

Extraction of Cell-Wall Polysaccharide Antigen from Streptococci

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ABSTRACT

SLADE, HUTTON D. (Northwestern University Medical School, Chicago, Ill., and Max-Planck Institut für Immunbiologie, Freiburg, Germany). Extraction of cell-wall polysaccharide antigen from streptococci. *J. Bacteriol.* **90**:667-672. 1965.—The carbohydrate grouping antigens in the cell walls of streptococci belonging to groups A, E, G, L, and T were extracted with 5% trichloroacetic acid at 90 C. The antigens were removed also from dry whole cells by extraction with trichloroacetic acid followed by treatment with phenol-water. Details of the methods are presented. The antigens obtained by use of either of these procedures were suitable for studies on immunological specificity and chemical structure. Quantitative enzymatic and chemical analyses of two group E antigens and one group T preparation showed the presence of L-rhamnose (22 to 44%), D-glucose (7 to 22%), D-galactose (T antigen only, 26%), glucosamine (2 to 16%), and galactosamine (T antigen only, 3%). In addition, analyses of A and G antigen preparations are presented. The protein and phosphate content of the A and E antigens were about 1% each. Quantitative precipitin curves of these antigens are presented.

The extraction of the group carbohydrate antigens from streptococci has been performed over the past 30 years by use of a variety of procedures. Since 1933, 0.05 N HCl (pH 2) at 100 C (Lancefield, 1933) has been used in clinical laboratories for the routine extraction of the antigen from packed wet cells. Fuller (1938) showed that formamide at 165 C could also be used on whole cells of group A. Krause and McCarty (1962) applied this procedure to group C cell walls, Curtis and Krause (1964) to group G and B cell walls, and Michel and Willers (1964) to group F cells. McCarty (1952) used *Streptomyces albus* enzymes to remove the carbohydrate antigen from group A cell-wall material, and Elliott (1960) found that the carbohydrate type-specific antigen was removed from group D streptococcal walls by the same enzymes.

Most of the group antigens of streptococci are located in the cell wall (Slade and Slamp, 1962; Smith and Shattock, 1964), and it is likely that the majority of the carbohydrate type antigens will be found there also. Ottens and Winkler (1962) showed this to be true for the group F type antigens, and Wittner and Hayashi (1965) for

group B type antigens. The compounds identified in the cell-wall group and type antigens of the streptococci are rhamnose, glucose, galactose, N-acetylglucosamine, and N-acetylgalactosamine.

For studies on the chemical composition of antigens, it is an advantage to have available a method for removal of the antigen which can be applied to whole cells, is not destructive to the structure of the antigen, can be used with large quantities of material, and yields a product of high purity without laborious methods of purification. The following report describes such a method. A summary of some of the properties of one of the antigens has been presented (Slade, Lüderitz, and Westphal, 1965).

MATERIALS AND METHODS

Growth of streptococci. The cells were grown in 2-liter quantities on Todd-Hewitt broth plus a glucose-salts mixture (Hess and Slade, 1955), washed three times with water, and lyophilized. The procedure for the preparation of cell walls has been reported (Slade and Slamp, 1960).

Serological procedure. To conserve antigen and antiserum, a microspectrophotometric method was used to determine the antigen-antibody nitrogen precipitated. To 25 μ liters of antigen diluted in 0.85% NaCl were added 25 μ liters of antiserum. The contents were mixed, and the tubes were tightly stoppered and incubated for 2 hr at 37 C and for 24 to 48 hr at 4 C. The tubes were centri-

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fused, the supernatant solutions were carefully withdrawn by use of micropipettes (Lang-Levy), and the antigen-antibody precipitates were washed three times with cold saline. The protein present was determined as follows by use of a modification of the procedure of Lowry et al. (1951). To the washed precipitates were added 30 μ liters of water and 325 μ liters of alkaline tartrate-CuSO₄ reagent. The contents were mixed by "buz-zing" and held at room temperature for 20 min. A 30- μ liter amount of Folin reagent was added, the solution was held for 20 min at room temperature, and the absorption was read at 750 m μ in cuvettes (1-cm light path and 350- μ liter volume) in a Zeiss spectrophotometer. Under these conditions, 25 μ g of crystalline bovine serum albumin had an optical density of 0.8 to 1.0. In some experiments, only 16 μ liters of antigen of the same concentration and 16 μ liters of antibody were used in a total volume of 50 μ liters.

For the preparation of the tartrate-CuSO₄ reagent, 4 g of NaOH, 20 g of Na₂CO₃, and 0.2 g of sodium tartrate were diluted to 1 liter. Before use, 49 ml of this solution were mixed with 1 ml of 0.5% CuSO₄·5H₂O. Folin reagent was obtained from A. H. Thomas and Co., Philadelphia, Pa.

Anti-group P serum was obtained through the courtesy of M. T. Parker, Streptococcus Reference Laboratory, London, England, and anti-group T serum from C. E. de Moor, Rijks Instituut voor de Volksgezondheid, Utrecht, The Netherlands. The remaining antisera were either obtained from the Communicable Disease Center, Atlanta, Ga., or prepared in our own laboratory (Slade and Slamp, 1962).

To obtain an approximation of the quantity of antigen present in extracts or precipitates, the procedure of Swift, Wilson, and Lancefield (1943) was used.

Chromatography. For the identification of sugars, 5 to 10 mg of antigen were hydrolyzed in 4 N H₂SO₄ at 105 C for 2 hr in sealed vials, neutralized with Ba(OH)₂, filtered, and dried under vacuum at room temperature. The hydrolyzed material was applied to Whatman 3 MM paper and run in 80% phenol down the long axis of the paper until the solvent reached the edge. After drying, a second solvent (butanol-pyridine-water, 6:4:3) was run on the short axis of the paper until the edge was reached. The paper was dried, dipped in aniline phthalate (aniline, 2 ml; phthalic acid, 3.3 g; acetone, 95 ml; water, 5 ml), and heated at 110 C for 12 min.

For the identification of amino acids and amino sugars, 5 to 10 mg of antigen were hydrolyzed in 6 N HCl at 105 C for 17 hr. The material was dried under a vacuum over NaOH and P₂O₅ at room temperature. The separation procedure was similar to the above, except that phenol was run on the short axis of the paper and 2,4-lutidine on the long axis. The papers were stained with 0.2% ninhydrin in 95% acetone-5% water and heated at 110 C for 3 min. Appropriate controls were run in all cases.

Determination of microquantities of sugars and

amino sugars. All determinations were carried out in tubes (7.5 by 1.0 cm), and all solutions were measured with Lang-Levy pipettes. D-Glucose was measured by glucose oxidase (Boehringer, Mannheim, Germany), D-galactose by *Pseudomonas* dehydrogenase (Wallenfels and Kurz, 1962), L-rhamnose by the *Lactobacillus* epimerase (Domagk and Zech, 1963), and hexosamines by the method of Lüderitz et al. (1964).

Protein. Protein was determined by the procedure of Lowry et al. (1951) with crystalline bovine serum albumin as the standard.

Amino acids. Amino acids were determined with a Beckman amino acid analyzer.

RESULTS

Ikawa (1961) showed that extraction of the cell walls of *Streptococcus faecalis* with 5% trichloroacetic acid at 90 C for 15 min gave a nondialyzable fraction equivalent to 29% of the wall. The reducing-sugar value of this fraction was 68% of the total weight, whereas the phosphorus and alanine values were 2% or less. The high reducing value of this fraction indicated to us that it might contain a considerable quantity of the group or type antigens or both. A study has been made on the cell walls of streptococci from several serological groups to determine whether trichloroacetic acid could be used for this purpose.

The following procedure was found suitable. A 500-mg amount of cell wall was extracted by continuous stirring with 50 ml of 5% trichloroacetic acid at 90 C for 15 min. The solution was cooled to room temperature and centrifuged at 10,000 \times g for 10 min; the extract was poured off. A second extraction was carried out in the same manner, and the two extracts were combined and dialyzed against several changes of water for 2 days at 4 C. The volume of the nondialyzable extract was reduced to about 5% under a vacuum at 45 C and centrifuged. Cold acetone was added (3.5 to 5 volumes), with stirring, directly to the concentrated extract. The solution was held at -15 C for 2 days to complete the formation of the flocculent precipitate. Most of the material settled to the bottom of the flask, and the supernatant fluid was poured off and then centrifuged to recover all of the material. The pellet, which was transparent and gelatinous, was dissolved in a small volume of water, centrifuged, and reprecipitated with acetone. The precipitate was washed twice with acetone and dried from ether.

Approximately 70% of the total material which could be removed from the walls by the trichloroacetic acid was obtained in the first extract, 20% in the second, and the remainder in the third. Two extractions were considered sufficient for routine use.

This procedure was applied to cell walls from

TABLE 1. Analysis of antigen extracted from streptococcal cell walls with trichloroacetic acid

Serological group	Strain	Yield of antigen fraction	Titer of antigen*	Antigen at equivalence point	L-Rhamnose	D-Glucose	D-Galactose	D-Glucosamine	Galactosamine
		%		μg	%	%	%	%	%
E	K129	21.7	1:2,560	1.2	44.2	22.0	0	2.2	0
E	5385	22.2	1:1,280	1.2	36.4	19.4	0	2.3	0
T	4965/61	22.5	1:160		22.6†	7.0	26.3	15.9	2.8
A	Kilborn	19.6	1:640	1.0	39.0†			24.3	
L	D167A	13.8	1:320						
G	9603	17.8	1:320		34.8†		16.0		18.3

* Against group-specific sera; precipitin method of Swift et al. (1943) was used.

† Determined by the method of Dische and Shettles (1948). This method is used to determine total rhamnose.

representative strains of five serological groups, with yields of antigen ranging from 14 to 22% of the cell wall, or 3 to 4% of the whole cell based upon a cell-wall weight of 20% (Table 1).

The titer of the antigens (Table 1, column 4) expresses the dilution of the original antigen solution containing 1 mg/ml on which a 1+ reaction was obtained in the precipitin test. At a dilution of 1:2,560, 0.4 μg of antigen would be present. It is apparent that differences will most likely be found to exist in the quality of the antigen extracted from various serological groups. However, the dilution titer is only an approximation of the relative strength of the various preparations. From the data in Table 1 it appears reasonable that any of the strains employed would yield antigens of sufficient purity for chemical and serological study.

Figure 1 shows the precipitin curve of the K129

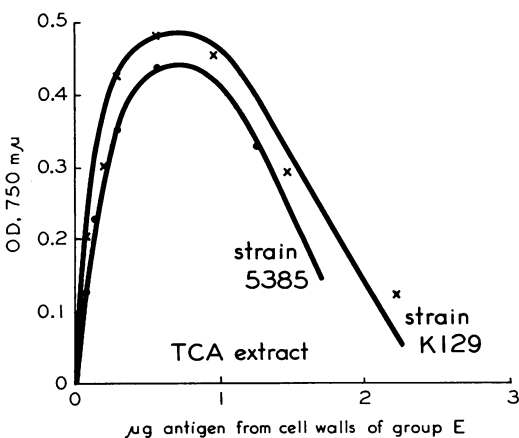


FIG. 1. Quantitative precipitin curves of group antigen preparations extracted with trichloroacetic acid from cell walls of group E, strains K129 and 5385. Antiserum and antigen, 16.2 μliters each, plus NaCl to 50 μliters , were used.

preparation. In this case, 16.2 μliters of antigen solution and the same volume of antiserum were used. The shape of the curve is typical for an antigen-antibody system. Under the conditions used, 0.7 μg antigen was required at the equivalence point. The molecular weight of the E (K129) preparation was only 8,000 to 10,000, and serological specificity was found to be associated with a β -glucosyl residue in the terminal position attached to L-rhamnose on the side chain (Slade, Lüderitz, and Westphal, 1965).

Table 1 shows the sugars, with their isomeric forms, and the amino sugars found in the group E antigen. Rhamnose, as determined by an enzyme specific for the L isomer, was present in K129 in

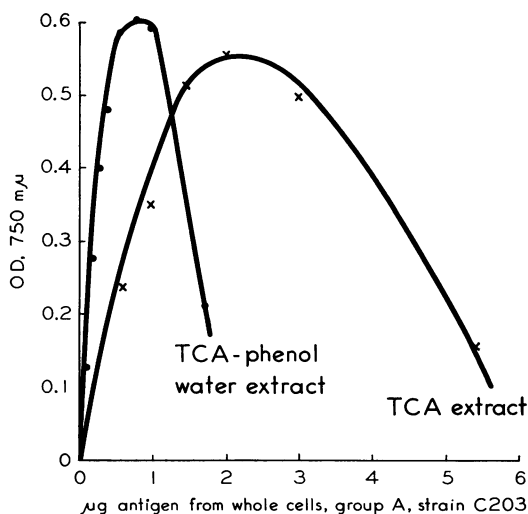


FIG. 2. Quantitative precipitin curves of group antigen preparations extracted from whole, dry cells of group A with trichloroacetic acid, followed by phenol-water extraction of the trichloroacetic acid-soluble material. Antiserum and antigen, 16.2 μliters each, plus NaCl to 50 μliters , were used.

the same quantity as the total rhamnose determined by chemical means, and 94.6% in 5385. It was concluded that substantially all the rhamnose in the E antigen is of the L configuration. Barkulis and Jones (1957) showed that cell walls of group A streptococci contain L-rhamnose only. No other information is available as to the configuration of the rhamnose present in the walls or antigens of the other serological groups of streptococci. It is likely, however, that the D form is not present. The presence of D-glucosamine, as determined by the specific enzymatic method of Lüderitz et al. (1964), is also shown in Table 1.

Galactose was shown to be a constituent of the cell walls of two-thirds of the serological groups of streptococci presently recognized (Slade and Slamp, 1962). Curtis and Krause (1964) found galactose in B and G preparations (the presence or absence of type carbohydrate antigens was not reported), and Wittner and Hayashi (1965) in the B type 1 antigen. D-Galactose made up 35% of the T antigen preparation (Table 1), a value which is close to that of the G preparation (23%). In spite of the high level of galactose in G, it does not appear to be concerned with serological specificity (Curtis and Krause, 1964). It will be of interest to determine whether a similar situation exists for the T antigen. No evidence is at hand to indicate that galactose other than the D isomer is present in the T or G antigens.

The quantities of rhamnose and glucosamine in the trichloroacetic acid-extracted A antigen material (Table 1) were similar to those found by Schmidt (1952) and McCarty (1952) for material obtained by different procedures. In addition, the analysis of the G preparation (Table 1) is similar to that of Curtis and Krause (1964), except that the present value of D-galactose is 35% lower than the value obtained by chemical means by the latter workers. The percentage of galactose in the total analysis in each case, however, is about the same. Different strains were used.

In the K129 preparation (Table 1), no type 1 antigen was found to be present (Slade et al., 1965). Moreira-Jacob (1956) found three type

antigens in this group. At present, the existence of carbohydrate type antigens has not been established for groups L or T.

The nitrogenous impurities in the preparations were very low. The compounds responsible in large measure for the impurities were those amino acids present in the mucopeptide structure of the cell wall. Amino acid analysis of the group T preparation (Table 1) gave the following results: glutamic acid, 0.08%; alanine, 0.07%; and lysine, 0.05%. In the case of group G, strain 9603 (Table 1), the values were glutamic acid, 0.02%; alanine, 0.03%; and lysine, 0.03%. The phosphorus content of the group E (K129) and group A preparations was 1.1%. It is unlikely that this small quantity of phosphorus represents glycerol- or ribitol-phosphate compounds or other compounds of similar structure. It is clear that, in these respects, the purity of the material was excellent and the preparations were well suited for studies on sugar and amino sugar composition and serological specificity.

Consideration was then given to the feasibility of using whole dry cells as a source of antigen rather than cell walls. The latter require considerable time in their preparation. The phenol-water extraction procedure (Westphal, Lüderitz, and Bister, 1952) has been used successfully with gram-negative cells of several genera to remove the cell-wall lipopolysaccharide. However, we found that extraction of 20 g of group A (strain C203) lyophilized cells with 350 ml of water and 350 ml of 90% phenol at 68 to 70 C resulted in the removal of only 2.7% of the cell. A 100- μ g amount of this material was required to produce a 1+ precipitin reaction. A repeat extraction was equally as poor.

A study was then made of the extraction of dry cells with trichloroacetic acid followed by phenol-water treatment of the trichloroacetic acid-soluble material. This procedure was found to be highly successful. The trichloroacetic acid procedure was the same as for cell walls. The addition of 3.5 volumes of acetone to the concentrated trichloroacetic acid extract brought out the anti-

TABLE 2. *Extraction of antigen from lyophilized cells of streptococci with trichloroacetic acid followed by phenol-water*

Cells		Acetone precipitate from trichloroacetic acid extract				Acetone precipitate from phenol-water extract			
Strain	Wt	Wt	Absorption at 260 m μ , 50 μ g/ml	Protein	Equivalence point	Wt	Absorption at 260 m μ , 50 μ g/ml	Protein	Equivalence point
	g	g		%	μ g	g		%	μ g
A, C203	11.0	0.75	0.22	12.4	2.3	0.16	0.03	1.1	0.8
E, Newson	10.7	1.05	0.60	13.0	1.0	0.61	0.02	1.7	0.9

gen as a brown, gummy material. After dissolving in water and reprecipitation with acetone, a transparent, gelatinous material, similar to that from cell walls, was obtained. Approximately 12% of the trichloroacetic acid-soluble material was dialyzable.

The phenol-water extraction was carried out as follows. To 1 g of acetone-precipitated trichloroacetic acid-soluble material were added 37 ml of water and 37 ml of 90% phenol. The material was stirred continuously at 68 to 70 C for 30 min, cooled to 4 C in ice, and centrifuged at $10,000 \times g$ for 10 min. The water layer was withdrawn, and the extraction was repeated after the addition of 35 ml of water. The two extracts were combined, dialyzed for 2 days, and concentrated to a small volume. To the concentrate were added 6 volumes of 90% ethyl alcohol and a few crystals of sodium acetate; this mixture was held at -15 C for 24 hr. The precipitate was sedimented by centrifugation, dissolved in water, and precipitated with 5 volumes of acetone. The acetone precipitation was repeated twice, and the antigen was dried from ether.

Typical data obtained on group A and E cells are given in Table 2. The overall yield of antigen was between 2 and 4%. A comparison of the quantity of antigen required in each case at the equivalence point indicated a difference in the apparent degree of purification achieved in the double extraction between strains from the various serological groups. Such a result was not unexpected and may reflect differences in quantity of antigen present and the relationship of antigen to other components of the cell wall. The most marked achievements in the phenol-water step were the reduction in protein and nucleoprotein content. It appears likely, however, that the presence of these impurities in the trichloroacetic acid extract did not interfere in the combination of antigen (Newson) in the extract with its antibody, as they may have done in the case of strain C203.

DISCUSSION

The methods described should be useful for obtaining antigens from the cell walls of many streptococcal groups and types. It is not anticipated that these preparations will possess any significant antigenicity; however, the availability of material of reasonable purity will encourage studies on the ability of chemically modified material to stimulate the formation of antibodies in animals and man.

It is of interest that Maxted and Fraser (1964) found that the extraction of group D cells with 0.2 N HCl at 100 C resulted in the formation of an alcohol-soluble, acetone-insoluble substance. This

substance, probably a form of the type polysaccharide antigen, combined with type antibody but did not form a precipitate. The type antigen from some strains was hydrolyzed in the HCl more easily than others. On the other hand, Moreira-Jacob (1956) used 0.2 N HCl to extract the E group and type antigens. These differences are probably a reflection of variations in chemical structure and composition of the various antigens in groups D and E. The successful use of 0.05 N HCl as an agent for the extraction of all group antigens has been adequately demonstrated over the years, but it is not known whether significant quantities of the antigens were altered in the process.

It is possible that some of the hapten material may have been altered in the trichloroacetic acid extraction procedure used in the present studies. In the case of group L (strain DS5440) and group O (strain 1360) cell walls, the yields of antigen have not been consistent. Variations in the method of preparation of cell walls would have a bearing on results of this kind.

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