The Sugar Composition of Streptococcal Cell Walls and its Relation to Haemagglutination Pattern

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SUMMARY

The sugar composition of cell walls of various streptococci and pneumococci was investigated by paper chromatography. Rhamnose was found in the walls of most streptococci bearing Lancefield group haptens but was not identified in the walls of pneumococci or most 'viridans' streptococci. In the group as a whole galactose and glucose were more regularly present than rhamnose. A significant correlation was found between the distribution of rhamnose and haemagglutination pattern, as determined by the presence of a widely distributed red cell sensitizing antigen and the production of an agent capable, like the receptor-destroying enzyme of the influenza virus, of modifying the antigenic properties of the red-cell surface.

INTRODUCTION

In recent years considerable advances have been made in our knowledge of the composition and structure of the bacterial cell wall. These advances were made possible by the development by Salton and others (Salton & Horne, 1951; Salton, 1952; Cummins & Harris, 1956) of satisfactory techniques for the preparation of purified cell-wall homogenates. On a priori grounds it seems likely that cell-wall composition would have considerable taxonomic value. This possibility is borne out particularly by the finding that the cell walls of Gram-negative bacteria show, as far as they have been examined, a wider range of amino acids and a higher lipid content than do those of Gram-positive organisms (Salton, 1952, 1958) and of generic differences amongst various Gram-positive bacteria in cell-wall sugar composition (Cummins & Harris, 1956, 1958).

An analysis of the cell-wall composition of various streptococci was reported by Cummins & Harris (1956). These workers found rhamnose to be a constituent of the cell walls of strains of streptococci of Lancefield groups A to G; from these results it seemed possible that rhamnose, which they found to be absent from the cell walls of staphylococci and certain other species, might be characteristic of the streptococci as a whole. They noted, however, differences between different strains of streptococci in their content of other sugars, and were in general of the opinion that the sugar and amino sugar composition of the cell wall was of taxonomic significance at species level. A quite different approach to the taxonomy of the streptococci was described by Stewart, Steele & Martin (1959). This proposed that these organisms might be classified by the haemagglutination technique on the
basis of the distribution of various red cell sensitizing antigens and of an agent—
cell-modifying agent (CMA)—possessing properties similar to those of the receptor-
destroying enzyme of the influenza virus.

The present communication reports the results of an investigation by paper
chromatography into the sugar composition of the cell walls of various streptococci
and pneumococci and considers in particular the relationship between cell-wall
sugar content and haemagglutination pattern.

METHODS

Organisms. At least one member of each Lancefield group A to Q examined was
from the National Collection of Type Cultures (N.C.T.C., Colindale, London, N.W. 9).
Two of the group K strains produced mucoid colonies on 5% (w/v) sucrose agar
and may therefore be classified as *Streptococcus salivarius*. The strains of groups R
and S examined were made available to us by the courtesy of Dr C. E. de Moor
(Utrecht). The majority of the 'viridans' and group K strains were laboratory
isolates from upper respiratory sources (throat swabs, sputa, saliva). They were
selected on the first instance on the basis of the production of a viridans change on
human blood agar incubated aerobically and were insensitive to 1 in 5000 ethyl
hydrocuprein, were deoxycholate insoluble and did not produce mucoid colonies
on sucrose agar. All strains classified as 'viridans' streptococci in addition gave
negative tube precipitin reactions with antisera for Lancefield groups A to N. The
Lancefield groups were determined on 48 hr. cultures in 0.5% (w/v) glucose digest
broth extracted at 100° with 0.05 N-HCl. The precipitating sera used were those
issued commercially by Burroughs Wellcome and Co.

Group O serum was also used routinely in our tests, but doubts about its speci-
cficity were raised by the finding of what appeared to be an excessively high pro-
portion of reactions with 'viridans' strains. On further investigation the serum was
found to react with a number of NCTC strains belonging to various other Lancefield
groups. On examination by the Ouchterlony plate diffusion technique against the
NCTC group O strain—8029—the serum was found to give a number of distinct
precipitation lines. Pending fuller investigations the designation group O has
therefore been withheld from all strains except NCTC 8029.

The pneumococci examined included one strain of each of types 2 (NCTC 7466),
14 and 16 and two strains of type 8. The remaining two strains were not typed.

Cell-wall analysis. Cultures for cell-wall analysis were obtained by overnight
growth at 37° in Hartley's digest broth containing 0.2% (w/v) NaHCO₃ and 0.5% (w/v)
glucose. Five hundred ml. of culture were usually sufficient for examination
except in the case of the pneumococci when twice this volume was found necessary
to give a satisfactory bacterial mass. The organisms were harvested by centrifuga-
tion at 3000 g and washed twice in 0.9% (w/v) saline.

The method used for the preparation of cell walls was essentially that of Cummins
& Harris (1956), involving disintegration of the organisms by shaking in a Mickle
disintegrator with no. 12 ballotini glass beads. Disintegration was considered
complete when no intact organisms could be seen in Gram-stained films. The glass
beads were removed by passage through a coarse sintered glass filter. The cell walls
were separated by differential centrifugation and purified by washing with distilled
water, followed by treatment with trypsin and ribonuclease. The cell walls were then sedimented by centrifugation, washed in water and resuspended in water. Peptic digestion was omitted since control experiments with a number of strains showed no difference between pepsin-treated cell walls and walls which had not been treated with pepsin.

Random samples were examined from time to time in a Beckmann spectrophotometer to detect the presence of contaminating nucleotide material from the cytoplasm (Salton & Horne, 1951; Barkulis & Jones, 1957). In no case was any peak observed in the 260 nm region.

*Conditions and treatment.* The method of hydrolysis employed followed that used by Cummins & Harris (1956) viz. treatment with 2N-H₂SO₄ at 100° for 2 hr. in sealed tubes. The hydrolysate was neutralized with Ba(OH)₂ and then dried over P₂O₅ in an Edwards centrifugal freeze-drying apparatus. Approximately 80 mg. dry weight of cell wall was used in each test; where this amount was not available the final volume of the hydrolysate was adjusted accordingly—a constant volume of this being applied to each chromatogram.

One-dimensional descending chromatograms on Whatman no. 4 paper with the solvent ethyl acetate + pyridine + water of Jennyn & Isherwood (1949) were found to resolve all the sugars present provided that the solvent front was allowed to run well off the paper. The sugars were revealed with aniline hydrogen phthalate, and hexosamines with the Elson and Morgan reagent. No attempt was made to separate individual hexosamines.

*Haemagglutination techniques.* Cultures for haemagglutination tests were obtained by growth for c. 18 hr. in Hartley’s digest broth. The cultures were centrifuged until visibly clear and the supernatant fluids thus obtained used for cell treatment. For the preparation of absorbing suspensions 48 hr. cultures in digest broth containing 0.5% (w/v) glucose were used. Human group O red cells, washed three times in saline before use and resuspended in saline to give a c. 5% suspension were used for cell treatment. Treatment was carried out by incubating mixtures of equal volumes of cell suspension and of supernatant fluid for 2 hr. at 37°. At the end of this time the cells were sedimented by centrifugation, washed in saline and resuspended to give a 5% suspension. For the haemagglutination tests proper one drop of cell suspension and one drop of serum dilution were mixed and the mixtures allowed to stand for 30 min. at room temperature; they were then examined for agglutination by inspection of the deposit with a x10 microscope ocular.

The serum used for the detection of the Hickey antigen was obtained by immunization of a rabbit with the deposit from a culture of *Streptococcus pyogenes* in 0.5% (w/v) glucose digest broth. The serum was heated to 50° for 30 min. and, in order to remove naturally occurring T agglutinins, was absorbed with red cells treated with the supernatant fluid of a digest broth culture of a ‘viridans’ streptococcus which produced the cell-modifying (receptor-destroying) enzyme. For test purposes the serum was used in a dilution of 1/20.

The serum used for the detection of cell-modifying activity was obtained by immunization of a rabbit with cells treated with allantoic fluid from chick embryos infected with the FMI strain (type A1) of influenza virus. The serum was heated to 56° for 30 min. and was heavily absorbed with normal red cells to remove species agglutinins. For test purposes it was used in a dilution of 1/40.
RESULTS

Cell-wall composition

The results of the cell-wall analyses are shown in Table 1. It will be seen that though in agreement with the findings of Cummins & Harris (1956) rhamnose was a component of the cell walls of the streptococci of groups A to G examined; its presence was not a general property of the streptococci since it was absent from the group O strain (NCTC 8029), from a number of group K strains, from the majority of the 'viridans' streptococci and from all the pneumococci. The two strains of Streptococcus salivarius classified as group K were rhamnose positive. The absence of rhamnose from the cell walls of pneumococci is consistent with the findings of Smith, Mills, Harper & Galloway (1957) who failed to detect this sugar in the cellular polysaccharide obtained by enzymic digestion of whole pneumococci.

Table 1. Cell-wall sugar composition of various streptococci

<table>
<thead>
<tr>
<th>Sugars</th>
<th>Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gal +</td>
<td>A (1), D (2), F (3), H (2), L (1), M (1), P (1), Q (1), R (2), S (2), 'Viridans' (11)</td>
</tr>
<tr>
<td>Gl +</td>
<td>E (1), H (1), N (1), 'Viridans' (1)</td>
</tr>
<tr>
<td>Gal -</td>
<td>B (1), D (1), G (1)</td>
</tr>
<tr>
<td>Gl -</td>
<td>A (4), C (1)</td>
</tr>
</tbody>
</table>

Gal = galactose; Gl = glucose. Hexosamine present in all strains. Figures in parentheses indicate number of strains examined.

Surprisingly, considerably less variation was found in the group as a whole in the distribution of galactose and glucose than in that of rhamnose. With the exception of four strains of Streptococcus pyogenes and one group C strain, all strains possessed one or other of these sugars and most possessed both. From a taxonomic point of view it is significant, however, that of the seven groups of which more than one strain was examined, intra-group variation in either galactose or glucose content was encountered in four groups, namely A, D, H and K. The discovery of a group A strain which possessed both galactose and glucose is of interest in view of the fact that previous strains of this species which have been reported were found to be deficient in both these sugars (Barkulis & Jones, 1957; Cummins & Harris, 1956; McCarty, 1952; Salton, 1958; Schmidt, 1952). Both galactose and glucose were regularly present in the pneumococci and appeared to be somewhat more consistently present, though not significantly so, in the 'viridans' streptococci than in the streptococci identified as possessing Lancefield group haptens. Unlike Cummins & Harris (1956), who detected mannose in the cell walls of one group E and two group D strains, we have failed to identify mannose in any of our strains.
Relationship of cell-wall sugar content to haemagglutination pattern

The relationship between cell-wall sugar content and haemagglutination pattern as determined by the reactions with the CMA and Hickey sera is shown in Table 4. Tests for the Hickey antigen were carried out in the first place with the supernatant fluids of digest broth cultures. Strains which gave negative results in these tests were then retested by an absorption technique. For this purpose the sediments from 48 hr. cultures in digest broth containing 0.5 % (w/v) glucose were used, the sediment from 20 ml. culture being used to absorb 1 ml. unabsorbed Hickey serum diluted 1/20. Absorption was for 30 min. at room temperature. In each case two consecutive absorptions of the serum were carried out, the same amount of culture being used for the second absorption. The unabsorbed serum and samples of serum after each absorption were then titrated against red cells treated with supernatant fluid of the Hickey (Streptococcus pyogenes) strain. The results obtained with this method were quite clear cut, most Hickey-positive strains removing the antibody completely after the first absorption, and Hickey-negative strains causing at most a twofold decrease in titre after two absorptions. A typical absorption result is shown in Table 2.

Table 2. Absorption of Hickey serum with representative strains of streptococci

Figures given are reciprocals of serum dilution causing agglutination.

<table>
<thead>
<tr>
<th>Test cocci</th>
<th>Treated with Hickey supernatant fluid</th>
<th>Untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reciprocals of serum dilution</td>
<td>1st absorption</td>
<td>2nd absorption</td>
</tr>
<tr>
<td>BI (CMA-positive viridans)</td>
<td>640</td>
<td>320</td>
</tr>
<tr>
<td>MM (CMA-positive group K)</td>
<td>320</td>
<td>320</td>
</tr>
<tr>
<td>NCTC 8029 CMA-positive group O</td>
<td>80</td>
<td>&lt; 40</td>
</tr>
<tr>
<td>44 CMA-negative group K</td>
<td>&lt; 40</td>
<td>.</td>
</tr>
<tr>
<td>Unabsorbed</td>
<td>640</td>
<td>&lt; 40</td>
</tr>
</tbody>
</table>

CMA = cell-modifying agent.

In the earlier work, in which the distribution of the Hickey antigen was studied only in the supernatant fluids of digest broth cultures (Stewart et al. 1959), a very high negative correlation was found between the distribution of the antigen and that of the cell-modifying enzyme. With the more rigorous technique used in the present work it was found, however, that strains which give negative results for the Hickey antigen in digest broth might yet be shown by the absorption technique to possess it. One such culture was the group O strain (NCTC 8029). On repeated testing of this strain, however, occasional positive haemagglutination reactions were obtained with the supernatant fluids of glucose digest broth cultures. (For tests with glucose digest broth the red cells were suspended in phosphate buffered saline at pH 7).
The distribution of the four haemagglutination patterns determined as described in the preceding paragraph amongst the various streptococci examined is shown in Table 3. It will be seen that, excluding certain group K strains the Hickey antigen was present in all the streptococci bearing Lancefield group haptens examined, and that all except two of these were CMA negative. This distribution is in sharp contrast to that found in the pneumococci, all of which were Hickey-negative CMA-positive. In contrast, the 'viridans' streptococci showed a much greater diversity of haemagglutination type.

Table 3. Haemagglutination patterns of strains examined

<table>
<thead>
<tr>
<th>Haemagglutination type</th>
<th>Hickey +</th>
<th>Hickey -</th>
<th>Hickey +</th>
<th>Hickey -</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMA -</td>
<td>26</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>CMA +</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CMA -</td>
<td>0</td>
<td>13</td>
<td>6</td>
<td>4</td>
</tr>
</tbody>
</table>

Streptococci of groups A to S (excluding K)

Group K streptococci 12 14 6 4

'Viridans' streptococci

Pneumococci 0 7 0 0

CMA = cell-modifying agent.

From the results shown in Table 4 it will be seen that there was a considerable correlation, amongst the streptococci, between the presence of rhamnose as a cell-wall component and the haemagglutination type. This is best seen in the relative frequencies of the Hickey-positive CMA-negative and Hickey-negative CMA-positive types in rhamnose-positive and rhamnose-negative strains. Thus of 49 rhamnose-positive strains 41 were Hickey-positive CMA-negative and 6 were Hickey-negative CMA-positive. The rhamnose-negative strains, on the other hand, showed a quite different haemagglutination pattern; of 29 of such strains examined 6 were Hickey-positive CMA-negative and 18 were Hickey-negative CMA-positive ($\chi^2 = 20.4, P < 0.001$). This correlation was even greater when the results with digest broth cultures alone were considered. In this case 40

Table 4. Relationship between streptococcal cell-wall sugar content and haemagglutination (HA) type

<table>
<thead>
<tr>
<th>Cell-wall sugars</th>
<th>Rhamnose</th>
<th>Galactose</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA type</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Hickey +</td>
<td>41</td>
<td>6</td>
<td>38</td>
</tr>
<tr>
<td>Hickey -</td>
<td>6</td>
<td>13</td>
<td>18</td>
</tr>
<tr>
<td>Hickey +</td>
<td>2</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Hickey -</td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

CMA = cell-modifying agent.
Results obtained with pneumococci excluded from this table.
of the rhamnose-positive strains were Hickey-positive CMA-negative and 6 were Hickey-negative CMA-positive, while of the rhamnose-negative strains 2 were Hickey-positive CMA-negative and 17 were Hickey-negative CMA-positive.

Of the 14 group K strains examined 9 were rhamnose-positive Hickey-positive CMA-negative, 4 were rhamnose-negative Hickey-negative CMA-positive, and one was rhamnose-positive Hickey-negative CMA-positive.

DISCUSSION

Our results have shown that rhamnose, though present in the cell walls of most streptococci possessing Lancefield group haptens, is not characteristic of the streptococci as a whole. In particular, it was found to be absent from the walls of the definitive group O strain examined, from those of certain group K streptococci and from those of the majority of viridans streptococci. The question therefore arises as to whether, amongst the streptococci, the presence of rhamnose as a cell-wall component is of importance as a criterion of phylogenetic relationship. Its presence in most streptococci bearing Lancefield group haptens and its absence from the pneumococci would suggest that it is. This view is reinforced by the finding that the distribution of rhamnose has a significant relation to haemagglutination pattern as determined by the presence of the Hickey antigen and the production of the cell-modifying enzyme. One is in fact tempted to see in this group of organisms two basic types from which others might have arisen by a process of hybridization namely: (1) a rhamnose-positive Hickey-positive CMA-negative type typical of the majority of the Lancefield group streptococci; (2) a rhamnose-negative Hickey-negative CMA-positive type characteristic of the pneumococci. These two types have in fact accounted for just over two-thirds of the strains of streptococci examined.

The status of group K strains as defined by these reactions is of interest. Group K streptococci have already been shown to be heterogeneous in respect of their capacity to produce mucoid colonies on sucrose agar—a property which is accepted as the definitive criterion of the species Streptococcus salivarius (Williams, 1956). In fact from the work of Williams the production of mucoid colonies on sucrose agar appears to be better correlated with various other physiological properties than is the possession of the K hapten. Group K strains are also heterogeneous in their haemagglutination patterns. The introduction of rhamnose as an additional parameter introduces still further heterogeneity into the group.

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REFERENCES


