) polysaccharide or with glycerol teichoic acid (22). The discrepancy between the molecular weights observed on SDS-PAGE and on gel filtration columns might also be explained by the hydrodynamic properties of the molecules. A large axial ratio would result in nonideal behavior on gel filtration and lead to an overestimation of the true molecular weight. Determination of the sedimentation coefficients would help clarify this.

Antigen II has been isolated as a proteolysis-resistant fragment of antigen I/II which has a consistent molecular weight (69,000 by gel filtration) and consists of 58% protein. This is broadly consistent with a molecular weight of the protein component of 48,000 on SDS-PAGE. Since a similar substance was obtained by treatment with different proteases, it is likely that free antigen II also exists naturally, at least in culture fluids, due to the action of endogenous proteases on extracellular antigen I/II. Although evidence for this has been observed (22), it does not appear that such proteolytic action is prominent, because no free antigen II was recovered during

the preparation of antigen I/II. The proteolysis resistance of antigen II despite its largely protein nature is reminiscent of antigen III, which is also a protein (20a). However, these two antigens are clearly distinguishable, both serologically and physicochemically. There was no evidence for the association of carbohydrate with the antigen II band on SDS-PAGE, as specific carbohydrate stains failed to react.

The separation of free antigen I from antigen I/II has not yet been achieved, either by using dissociating agents or by enzymatic treatment. However, evidence for the existence of free antigen I moieties has been obtained. Fractions which react with antiserum to antigen I/II but not with antiserum to antigen II have been eluted from diethylaminoethyl cellulose (Russell, manuscript in preparation), and on SDS-PAGE of gel filtration-purified antigen I/II band C also reacted in this way. Whether these substances represent partial breakdown products of antigen I/II or the presence of antigen I determinants on other carrier molecules is being investigated.

Immunofluorescence with antisera to antigens I/II and II has shown that these antigens are located at the cell surface of *S. mutans* and perhaps in the cell wall. A cellular location was deduced previously by absorbing antisera with whole organisms (22), and antigen I/II, as well as antigen III, can be extracted from cells with urea (20a).

Immunofluorescence has also confirmed and extended the original observations on the serotype distribution of antigens I/II and II (22). Antiserum to antigen II reacted with *S. mutans* serotypes *c*, *e*, and *f*, but not with other serotypes. Remarkably however, antiserum to antigen I/II reacted not only with *S. mutans* strains of all serotypes except *b*, but also with representatives of other oral streptococci, although such cross-reactions were weak. *S. mutans* serotypes *c*, *e*, and *f* are related genetically (7), serologically (3), and biochemically (20), so it was perhaps not surprising that they all possessed antigens I and II; they also share antigen III (20a). It also appeared that serotypes *a*, *d*, and *g*, which are serologically cross-reactive (20), possessed antigen I or a related antigen, but not antigen II. This was further evidence that the antigen I determinant could exist separately from antigen II. Furthermore, it raises questions as to what, if anything, replaces antigen II in these serotypes and what other antigens may be present in serotype *b*, which appears to lack antigens I and II, although it possesses antigen III (20a). It is also remarkable that antigen I, although not antigen II, seemed to be present in *S. sanguis* OMZ9, *S. salivarius* HHT, and *Streptococcus* CHT. Cross-reactions between different species of oral streptococci have been noted frequently. Undoubtedly, many of these can be ascribed to glycerol teichoic acids (6). Other workers have reported serological cross-reactions between serotypes of *S. mutans* (4, 9) or between *S. sanguis* and *S. mutans* (11), but the properties of these cross-reactions do not appear to agree with explanations based on antigens I/II and II. Recently, another protein antigen was identified in *S. mutans* serotype *c* (23). Although its molecular weight and isoelectric point are close to those of antigen I/II, it appears to be totally susceptible to proteolysis, and its serotype distribution is slightly different from that of antigen I/II.

Antigens I/II and II are highly immunogenic when injected into rabbits and monkeys. Antibodies to both determinants have been found in rhesus monkeys which have been immunized with whole cells or cell wall preparations of *S. mutans* and show protection against dental caries (Russell et al., in press). The interpretation that these antigens may be involved in immunity to dental caries can now be examined directly, and experiments toward that end are in progress.

ACKNOWLEDGMENTS

We thank Christine Powell for her excellent technical assistance and P. Toseland and N. Dalton for the amino acid analyses.

This work was supported by grants from the Medical Research Council and the National Research Development Corp.

LITERATURE CITED

1. **Andrews, P.** 1965. The gel-filtration behaviour of proteins related to their molecular weights over a wide range. Biochem. J. **96**:595–606.
2. **Bowden, G. H., J. M. Hardie, and E. D. Fillery.** 1976. Antigens from *Actinomyces* species and their value in identification. J. Dent. Res. **55**(Special Issue A):A192–A204.
3. **Bratthall, D.** 1970. Demonstration of five serological groups of streptococcal strains resembling *Streptococcus mutans*. Odontol. Revy **21**:143–152.
4. **Bratthall, D., and B.-M. Petterson.** 1976. Common and unique antigens of *Streptococcus mutans*. J. Dent. Res. **55**(Special Issue A):A60–A64.
5. **Chen, P. S., T. Y. Toribara, and H. Warner.** 1956. Micro-determination of phosphorus. Anal. Chem. **28**: 1756–1758.
6. **Chorpenning, F. W., H. R. Cooper, and S. Rosen.** 1975. Cross-reactions of *Streptococcus mutans* due to cell wall teichoic acid. Infect. Immun. **12**: 586–591.
7. **Coykendall, A. L.** 1977. Proposal to elevate the subspecies of *Streptococcus mutans* to species status, based on their molecular composition. Int. J. Syst. Bacteriol. **27**: 26–30.
8. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
9. **Grenier, E. M., W. C. Eveland, and W. J. Loesche.** 1973. Identification of *Streptococcus mutans* serotypes in dental plaque by fluorescent antibody techniques.

Arch. Oral Biol. **18**:707–715.

10. **Hardie, J. M., and G. H. Bowden.** 1974. Cell wall and serological studies on *Streptococcus mutans*. Caries Res. **8**:301–306.
11. **Hardie, J. M., and G. H. Bowden.** 1976. Some serological cross-reactions between *Streptococcus mutans*, *S. sanguis* and other dental plaque streptococci. J. Dent. Res. **55**(Special Issue C):C50–C58.
12. **Hladny, J., K. H. Schleiffer, and O. Kandler.** 1972. Die Aminosäuresequenz der threoninhaltigen Mureine einiger Streptokokken. Arch. Mikrobiol. **85**:23–38.
13. **Joseph, R., and G. D. Shockman.** 1975. Synthesis and excretion of glycerol teichoic acid during growth of two streptococcal species. Infect. Immun. **12**:333–338.
14. **Knox, K. W., and A. J. Wicken.** 1973. Immunological properties of teichoic acids. Bacteriol. Rev. **37**:215–257.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
16. **Lehner, T., S. J. Challacombe, and J. Caldwell.** 1976. Immunological basis for vaccination against dental caries in rhesus monkeys. J. Dent. Res. **55**(Special Issue C):C166–C180.
17. **Linzer, R.** 1976. Serotype polysaccharide antigens of *Streptococcus mutans*: composition and serological cross-reactions, p. 91–99. *In* W. H. Bowen, R. J. Genco, and T. C. O'Brien (ed.), Immunologic aspects of dental caries. Information Retrieval Inc., Washington, D.C.
18. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
19. **Markham, J. L., K. W. Knox, A. J. Wicken, and M. J. Hewett.** 1975. Formation of extracellular lipoteichoic acid by oral streptococci and lactobacilli. Infect. Immun. **12**:378–386.
20. **Perch, B., E. Kjems, and T. Ravn.** 1974. Biochemical and serological properties of *Streptococcus mutans* from various human and animal sources. Acta Pathol. Microbiol. Scand. Sect. B **82**:357–370.

20a. **Russell, M. W.** 1979. Purification and properties of a protein surface antigen of *Streptococcus mutans*. Microbios **25**:7–18.

21. **Russell, M. W., J. Caldwell, and T. Lehner.** 1979. Antibody response to *Streptococcus mutans* antigens during immunisation against dental caries, p. 217–218. *In* M. T. Parker (ed.), Pathogenic streptococci. Reedbooks, Chertsey, England.
22. **Russell, M. W., and T. Lehner.** 1978. Characterisation of antigens extracted from cells and culture fluids of *Streptococcus mutans* serotype *c*. Arch. Oral Biol. **23**: 7–15.
23. **Russell, R. R. B.** 1979. Wall-associated protein antigens of *Streptococcus mutans*. J. Gen. Microbiol. **114**:109–115.
24. **Schmidt-Ullrich, R., W. S. Thompson, and D. F. H. Wallach.** 1977. Antigenic distinctions of glycoproteins in plasma and mitochondrial membranes of lymphoid cells neoplastically transformed by simian virus 40. Proc. Natl. Acad. Sci. U.S.A. **74**:643–647.
25. **Showe, M. K., E. Isote, and L. Onorato.** 1976. Bacteriophage T4 prehead proteinase. Its cleavage from the product of gene 21 and regulation in phage-infected cells. J. Mol. Biol. **107**:55–69.
26. **Studier, F. W.** 1973. Analysis of bacteriophage T7 early RNAs and proteins on slab gels. J. Mol. Biol. **79**:237–248.
27. **Tsuji, A., T. Kinoshito, and M. Hoshino.** 1969. Analytical chemical studies on amino sugars. II. Determination of hexosamines using 3-methyl-2-benzothiazolinone hydrazine hydrochloride. Chem. Pharm. Bull. **17**:1505–1510.