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Antigens of *Streptococcus sanguis*

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An antigenic analysis of the alpha-hemolytic streptococci isolated from dental plaque was performed by use of antisera against a strain of *Streptococcus sanguis* (M-5) which was isolated from dental plaque. Immunoelectrophoretic and Ouchterlony tests of Rantz and Randall extracts of 45 strains gave positive reactions with the M-5 antisera. These strains represented 60% of the strains tested. The number of antigens which could be identified in these extracts varied from one to five and were designated *a* to *e*. The *a* antigen was found in 36 of the strains tested, including reference strains of *S. sanguis* and the group H streptococci. The strains reacting with the M-5 antisera were divided into two majors types: type I consisted of 23 strains in which the *a* antigen was found alone or with one or more of the *c*, *d*, and *e* antigens; type II consisted of 13 strains in which both the *a* and *b* antigens were found with or without one or more of the *c*, *d*, and *e* antigens. The remaining strains contained, either singly or in combination, the *b*, *c*, *d*, and *e* antigens but not the *a* antigen. Biochemical tests of representatives of each serotype and reference strains indicated that strains reacting with M-5 antisera were *S. sanguis*. These findings suggest that *S. sanguis* strains share common physiological and serological properties.

The history of studies of the alpha-hemolytic streptococci is replete with examples suggesting that they are a heterogeneous group of organisms, both physiologically and serologically (13, 15, 22, 23, 28). However, recent extensive physiological studies (8) on the classification of the alpha-hemolytic streptococci, particularly those colonizing the teeth, indicated that several distinct species could be recognized. The most important of these species in terms of prevalence and relation to dental disease were *Streptococcus sanguis* and *S. mutans*. Although recent reports indicated that the latter species can be divided into several serological groups (4, 6, 29), the serology of *S. sanguis* has still not been defined satisfactorily.

The early studies of *S. sanguis* strains suggested that they contained the Lancefield group H antigen (11). Group H was originally described as containing strains from the human pharynx that caused beta-hemolysis on blood agar but, in contrast to strains of Lancefield groups A, B, and C, did not produce a soluble hemolysin (12). The strains of *S. sanguis* described recently have usually been isolated from dental plaque and produced alpha-hemolysis. These strains were similar to strains isolated from the blood of patients with endocarditis (25) and were said to possess type antigens rather than a group antigen (24). Since authentic group H antisera have not been available generally, it has proved difficult to determine whether *S. sanguis* is indeed associated with Lancefield group H.

The studies described in this report were initially undertaken to develop a serological classification which could be generally applied to the alpha-hemolytic streptococci isolated from dental plaque. During these investigations, it was observed that extracts of 60% of the organisms tested reacted with antisera against an oral streptococcus designated M-5. Among the strains which reacted with these antisera were reference strains of *S. sanguis* and group H streptococci. The object of these investigations was to apply the high resolution techniques of gel diffusion to studies of *S. sanguis* to determine whether common antigens useful in identifying these organisms could be found.

MATERIALS AND METHODS

Bacterial strains and media. Strains of streptococci were isolated from the dental plaque of human patients. The plaque samples were serially diluted in 0.15 M NaCl prior to plating on Mitis-Salivarius Agar (Difco). Duplicate plates were incubated aerobically and anaerobically at 37 C for 48 hr, and three or four colonial variants were chosen for subculture. The primary emphasis was to select a limited number of

colonial variants from a number of patients rather than exhaustively studying strains isolated from each patient. The 62 strains isolated from 26 patients were preserved by lyophilization after ascertaining that each culture represented a single colony isolate by repeated selection of single colonies.

In addition to oral isolates, the following reference strains were used: *S. mitis* ATCC 903, 9811, and 6249; *S. sanguis* ATCC 10556, 10557, and 10558; group H streptococcal strains Challis-6, Wicky (We-4), F90A, K208, and Blackburn (the latter strains supplied by J. Ranhand and Roger Cole, National Institute of Allergy and Infectious Disease, Bethesda, Md.); and *S. mutans* FA-1, PK-1, LM-7, HS-6, and GS-5 (supplied by R. J. Gibbons, Forsyth Dental Center, Boston, Mass.). Brain heart infusion broth (Difco) was used to grow cultures for the preparation of antisera and for the extraction of antigens.

Preliminary descriptions of isolated strains were based on four factors: colonial morphology on Mitis-Salivarius Agar, colonial morphology and type of hemolysis on 5% horse blood agar, cellular morphology and arrangement in Gram-stained smears, and a negative catalase test. Subsequent to serological analysis, further identification of selected oral isolates and reference strains was performed. The following tests were used: growth in 4% NaCl; hydrolysis of esculin and arginine; final *p*H in 1% glucose and sucrose broths; the fermentation of inulin, lactose, mannitol, melibiose, raffinose, salicin, sorbitol, and trehalose; and the production of an ethanol-insoluble substance in 5% sucrose (dextran). The absence of ethanol-insoluble substances in the supernatant fluid of cells grown in 5% glucose broth was used as a control for the latter test. These tests represented an abbreviated series of biochemical tests adapted from the extensive physiological studies of Carlsson (8) and DeStoppelaar (10). The methods employed for the medium preparation and tests were those described by Carlsson (8).

Serological methods. Vaccines were prepared from cells grown in brain heart infusion broth for 18 hr and washed three times in 0.15 M NaCl. Cell suspensions (0.4 mg/ml in 0.15 M NaCl containing 0.2% Formalin) were injected intravenously into female albino rabbits twice weekly for 4 weeks, starting at 0.2 ml and increasing to 1.0 ml for the last four injections. Rabbits were bled by heart puncture 5 days after the last injection. Preimmune sera were obtained from all rabbits, and commercial antisera against Lancefield groups A through N (Difco) were used as controls.

Washed cells were extracted in 0.15 M NaCl at 121 C according to the method of Rantz and Randall (21). Extracts were also prepared from supernatant fluids of cells disrupted at 50,000 psi in a Ribi Cell Fractionator (Ivan Sorvall, Norwall, Conn.). The extracts were dialyzed and lyophilized; these crude antigen preparations were dissolved in water at concentrations of 40 mg/ml. This high concentration was employed because both weak and strong bands could be detected; dilution of the extracts often resulted in loss of the weaker reactions, presumably because the concentration of these antigens was low in the original extract. Controls for specificity were Rantz and Randall extracts of *Lactobacillus casei* strain L324M, *S. faecalis* strain S161, and *Escherichia coli* strain 121. In addition, commercial antigen extracts of Lancefield groups A through N (Difco) were used as controls. The modification of the Ouchterlony technique described by Campbell et al. (7), employing borate buffers, *p*H 8.4, and an ionic strength of 0.1 μ, was used to resolve the mixture of streptococcal antigens. Increased sensivitity was achieved by the incorporation of 0.0125% $CdSO_4$ in the agar (9). A 1:10 dilution of the borate buffer was employed in the buffer chambers for immunoelectrophoretic studies; the voltage used was 11 v/cm.

In addition to the Rantz and Randall method, the following extraction procedures were used in this study: 10% trichloroacetic acid (2, 26), phenol-water (27), formamide (14), 0.1 N NaCl, 0.01 N HCl (16), 1 N NaOH, and deoxycholate (3). In all cases, the extracts were dialyzed against water and lyophilized.

RESULTS

Selection of antisera. Antisera against 20 strains were screened by Ouchterlony techniques with extracts (*see below*) of the same cells used as a source of antigen. With the exception of strain M-5, an isolate from dental plaque, the antisera were relatively specific for the immunizing strains and therefore were not suitable for grouping. In contrast, the antiserum against strain M-5 reacted with several antigens which were also found in many extracts of heterologous oral streptococci. Injection of Formalin-treated M-5 vaccines has consistently yielded similar Ouchterlony and immunoelectrophoretic patterns in 18 rabbits immunized with this vaccine. Therefore, extracts of strain M-5 and homologous pooled antisera were employed for further investigation.

The controls for gel diffusion tests were concentrated growth medium, extracts of *L. casei*, *S. faecalis*, and *E. coli*, and the commercial streptococcal antigen preparations; none of these antigens reacted with the M-5 antisera. In addition, neither the preimmune sera nor commercial streptococcal antisera reacted with the M-5 extract. Extracts of *S. mutans* also failed to react with the M-5 antisera.

Extraction of the antigens. Extracts for examination by Ouchterlony and immunoelectrophoretic methods were obtained by the procedures described in Materials and Methods. The supernatant fraction of disrupted cells yielded a variety of antigens which were detected in gel diffusion analysis. However, the patterns were so complicated that even with the resolution achieved by immunoelectrophoresis interpretation was difficult.

The extracts obtained by the autoclave method of Rantz and Randall (21) gave fewer bands than the supernatant fraction of dis-

rupted cells. The other methods described in Materials and Methods either did not extract all the antigens detected in the Rantz and Randall extracts or caused a loss in their activity.

Antigenic analysis of strain M-5. Immunoelectrophoretic studies of the Rantz and Randall extracts of M-5 with homologous antisera revealed five precipitin bands (Fig. 1A). The precipitin bands which gave the strongest reaction (based on density) were designated *a* and *b*; the *a* antigen showed a greater mobility toward the positive pole than the *b* antigen. The antigens showing weaker reactions were labeled *c*, *d*, and *e*. The *c* and *d* antigens were neutral or had a slight positive charge; the *e* antigen showed a broad precipitin band from the center well toward the negative pole. It was possible that partial antigenic relationships existed between some of these weakly reactive antigens, but these relationships could not be evaluated fully because of the low intensity of some of the precipitin bands. As shown in Fig. 1B, the immunoelectrophoretic pattern of the extract of strain M-5 closely resembles the pattern shown by an extract of *S. sanguis* ATCC 10558. The identity of these reactions was confirmed by Ouchterlony tests.

Serological analysis of Rantz and Randall extracts with M-5 antisera. The extracts of 45 of the 75 streptococcal strains examined reacted to some extent with the M-5 antisera. In 13 of these strains, shown in Fig. 2A, only the *a* antigen was detected. The immunoelectrophoretic precipitin band shown in Fig. 2B for *S. mitis* ATCC 903 was typical for these strains. Although differences in intensity of reactions were observed among the extracts, these differences could not be evaluated in quantitative terms. However, it is possible that quantitative variation of the antigens does occur in different strains.

Another group of nine strains (Fig. 3A) contained one or more of the *c*, *d*, and *e* antigens in addition to the *a* antigen. The typical immunoelectrophoretic pattern for these strains is that shown for strain B-4 in Fig. 3B. Since the *a* antigen was the most common and easily identifiable antigen detected in these first two groups, they have been combined and designated type I.

A third group of six strains contained both *a* and *b* antigens (Fig. 4A and B) in addition to one or more of the *c*, *d* and *e* antigens. The *a* and *b* antigens in the Ouchterlony pattern are identified by reference to the bands formed, respectively, by strains 903 and 10556. The latter extract was obtained from *S. sanguis* ATCC 10556, in which the *b* antigen was the only antigen detected (Fig. 4C). One oral streptococcal extract also showed only the *b* antigen. In the strains shown in Fig. 4A and B, the intensity of the reaction with the *a* and *b* antigens often

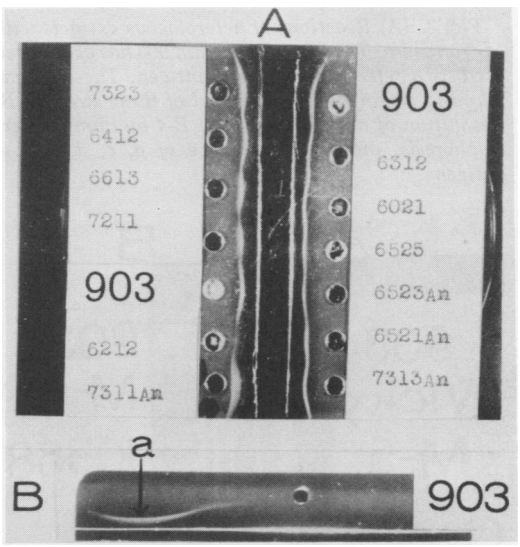

FIG. 2. *(A) Reactions of heterologous extracts with M-5 antisera. S. mitis ATCC 903 was used as a reference strain for the a antigen and is designated in large heavy type. The smaller type refers to oral isolates. (B) Immunoelectrophoretic pattern of an extract of strain 903 with M-5 antisera, identifying the negatively charged a antigen.*

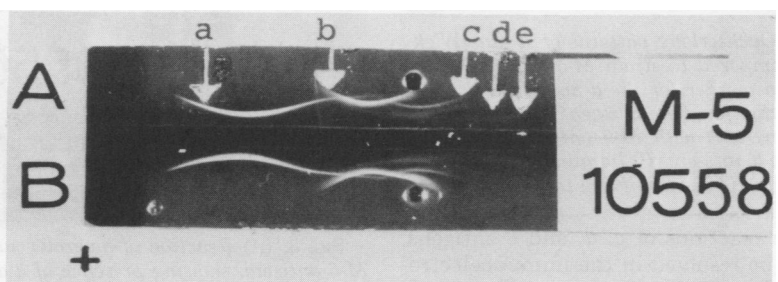

FIG. 1. *(A) Immunoelectrophoretic patterns of extracts of M-5. (B) Immunoelectrophoretic pattern of S. sanguis ATCC 10558.*

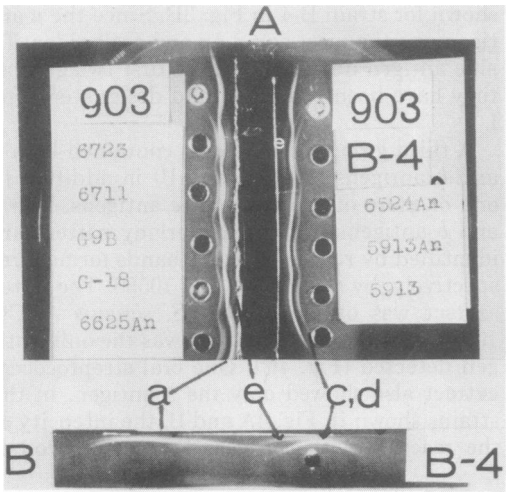

FIG. 3. *(A) Reactions of heterologous extracts with M-5 antisera. The extract of strain 903 has again been used as a reference for the a antigen. The antigens found in strain B-4 were typical of these strains. (B) Resolution of antigens of strain B-4 by immunoelectrophoresis, indicating presence of a, c, d, and e, antigens.*

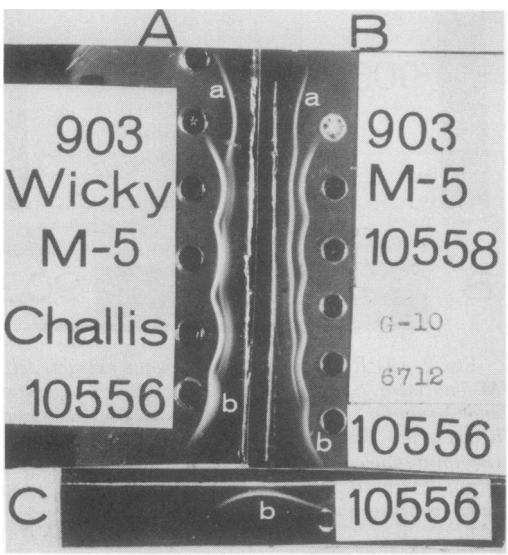

FIG. 4. *(A) Ouchterlony patterns of strains Wicky and Challis compared to strain M-5. Strain 903 has been used as a marker for the a antigen and strain 10556 as a marker for the b antigen. (B) Reactions of heterologous extracts with M-5 antisera, indicating presence of the b antigen. (C) Immunoelectrophoretic pattern of strain 10556 identifying the b antigen.*

obscured the reactions of *c*, *d*, and *e* antigens. These could be resolved in the immunoelectrophoretic studies of these extracts. The Ouchterlony pattern of extracts of *S. sanguis* strains Challis and Wicky is also shown in Fig. 4A. The double bands for the *a* antigen in the latter extracts were also seen in the immunoelectrophoretic patterns. Whether this pattern was an artifact or real could not be ascertained; otherwise, these strains appeared to be serologically similar to those shown in Fig. 4B. Thus, all of these strains appeared to be antigenically most closely related to strain M-5.

The immunoelectrophoretic patterns of the strains shown in Fig. 5A appeared to be identical to that shown for strain 6223 (Fig. 5B), in which the *a* and *b* antigens were the only ones detected. Despite the differences in the presence or absence of *c*, *d*, and *e* antigens, the 12 strains shown in Fig. 4 and 5 all gave bands with the *a* and *b* antigens. Therefore, these 12 strains were considered to be a serological unit which was designated type II.

In extracts of the remaining nine strains (Fig. 6), the antigens detected were *c* or *d*, or both. The immunoelectrophoretic pattern of an extract of *S. sanguis* ATCC 10557 was typical for the majority of these strains. However, two strains, M-21 and B-15, appeared to contain small quantities of *b* antigen. The reaction of this group of strains was generally much less intense than those observed for strains of types I and II. However, in the absence of other demonstrated antigenic relationships, even these reactions could prove useful. This last group was considered to be heterogeneous and

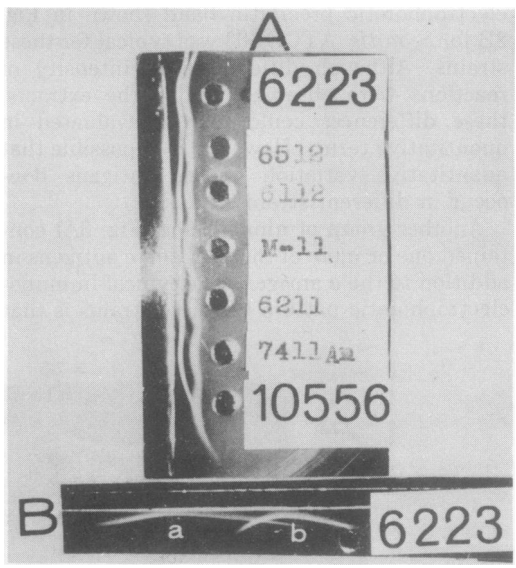

FIG. 5. *(A) Reaction of heterologous extracts with M-5 antisera, showing presence of a and b antigens. (B) Typical immunoelectrophoretic pattern for strains shown above.*

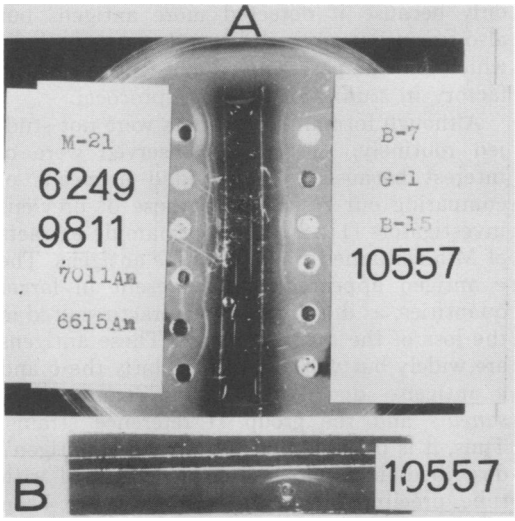

FIG. 6. *(A) Reactions of heterologous strains with M-5 antisera. (B) Immunoelectrophoretic pattern of strain 10557 which was typical for most strains shown above.*

included reference strains *S. mitis* 9811 and 6249, and *S. sanguis* 10557. Tentatively included among this group was *S. sanguis* strain 10556 and an oral isolate in which only the *b* antigen was detected.

A summary of the serological data is shown in Table 1. The designation of "constant" and "variable" was used to avoid terms which would connote quantitative evaluation, which was not possible with the techniques used. It was obvious, however, that the *a* antigen was the most widely distributed among these strains and that strains of types I and II, at least, were antigenically similar.

Reactions with other representatives of group H streptococci. Subsequent to the data shown in Table 1, cultures of group H streptococcal strains K208, F90A, and Blackburn were obtained. Rantz and Randall extracts of these organisms were tested with M-5 antisera. Strain K208 did not react with the M-5 antisera. Cole has found that this strain reacts only with homologous antisera (Roger Cole, *personal communication*). Strain F90A contained the *a* and *e* antigens (Fig. 7A), and strain Blackburn contained the *a*, *b*, and *e* antigens (Fig. 7B). Thus, strain F90A would conform antigenically to the type I group, whereas strain Blackburn would be placed in the type II group.

It is also of interest that the *e* antigen found in Rantz and Randall extracts of strain M-5 is identical both electrophoretically and in Ouchterlony tests to an antigen extracted from these cells by the formamide procedure. This method also extracts some of the *a* and *b* antigens (Fig. 8).

Physiological analysis. Based upon previously published physiological characteristics established for some of the reference strains (8), it was conceivable that only strains of *S. sanguis* were reacting with M-5 antisera. To test this possibility, all of the ATCC reference strains, five selected oral isolates representing each serotype, and all of the *S. mutans* strains were examined physiologically.

Strain M-5 was found to be physiologically most similar to *S. sanguis* strain 10558. Neither strain grew in broth containing 4% NaCl. Both strains hydrolyzed esculin and the final *p*H in 1% glucose broth was 4.5; both strains produced an ethanol-insoluble precipitate in 5% sucrose broth (presumably dextran); neither strain fermented mannitol, sorbitol, melibiose, or raffinose. Both strains fermented inulin, lactose, salicin, sucrose, and trehalose. Strain 10558 released NH_3 from arginine whereas strain M-5 did not.

The strains which reacted with M-5 antisera were variable in some physiological properties. However, all of the strains produced dextrans in 5% sucrose broth and none of the strains fermented mannitol and sorbitol.

Some of the reference strains (*S. sanguis* 10556, 10557, and 10558; *S. mitis* 903 and 9811)

TABLE 1. *Summary of serological types observed with M-5 antisera*

Serological type	Constant antigens	Variable antigens	No. of strains
I	*a*	*c, d, e*	22
II	*a, b*	*c, d, e*	12
Heterogeneous		*b, c, d*	11

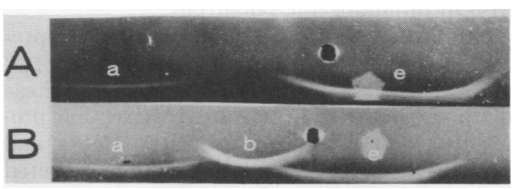

FIG. 7. *(A) Immunoelectrophoretic pattern of strain F90 A, showing a and e antigens. (B) Immunoelectrophoretic pattern of strain Blackburn, indicating presence of a, b, and e antigens.*

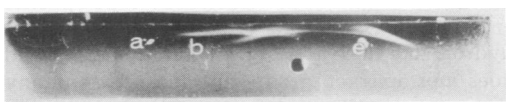

FIG. 8. *Immunoelectrophoretic pattern of formamide extract of strain M-5, indicating presence of b, e, and some a antigen.*

had been studied previously by Carlsson (8), who suggested that the last two strains more closely resembled *S. sanguis*. The physiological reactions of all of these reference strains were the same as those reported by Carlsson. The reactions of *S. mutans* strains also matched the published characteristics of this species (8, 10). The results of these studies suggested that only strains resembling *S. sanguis* reacted with M-5 antisera.

DISCUSSION

The major goal of these studies was the identification of antigens which might be important in the serology of *S. sanguis*. It might be argued that the use of a single strain as a source of antisera minimizes the usefulness of the results, but such arguments stem from the necessity of early serologists to explore the complex antigenic mosaics of bacterial cells indirectly by means of cross-absorptions. The ability to demonstrate directly the patterns of complex antigenic mixtures by gel diffusion eliminates the necessity for such indirect methods.

The use of specific strains for the production of reference antisera is not unusual, since it is often impossible to predict whether a given strain or animal will produce type- or group-specific sera (22). One reason that controversy still surrounds the serology of *S. sanguis* and its relation to group H might be the difficulty in producing uniform antisera. Antisera prepared by injecting Formalin-treated M-5 cells have consistently yielded the same precipitin pattern in gel diffusion. In contrast, vaccines prepared from viable cells, cell homogenates, and heat-killed cells did not detect all of the antigens (*unpublished data*). It is likely that a suitable search would reveal other strains equal to M-5 in immunogenic potential.

Studies of *S. sanguis* and group H streptococci have employed either Lancefield or formamide extracts (11, 12, 24). In addition to these methods, other routinely used extraction procedures were tried, including the Rantz and Randall procedure. The latter method detected five antigens in homologous extracts, whereas the other techniques detected fewer antigens. There is ample evidence that many of these routine methods can affect specific kinds of antigens; e.g., HCl destroys type-specific antigens in *S. faecalis* (18), teichoic acids are hydrolyzed in NaOH (1), trichloroacetic acid does not extract group antigens from many streptococci (17), and formamide destroys the serological activity of the type antigens of group D streptococci (5). The Rantz and Randall method was chosen for routine analysis not only because it detected more antigens but also because it seemed illogical to use methods which in the past proved not entirely satisfactory in studies of these streptococci.

Although formamide extracts were not studied routinely, the results observed were of interest because they provided a means of comparing our results with those of previous investigators (11, 24). The formamide extracts of M-5 contained the *a*, *b*, and *e* antigens. The *e* antigen appeared to be present in larger quantities, as dilution of the extract resulted in the loss of the *a* and *b* bands. These antigens are widely but variably (particularly the *b* and *e* antigens) distributed among strains of *S. sanguis* and the group H reference strains. Thus, it is possible that the "group H antigen" described in previous studies performed with tube precipitin techniques might have been due to reactions with one or more of the *a*, *b*, and *e* antigens.

Because of its wide distribution, the most likely choice for the group antigen of *S. sanguis* based on the present studies is the *a* antigen; this antigen is probably a glycerol teichoic acid (B. Rosan, Int. Ass. Dent. Res. Proc., 1972, p. 265). The remaining antigens are thought, at present, to represent type-specific antigens. The distribution of these type antigens among strains containing the *a* antigen indicated at least three categories: those strains which contain the *a* antigen but not the *b* antigen (type I), those containing the *a* and *b* antigens (type II), and strains in which the *a* antigen is absent. The presence of more than one type antigen in some strains is not surprising; Farmer reported similar findings (11), and streptococci belonging to other serological groups frequently have more than one type-specific antigen (19). It is hoped that the proposed serological classification for *S. sanguis*, though still preliminary, might prove useful both in clinical studies of the pathogenesis of dental disease and in studies of the genetics of these organisms (20).

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