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Relationship of the Cell Wall Composition of Group H Streptococci and *Streptococcus sanguis* to Their Serological Properties

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Previous studies indicated the *a* antigen was widely distributed among strains of *Streptococcus sanguis* and the group H streptococci. The cell walls of strains containing this antigen had moderate to large quantities of rhamnose, small amounts of phosphorus, and little to no ribitol. The molar ratios of the peptidoglycan amino acids and hexosamines suggested a di-alanyl cross bridge. The homogeneity of the walls of these strains suggested that serological group H can be considered synonymous with *S. sanguis*. In contrast those strains that did not contain the *a* antigen had only small amounts of rhamnose in their cell walls, galactosamine and ribitol were always detected, and large quantities of phosphorus were present. The molar ratios of the peptidoglycan components in the latter strains suggested a direct alanyl-lysl cross bridge. Although many of these strains had been classified either as *S. sanguis* or group H streptococci because of minor serological cross-reactions and similar biochemical properties, the distinct differences between the composition of their cell walls and those of *S. sanguis* and group H streptococci indicate that they do not belong in this species or group. The cell walls of strains containing the *a* antigen are relatively homogeneous and therefore it is suggested that cells containing this antigen be considered Lancefield serological group H.

In previous studies of the serology of *Streptococcus sanguis* five antigens designated *a* to *e* were found in autoclaved extracts of whole cells (16). The *a* antigen was the most widely distributed. Subsequent studies of reference strains of Lancefield group H and other reference strains of *S. sanguis* confirmed these earlier observations and it was speculated that the *a* antigen might represent the group H antigen (16a).

Although not a prerequisite for defining a serological group, the taxonomic importance of a serological group is considerably enhanced if other biological properties are correlated with the serological relationships. Cole et al. (3a) have shown recently that the proposed serological classification for *S. sanguis* and group H streptococci is closely correlated with the adsorption of bacteriophage, susceptibility to group C phage lysin, lysogeny, and the presence of certain neutral sugars in the cell walls.

The chemical composition of the cell walls of the viridans streptococci including organisms designated *S. sanguis*, group H streptococci, and *S. mitis (S. mitior)* has been investigated by several groups (4, 20; J. M. Hardy, Ph.D. thesis, Univ. of London, London, 1975). Most studies involved qualitative analysis of the neutral sugars of the cell walls; the results of these studies suggested that the strains could be divided into two to three groups based on the presence or absence of rhamnose, glucose and/or galactose, and ribitol. Although Slade and Slamp (20) and more recently Hardie (Ph.D. thesis, 1975) attempted to relate serological activity to cell wall composition, they were only partially successful. Part of the difficulties stemmed from their inability to establish a common antigen among the group.

Since the *a* antigen appeared to exhibit the kind of specificity required for a group antigen, a survey of the chemical composition of the cell walls of strains of *S. sanguis* and group H streptococci was undertaken. The cell walls determine not only the biological properties described by Cole et al. (3a) but also are believed to be important in the rapid and specific adhesion of *S. sanguis* to tooth surfaces. The purpose of these studies was to establish the relationship of the cell surfaces to the serological properties and thus provide a firm founda-

tion for the taxonomic status of *S. sanguis* and the Lancefield group H streptococci.

MATERIALS AND METHODS

Strains and media. The strains studied, their sources, serotype, and the antigenic composition are listed in Table 1. Cole et al. (3a) have recently traced the genealogy of many of the reference strains of group H and *S. sanguis* and thus where possible the various designations used for the strains have been shown in Table 1. The strain designations used in the text are those which would be familiar to most investigators rather than designations applied by individual laboratories. It was because of this latter type of labeling that it was not known that many of the strains had the same origin though they were obtained from different sources. The strains received from Cole were subjected to biochemical screening tests by R. Facklam, Streptococcus Laboratory, Center for Disease Control, Atlanta. These strains, as well as the oral isolates (16), were identified as *S. sanguis*. Cultures were routinely checked for purity cytologically, culturally, and serologically.

The cells used for the preparation of the walls were grown at 37 C for 18 h in 50 liters of brain heart infusion broth (Difco) containing 0.5% glucose; the cells were harvested by centrifugation in a Sharples continuous flow centrifuge and washed three times in 0.15 M NaCl. The yield of cells was approximately 0.5 g/liter (dry weight) for each strain.

Preparation of the cell walls. The packed cells were suspended in 300 to 400 ml of 0.15 M NaCl and disrupted in a Ribi cell fractionator (Ivan Sorvall, Inc., Newtown, Conn.) at 55,000 lb/in^2. The temperature at the needle valve was maintained between 5 to 15 C. The crude cell walls were separated from the cell sap by centrifugation at 16,300 $\times$ g. At this stage two distinct layers could be discerned; a light-gray upper layer and a dark-gray lower layer. Examination of the layers by electron microscopy indicated that the upper layer contained mostly cell walls, mixed with some whole cells and cytoplasmic debris. The lower layer contained a mixture of whole cells and some cell walls. Careful removal of the upper layer and recentrifugation concentrated the cell wall layer; the procedure was repeated several times until electron microscope examination indicated the absence of whole cells. Although relatively much cleaner than the original preparation, cyto-

TABLE 1. *Sources of strains*

Strain	Alternate designation	Source	Serotype	Antigenic composition
B-4	—[a]	Plaque	1	*a, c, d*
G9B	—	Plaque	1	*a, c, d*
72x40	FW35	Cole[b]	1	*a, c, d*
72x43	FW225	Cole	1	*a, c, d*
66x49	F90A (Am. H)[c]	Cole	1	*a, e*
903	ATCC *S. mitis*	ATCC[d]	1	*a*
72x42	FW213	Cole	1	*a*
M-5	—	Plaque	2	*a, b, c, d, e*
10558	NCTC 7865[e]	ATCC	2	*a, b, c, d, e*
Blackburn	NCTC 10231	Cole	2	*a, b, c, d, e*
Challis	NCTC 7868	Cole	2	*a, b, c, d, e*
Wicky		Cole	2	*a, b, c, d, e*
72x29	Channon, NCTC 7869	Cole	2	*a, b, c, d, e*
6312	—	Plaque	2	*a, b*
6623	—	Plaque	2	*a, b*
10556[f]	NCTC 7863	ATCC	2	*b*
Enole	—	Kashket[g]	2	*a, b*
6249	—	ATCC	Heterogeneous[i]	*c, d*
9811	ATCC *S. mitis*	ATCC	Heterogeneous	*c, d*
10557	NCTC 7864	ATCC	Heterogeneous	*c, d*
72x35	NCTC 7864	Cole	Heterogeneous	*c, d*
72x41	*S. sanguis* II	Cole	Heterogeneous	*c, d*
71x50[h]	K208 (Am. H)	Cole		*e*

[a] No other designation.
[b] These cultures were provided by R. Cole, National Institute of Allergy and Infectious Diseases.
[c] American group H.
[d] American Type Culture Collection.
[e] National Type Culture Collection.
[f] This strain was originally placed in heterogeneous group but cell wall data suggest it belongs in serotype 2.
[g] This culture provided by Shelby Kasket, Forsyth Dental Infirmary, Boston, Mass.
[h] Does not react with M-5 antiserum.
[i] Heterogeneous group.

plasmic debris was still evident. This debris was removed by treatment with ribonuclease (Sigma Chemical Co., St. Louis, Mo.; 0.02 mg/ml), deoxyribonuclease (II, Sigma; 0.02 mg/ml), and trypsin (type III, Sigma; 0.40 mg/ml), according to the method of Cummins and Harris (6). This procedure removed protein surface antigens (9) and consequently these walls consisted primarily of peptidoglycan and polysaccharide components. Methods in which an attempt was made to obtain complete cell wall, including protein, did not yield electron microscopically clean wall preparations.

Chemical analysis. Phosphorus was determined by the method of Chen et al. (3); rhamnose was determined by the Dische and Schettles technique (7); the Rondle and Morgan (14) procedure was used for colorimetric estimation of hexosamines. Analyses for amino acids and hexosamines were performed by the method of Spackman et al. (22) as modified by Rosan (15). In this study an attempt was made to define the optimal conditions of hydrolysis for the determination of amino acids, hexosamines, and neutral sugars in cell wall. The walls of three strains were hydrolyzed in 6 N HCl at 100 C, 105 C, and 110 C at three time periods. Analysis of the data indicated that amino acid and hexosamine compositions obtained from cell walls hydrolyzed in 6 N HCl for 18 h at 100 C would be suitable for these studies. Multiple determinations (duplicate to quadruplicate) were done on 10 cell wall preparations. The average deviations ranged from 0.005 μmol/mg to 0.072 μmol/mg. The highest deviations were observed for alanine 0.072 at μmol/mg, lysine at 0.062 μmol/mg, and glucosamine at 0.038 μmol/mg. The deviations of all the other amino acids and hexosamines were 0.010 μmol/mg or less.

In addition to the colorimetric analysis for rhamnose, other neutral sugars and polyols were analyzed by gas chromatography. A modification of the alditol acetate method of Sawardeker et al. (17) was used. In this technique the sugars were reduced to the corresponding sugar alcohols, which were then acetylated. The modifications were primarily in the phases involving evaporation of methyl borate and extraction of the alditol acetates. After reduction of the sugars in the hydrolysate with $NaBH_2$ (an internal standard of inositol at a concentration of 4% is added before hydrolysis) the borate was removed by the following procedure. Glacial acetic acid was added and the sample evaporated. A second wash consisting of 25% methanol in glacial acetic acid was also evaporated, the third wash was 50% methanol, the fourth 75%, the fifth 90%, and finally 100% methanol was used. After the final evaporation of the methyl borate, the acetylation mixture of acetic anhydride and pyridine (dried over NaOH, 1:1) was added and the sample was refluxed at 80 C for 2 h. After acetylation, 1 ml of water and 3 ml of $CHCl_3$ were added and the tube was shaken; to this mixture 5 ml of 0.1 N HCl was added and the tube was again shaken. The aqueous layer was removed and discarded; this procedure was repeated twice. The same procedure was followed with a solution of 1 M $NaHCO_3$ and repeated three times. Finally, the $CHCl_3$ layer was extracted with 5 ml of water three times; the chloroform was evaporated over N_2 and the preparation was dissolved at a concentration equal to 50 μg of cell wall/μl.

Approximately 1-μl samples were injected into a Hewlett-Packard model 5700 gas chromatograph. Stainless-steel columns (0.32 by 183 cm) packed with Chromsorb 100/120 mesh coated with 3% OV225 (Applied Science Laboratories, State College, Pa.) were used for chromatography (9). The conditions for analysis were the following: (i) injection temperature, 250 C; (ii) flame ionization detector, 250 C; the oven was temperature-programmed from 160 to 200 C at 2 C/min; (iv) N_2 was used as a carrier gas at a flow rate of 60 ml/min.

Hydrolysis curves for maximal release of reducing sugars from the cell wall were determined using the method of Thompson and Shockman (23). This study used only strain M-5 cell wall and compared reducing sugars released at 1 and 2 N HCl at 100 C in vacuo for up to 8 h. It was observed that maximum release of reducing sugars was achieved by 8 h in 1 N HCl. However, it was apparent that these conditions did not cause the complete release of polyols in some strains. Studies of pneumococcal C polysaccharides (M. J. Bonner, Ph.D. thesis, Univ. of Penn., Philadelphia, 1974) indicated that up to 18 h of hydrolysis was required for maximal release of these components. However, these conditions resulted in too great a loss of other neutral sugars to be useful in cell wall surveys. Thus, the results for glycerol and ribitol were not quantitative. The gas chromatography assay was done in triplicate and the average deviations were generally less than 1%.

Serological methods. The autoclave method of Rantz and Randall (13) was used to extract antigens from whole cells. The techniques for vaccine preparation, antisera production, and gel diffusion methods have been previously reported (16).

RESULTS

Neutral sugar and polyol composition. In Table 2 the percentage of composition of neutral sugars, polyols, and phosphate of the cell wall is shown. Although there is a great variation among the strains in content of individual sugars, it is clear that two major groupings can be delineated. Those strains whose walls contain moderate to large quantities of rhamnose (14 to 30%), 0.6 to 1.0% phosphorus (except strain 6312), and little to no detectable ribitol (appears as anhydroribitol in gas chromatogram after hydrolysis [1]) belong to serotypes 1 and 2. In contrast, the strains in the heterogeneous group contained 1 to 5% rhamnose; detectable quantities of ribitol, 2 to 3% phosphorus, and galactosamine are found routinely. The presence of trace amounts of glycerol in nearly all the strains might reflect the presence of contaminating membrane lipoteichoic acids which have been reported even in highly purified cell walls (26).

Strains NCTC 7864 and 10557 were analyzed

TABLE 2. *Composition of cell walls*[a]

Strain[b] (serotypes 1 and 2)	Rhamnose[c]	Component glucose	Galactose	Ribitol	Glycerol	Phosphorus
B-4	24.4	3.7	1.0	ND[d]	+	0.93
G9B	23.6	1.9	0.7	ND	+	0.70
FW35	26.4	3.4	1.5	ND	+	0.67
FW225	16.7	4.0	3.8	ND	+	0.42
F90A	19.5	6.9	4.0	+	+	0.80
903	15.6	8.1	2.5	ND	+	0.61
FW213	32.5	0.7	ND	+[e]	+	0.74
M-5	18.5	5.5	0.3	ND	+	1.02
10558	23.4	3.3	0.2	+	+	0.64
Blackburn	24.1	2.1	0.1	ND	+	0.93
Challis	20.9	2.2	ND	ND	+	0.96
Wicky	13.8	1.7	0.9	+	+	0.99
Channon	25.7	0.6	0.1	ND	+	0.67
6312	24.9	15.4	ND	ND	+	1.40
6623	18.4	14.9	0.3	ND	+	0.99
10556	18.3	13.4	0.2	+	+	0.70
Enole	17.4	14.4	ND	ND	+	0.61
Heterogeneous						
6249	0.6	0.6	0.7	+	+	1.70
9811	4.9	1.0	1.4	+	+	3.68
10557	2.1	1.1	1.0	+	+	2.34
7864	2.1	4.3	3.9	+	+	2.53
S5723	2.0	1.5	1.2	+	+	2.08
K208	0.3	4.1	2.1	+	+	2.66

[a] Percent composition.
[b] Common designations used.
[c] Based on colorimetric determination.
[d] ND, Not detected.
[e] +, Detected in small amounts and generally the ribitol in types 1 and 2 was less than 0.5% and glycerol was 0.3%; in the heterogeneous group, glycerol was also about 0.3% whereas ribitol was 0.5% or greater.

before learning that both strains were originally derived from the same source and represented *S. sanguis* type II strains (5, 12); strain S 5723 also was a type II. Thus it was of interest that all three strains yield similar analytical data for neutral sugars, phosphorus, and amino acids.

The quantities of glucose and galactose seem to be quite variable among the strains. Strains 6623 and 6312 and Enole which have the *a* and *b* antigens contain approximately 15% glucose; strain 10556 which contains only the *b* antigen is very similar in composition to the latter strains and therefore chemically, at least, more closely resembles the strains of serotype 2 rather than the heterogeneous group into which it had been formerly placed (16). It is of interest that strain ATCC 10556 is the same as strain NCTC 7863; as shown in Fig. 1, the latter strain still contains the *a* and *b* antigens. The *a* antigen is a glycerol teichoic acid (Rosan, Int. Assoc. Dent. Res. Abstr. 873, 1972); strain 10556 contains the polyol backbone of the antigen but apparently has lost the determinant groups or has undergone some change in configuration which prevents it from reacting with the reference M-5 antisera. In any event, it seems clear that the cell walls of serotypes 1 and 2 can be distinguished from the cell walls of the heterogeneous group on the basis of rhamnose, phosphorus, and lack of detectable ribitol in their cell walls.

Amino acid and hexosamine composition. The initial studies of hydrolysis of cell wall used total hexosamine release to determine optimal conditions of hydrolysis. Hydrolysis was performed in 4 N and 6 N HCl and 100 C for varying periods of time. Both concentrations of acids yielded essentially the same results and indicated that most of the amino sugars were released after 2 h; no significant losses occurred up to 8 h. However, these conditions did not hydrolyze the peptides completely, thus we sought to determine hydrolytic conditions that would allow estimation of both hexosamines and amino acids at the same time.

To determine the optimal hydrolytic conditions we used the cell walls of strains 903, 6249, and Challis as representative of the different antigenic composition of the strains; 903 contained only the *a* antigen, 6249 the *c* and *d* antigens, and Challis contained the *a*, *b*, *c*, *d*, and *e* antigens. The results indicate that no consistent pattern of amino acid or hexosamine release and/or degradation is evident among these strains after hydrolysis. The differences

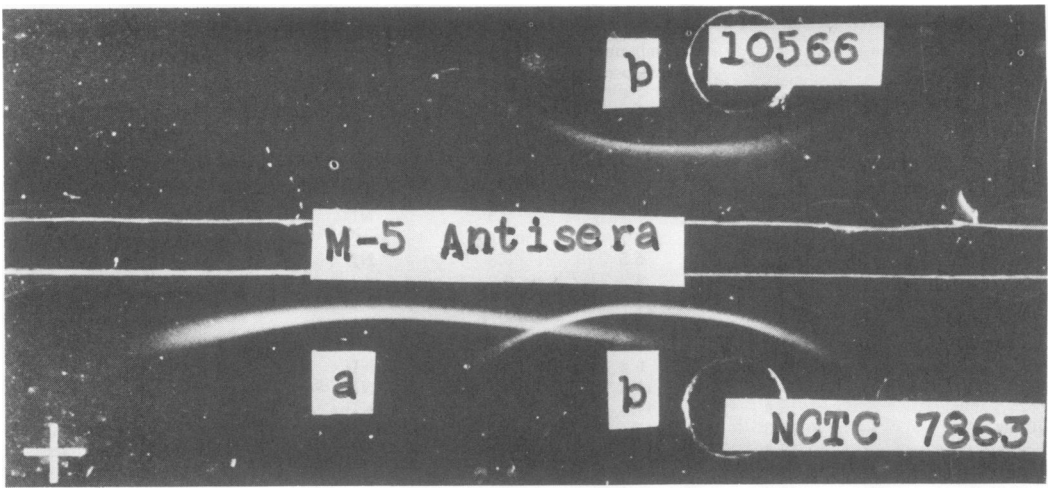

FIG. 1. *The top well contains autoclave extract of ATCC strain 10556 which was originally derived from NCTC strain 7863 (bottom well). The latter strain still contains determinants on the a antigen which are detected by M-5 antisera (center well) (16).*

in hydrolysis curves which are observed did not appear to be related to the specific amino acid or hexosamine, but rather to the cell wall itself. Thus, in Fig. 2c the release of glutamic acid and muramic acid for strain 903 is depicted. In this strain the muramic acid does not appear to level off but continues to increase during hydrolysis as does glutamic acid; the glutamic acid reaches approximately the same concentration under all three hydrolytic conditions. This behavior is clearly different from the two other strains studied; in strains Challis and 6249 (Fig. 2a and b) the muramic acid concentration reached a maximum and stayed constant during all three hydrolytic conditions. At 100 C both strains showed higher concentrations of muramic acid, suggesting less degradation of this hexosamine in cell walls at this temperature. The behavior is quite different from strain 903 which shows no loss of muramic acid at the higher temperatures. In fact there appears to be less muramic at 100 C in strain 903 than at either 105 or 110 C. In strain Challis (Fig. 2a) glutamic acid appears to be increasing at both 100 and 105 C; some degradation seems to occur at 110 C. Strain 6249 (Fig. 2b) shows a similar pattern, except at 100 C the concentration of glutamic acid reaches a maximum and remains stable. The curves for the other amino acids in each preparation were generally similar to the glutamic acid and the curves for the hexosamines parallel the muramic acid. Thus, it appears that no single pattern of decomposition of individual amino acids or hexosamines occurs during hydrolysis of cell walls. Indeed it seems as if each cell wall had a characteristic hydrolysis curve. The results suggested extrapolation of data to zero time or application of other correction factors would not be a valid method of interpreting the data from the cell walls. With the exception of strain 903, hydrolysis at 100 C for 18 h seemed to offer the best conditions for obtaining reasonable estimates of the amino acid and hexosamine content of the cell walls and therefore these conditions were chosen as the standard for comparisons.

To aid in interpretation of the results, the amino acid and hexosamine composition was divided into the major and minor components; the former represented amino acid and hexosamines most closely associated with the peptidoglycan. It was not clear whether some minor amino acids were components of the peptidoglycan in some instances, whether they represented portions of surface proteins, or membrane proteins not removed by the Cummins and Harris procedure (6).

In Table 3, the range of concentration of peptidoglycan components is shown in serotypes 1 and 2 and for the heterogeneous group. No division was made between serotypes 1 and 2 because no consistent differences in composition were noted. However, it is clear from the data that both the alanine and glucosamine content of serotypes 1 and 2 is greater than found in the heterogeneous group. These patterns become even more evident if the molar ratios relative to glutamic acid are compared as was done in Table 4.

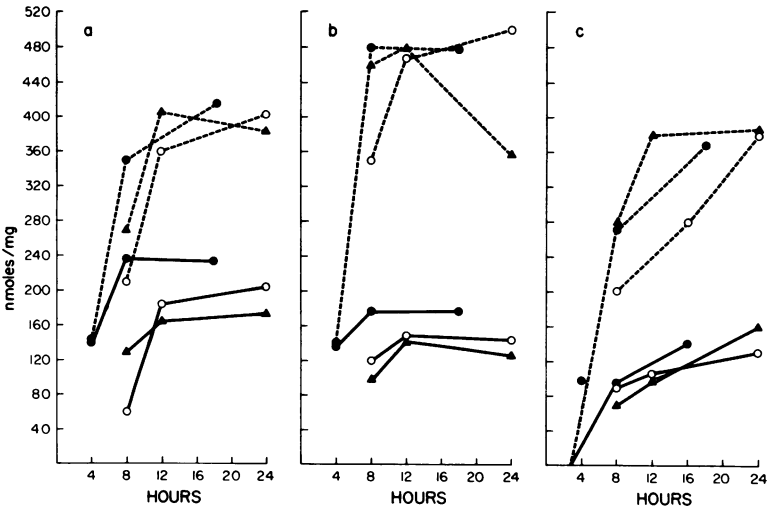

FIG. 2(a). *Hydrolysis curves for strain Challis (- - -) glutamic acid; (——) muramic acid; (●) 100 C; (○) 105 C; (▲) 110 C. (b) Strain 6249. (c) Strain 903.*

TABLE 3. *Major amino acids and hexosamines of cell wall*

Component	Serotypes 1 and 2 (μmol/mg)	Heterogeneous
Glutamic acid	0.36–0.50[a]	0.40–0.53
Lysine	0.31–0.49[b]	0.39–0.54
Alanine	1.12–1.75	0.61–0.73
Muramic acid	0.20–0.30[c]	0.15–0.31
Glucosamine	0.42–0.72[d]	0.25–0.39

[a] Fourteen out of 16 strains ranged between 0.40 to 0.50.
[b] Strain Wicky had a value of 0.58 μmol/mg.
[c] Strain 903 had a low value of 0.14 μmol/mg.
[d] Fourteen out of 17 strains were between 0.60 to 0.72 μmol/mg.

TABLE 4. *Molar ratios of major amino acids and hexosamines relative to glutamic acid*

Component	Serotypes 1 and 2	Heterogeneous
Lysine	0.72–1.21	0.77–1.23
Alanine	3.13[a]–3.63[b]	1.21–1.78
Muramic acid	0.48–0.66	0.37–0.60
Glucosamine	1.21–1.70[c]	0.45–0.76

[a] The molar ratios of alanine in strains Wicky, FW213, FW225, and F90A1 were 2.35, 2.69, 2.85, 2.75, respectively. These lower values of alanine were associated with larger amounts of glycine (Table 5).
[b] Strain 903 had a molar ratio of 4.70.
[c] Strain Channon had a molar ratio of 0.95, whereas strain FW225 had a molar ratio of 1.97.

The major difference appears in the ratio of alanine and glucosamine to glutamic acid. The ratios of alanine probably represent differences in the cross bridge (18), whereas gucosamine probably reflects differences in the glucosamine content of other cell wall polysaccharides (Rosan, J. Dent. Res. 54: Abstr. 522, 1975). The pattern of differences in the amino acid and hexosamine composition appears to segregate the strains into the same two categories noted for rhamnose, phosphate, and ribitol. Strains FW225, FW213, and Wicky had smaller amounts of alanine than other strains within serotypes 1 and 2; these strains, however, had relatively large concentrations of glycine in their cell walls (Table 5). Strain F90A also contains more glycine and less alanine than found in the other strains.

In contrast to the distinct differences in patterns of the major amino acid and hexosamines, the minor amino acid content does not form any detectable pattern that could distinguish serotypes 1 and 2 from the heterogeneous group. The values for components shown in Table 5 suggest a relative uniformity between the two groups. However, two strains, F90A and 903, contain more of the acidic amino acids than usually found in other strains. Strain Wicky also contains significant quantities of galactosamine. The latter amino sugar, though present in some strains of serotypes 1 and 2, is found in all strains of the heterogeneous group, giving further credence to the idea that these organisms are distinct from serotypes 1 and 2.

Although the range of values of the minor amino acids appears large, i.e, aspartic acid 0.02 to 0.09 μmol/mg, the order of magnitude is about the same. Even these figures are somewhat misleading because the range was considerably reduced if two to three strains were

TABLE 5. *Composition of minor amino acids and hexosamines in cell walls*

Amino acids	Serotypes 1 and 2	No. of strains within this range	Heterogeneous	
Aspartic acid	0.02–0.09	15/17[a]	0.06–0.10	6/6
Threonine	0.01–0.05	15/17[b]	0.03–0.05	6/6
Serine	0.02–0.05	15/17[c]	0.02–0.06	6/6
Glycine	0.06–0.10	12/17[d]	0.07–0.13	6/6
Valine	0.02–0.10	13/17[e]	0.05–0.08	6/6
Isoleucine	0.01–0.03	17/17	0.01–0.03	6/6
Leucine	0.02–0.06	16/17[f]	0.03–0.05	6/6
Tyrosine	0.01–0.02	13/17[g]	0.01	5/6[g]
Phenylalanine	0.01–0.02	13/17[g]	0.01–0.02	6/6
Galactosamine	0.02–0.03	4/17[h]	0.01–0.03	5/6[h]
Histidine	0.01–0.08	17/17	0.04–0.05	

[a] Strains F90A, 0.15 μmol/mg, strain 903, 0.12 μmol/mg, respectively.
[b] Strain F90A, 0.10 μmol/mg; 903 0.07 μmol/mg.
[c] Strain F90A, 0.09 μmol/mg; 903 0.08 μmol/mg.
[d] Strains Wicky, 0.36 μmol/mg; F90A, 0.15 μmol/mg; 903, 0.12 μmol/mg; FW213 0.42 μmol/mg – FW225, 0.30 μmol/mg.
[e] Strains FW225 had less than 0.01 μmol/mg and no valine was detected in strain 6623.
[f] No leucine detected in strain FW225.
[g] No tyrosine or phenylalanine was detected in strain FW225 and only small amounts <0.01 were found in strains Challis, 6312, and 6623 and 10557.
[h] Strain Wicky contained 0.20 μmol/mg of galactosamine, 6249 contained 0.12 and strain Channon 0.45 μmol/mg.

TABLE 6. *Amino acid and hexosamine composition of strains Blackburn and Challis*[a]

Amino acid	Blackburn	Challis[a]
Aspartic acid	0.08	0.08
Threonine	0.04	0.03
Serine	0.05	0.02
Muramic acid	0.22	0.23
Glutamic acid	0.42	0.42
Glycine	0.08	0.09
Alanine	1.43	1.62
Valine	0.08	0.03
Isoleucine	0.02	0.01
Leucine	0.05	0.03
Tyrosine	0.01	+
Phenylalanine	0.02	+
Glucosamine	0.66	0.71
Galactosamine	ND[b]	+
Lysine	0.37	0.38
Histidine	+[c]	0.03
Arginine	ND	ND

[a] Micromoles per milligram.
[b] ND, Not detected.
[c] +, Less than 0.01 μmol/mg.

omitted from the summary. However, because different strains were being studied, it was not considered valid to give an average or median values. A comparison of the composition of strains Challis and Blackburn are shown in Table 6; these strains were chosen because the values are typical of serotypes 1 and 2 and because they give some indication of the precision of the determination. As seen in Table 6, except for alanine which differs by 7%, the concentrations of the major amino acids and hexosamines are within 1% of each other; even the quantities of minor amino acids are similar. It is also of interest that Slade and Slamp (21) analyzed the peptidoglycan of strain Challis and, except for glucosamine, obtained similar results. The difference in glucosamine probably reflected the extraction of polysaccharides containing this amino sugar from their peptidoglycan preparation with trichloroacetic acid.

The concentrations of the minor amino acids in both serotypes 1 and 2 and the heterogeneous group appear similar and therefore these amino acids do not appear to differentiate the groups. Suffice to say, these amino acids are found regularly in all strains and thus are associated with the cell walls; with the possible exception of glycine in some strains the significance of these minor amino acids in the cell wall is not known.

It is evident that strains Wicky, F90A, 903, FW213, and FW225 contain fairly large quantities of glycine. These strains also contain less alanine than do the other strains in serotypes 1 and 2; thus it is possible that some of the glycine in these strains may be a component of the peptidoglycan.

DISCUSSION

The results of these studies indicate clearly that at least two major groups may be defined among the group H streptococci and *S. sanguis* on the basis of cell wall composition. Group I contains moderate to large quantities of rhamnose, little or no detectable ribitol, and usually less than 1% phosphorus. The molar ratio of alanine-glutamic acid in this group ranges from 3 to 4:1. Group II contains only small amounts of rhamnose, small but consistently detectable levels of ribitol, galactosamine, larger concentrations of phosphorus, and a molar ratio of alanine-glutamic of 0.6 to 0.7:1. It is assumed that the ribitol and the large phosphate concentration reflect the presence of a ribitol teichoic acid in the walls of the heterogeneous group which is either absent or present in only small quantities in serotypes 1 and 2.

In studies of the cross bridge of strains of *S. sanguis*, Schleiffer and Kandler (18) reported that strains designated as type I had di- or trialanyl cross bridges, whereas strains desig-

nated type II had a direct alanyl-lysl cross bridge in the peptidoglycan. The designation of type I and II stem from the observations of Washburn et al. (24) and Porterfield (12) of serological types among the group H streptococci. Types I and I/II were said to contain the group H antigen, whereas the group antigen was not found in type II strains. The ATCC strains 10556, 10558, and 10557 (Table 1) are equivalent to type I, I/II, and II, respectively; the cell walls of the first two strains have molar ratios of alanine-glutamic acid of 3 to 4:1, suggesting the di-alanyl-lysl cross bridge by Schleiffer and Kandler for type I strains, whereas in the type II strain the molar ratio of alanine to glutamic acid approached the 1:1 ratio which would be associated with the direct alanyl-lysl cross bridge found in the type II strains. Since the molar ratios of alanine to glutamic acid in all of group I strains was approximately 3:1 it is probable that these strains also have the di-alanyl cross bridge in their peptidoglycan. In a similiar fashion, it was also concluded as probable that the strains in group II had a direct alanyl-lysl cross bridge. It is perhaps germaine to point out that with the exception of the peculiar behavior of strain 10556 explained previously, all these strains contained the *a* antigen; thus, these results confirm the earlier studies concerning the distribution of the group H antigen among *S. sanguis* type I and type I/II. The correspondence between the present studies and those of other investigators is also exemplified in the neutral sugar composition.

In Table 7, an attempt has been made to summarize the neutral sugar composition found in these studies with those of other investigators. Only well-defined named reference strains were used for the comparisons. In preparing this summary it was realized that some investigators initially attached a great deal of importance to the presence or absence of glucose and/or galactose in the walls. However, a comparison of the data among all the investigators made it quite clear that the presence or absence of these sugars was less significant than originally thought as studies of the same strains by different investigators gave variable results. This finding was not really surprising in light of the relatively small amounts of some of these sugars which appeared to be present in some strains. If one considers the presence of moderate to large amounts of rhamnose and little to no detectable ribitol (group I), as distinguished from strains containing little to no rhamnose and detectable ribitol (group II) as the major categories to be examined, then it was clear that there was a correspondence of data from all investigators (most did not determine phosphorus and thus this parameter has been omitted). The strains belonging to group II appeared to be sufficiently distinct from group I to merit a different taxonomic category. Indeed Coleman and Williams (5) have suggested that some of these organisms which also have some distinct biochemical properties be considered to represent *S. mitior (S. mitis)*. With the exception of strain K208 which has some interesting serological relationships (Rosan, J. Dent. Res.

TABLE 7. *Summary of groups of Streptococcus sanguis and group H streptococci based on cell wall*

Strain and synonyms	Cole[a]	Coleman and Williams (2)	Hardie (8)	Slade and Slamp (18)	Rosan
ATCC 10556 (NCTC 7863-*S. sanguis*)	I	I	I	I	I
Blackburn (NCTC 10231)	I	I	I	I	I
ATCC 10558 (NCTC 7865, s.b.e. I/II)	I	I	I	I	I
Channon (NCTC 7869)	I	NT[b]	I	I	I
F90A (Perryer, ATC 12396)	I	I	I	I	I
Challis (NCTC 7868)	I	I	I	I	I
Wicky (NCTC 9124)	I	I	I	I	I
ATCC 9811 *(S. mitis)*	NT	II	II	II	II
ATCC 10557 (NCTC 7864, *S. sanguis* II)	II	II	II	II	II
S 5723	II	NT	II	NT	II
6249	NT	NT	NT	II	II
K208	II	II	II	II	II
S. sanguis II (NCTC 7864)	II	II	II	II	II

[a] Reference 3a.
[b] NT, Not tested.

54:Abstr. 523, 1975) with strain F90A (the latter two strains have been spoken of as representing "American group H" (R. Cole, personal communication), this idea has much merit. It would provide for the first time distinct characteristics with which to identify *S. mitior*, i.e., absence of rhamnose, presence of ribitol, and high cell wall phosphorus and galactosamine. In the past, *S. mitior* has been identified essentially by a process of elimination. It begins to appear that in the very near future the heterogeneity which was once considered the sine qua non of the viridans streptococci will soon give way to distinctive biological and serological properties which characterize at least four species: *S. mutans, S. sanguis, S. mitis,* and of course, *S. salivarius* which was so well characterized many years ago by Sherman et al. (19).

It would be remiss to conclude this report without some discussion of obvious technical problems involved in the analytical methods and interpretation of data which were employed in these studies. An intense effort to standardize hydrolysis procedures and/or determine factors that could be used to correct for hydrolytic losses was not considered successful. Although some of these problems perhaps could be attributed to technical artifacts, these artifacts must be reproducible because the precision of the analytical data obtained with such strains as Blackburn and Challis was quite good as was the data from strains ATCC 10557 and *S. sanguis* II both of which were originally derived from NCTC 7864. Thus, the techniques used seemed to offer a degree of precision. Is is possible to make judgments of accuracy?

It is obvious that the molar ratios of the muramic acid to glutamic acid and the peptidoglycan do not approach unity as is usually expected for these components. Although this discrepancy could be corrected easily by use of a factor, the data for the hydrolysis do not seem to justify the use of such a correction factor. Indeed, no general agreement seems to exist on the extent of degradation of hexosamines during hydrolysis. In the studies of Schleiffer and Kandler (18) the correction factor was calculated from the degradation of a standard sample of muramic acid. Studies of the loss of muramic acid at the conditions used in their studies (6 N HCl, 100 C, 18 h) indicated a 63% loss (Rosan, unpublished observations), a figure which seems grossly inflated relative to the hydrolysis curves shown in Fig. 2 a, b, and c; on the other hand, Slade and Slamp (21) suggested a 15% loss for muramic acid under conditions of hydrolysis similar to those used in the current report. Other investigators have extrapolated hydrolysis curves to zero time (11) or multiplied observed results by approximately 1.3 (25) to correct for losses of hexosamine. In most instances, such corrections do result in bringing muramic acid, glutamic acid ratios closer to the presumed theoretical value of 1:1. Indeed, in a study of cell walls of *S. sanguis,* strain M-5, extracted with both formamide and trichloroacetic acid to prepare "pure" peptidoglycan, the molar ratios relative to glutamic acid were found to be lysine-alanine muramic acid-glucosamine, 1:3.5:0.63:1 (Rosan, and Hornick, unpublished observations) suggesting that in absence of nonpeptidoglycan structures the ratios do approach the theoretical values. If the low molar ratios of muramic acid in whole cell walls are found to be accurate, it might reflect the presence of extra peptide units which could conceivably function to join nonpeptidoglycan components of the cell wall to the glycan strands via additional peptide units. Such a structure could account for the observation that many cell wall polysaccharides and teichoic acids contain peptide components (2, 8).

These observations suggest that a thorough analysis of cell wall composition can be performed with adequate precision; however, the accuracy with which some of the components, e.g., muramic acid, can be determined after hydrolysis requires further study.

Despite the caution which must be observed in measuring the exact concentrations of some components in the cell wall, there is no difficulty in interpreting the clear relationships of the presence of the *a* antigen with a distinct cell wall composition. This wall is characterized by rhamnose, probably a di-alanyl cross bridge in the peptidoglycan, relatively low phosphorus, and little or no detectable ribitol. Not only do these walls show similar chemical properties but as indicated the strains possess common biological properties, i.e., phage absorption and sensitivity to phage lysin. Indeed, Coykendall and Specht (J. Dent. Res. 54: Abstr. L293, 1975) have shown by deoxyribonucleic acid hybridization that the few strains of this group tested had a significant degree of genetic relatedness. These observations suggest strongly that these strains form a homogeneous group biologically, genetically, structurally, and serologically, and therefore it is suggested that the antigen defining this group, the *a* antigen, be recognized as the Lancefield group H antigen.

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