

# Immunochemistry of the Streptococcal Group R Cell Wall Polysaccharide Antigen

PANDU SOPREY<sup>1</sup> AND HUTTON D. SLADE

*Department of Microbiology, Northwestern University Medical School, Chicago, Illinois 60611*

Received for publication 21 July 1971

The group R streptococcal group antigen has been shown to be a polysaccharide located at the surface of the cell wall of the organism. The antigen was extracted from cell walls in 0.05 N HCl or 5% trichloroacetic acid at 100 C, from whole cells at room temperature in 0.85% NaCl or 0.1 M acetate (pH 5.0), and by sonic oscillation. The antigen is largely destroyed when extracted from whole cells in 0.05 N HCl at 100 C. Acetate is recommended for routine extraction. The antigen extracted by sonic treatment was separated into six immunologically active fractions on diethylaminoethyl-Sephadex. The fractions were found to possess a common antigen which exhibited similar properties on immunodiffusion and immunoelectrophoresis. The purified antigen did not react with any other streptococcal group antisera. Adsorption of group R serum with the antigen removed all antibodies against whole cell antigen extracts of R cells. Chemical and enzymatic analysis of three fractions showed that the antigen was composed of D-glucose, D-galactose, rhamnose, and glucosamine. No significant quantities of phosphorus, glycerol, ribitol, or muramic acid were present. Significant inhibition of the quantitative precipitin determination by D-galactose and stachyose indicated that galactose in terminal alpha linkage was the immunodominant hexose in the antigen. D-Glucose and D-glucosamine possessed a partial inhibitory activity. N-acetyl-D-glucosamine and L-rhamnose did not produce significant inhibition. The results indicate that the R antigen is an immunologically specific structure which serves as a reliable means of identification of these streptococci as a serological group.

Group R streptococci are beta-hemolytic, bacitracin-sensitive organisms which have been isolated from septicemic infections in pigs (2), and meningitis and septicemia in man (9). The cell walls of the R streptococci are known to contain rhamnose, glucose, and galactose (1, 11, 14), and acid extracts of the walls give positive precipitin reactions with anti-whole cell sera (2). No information is available on the chemical composition, structure, and immunological specificity of the R group antigen, although it would appear likely that the antigen is a polysaccharide located in the cell wall. In view of the importance of such polymers to the structure and composition of the wall, and the antigenicity of the cell, it was of interest to examine the location and nature of the streptococcal group R antigen. A preliminary report has been presented (P. R. Soprey and H. D. Slade, *Bacteriol. Proc.*, p. 73, 1971).

## MATERIALS AND METHODS

**Streptococcal cultures and extracts.** The streptococcal strains were kindly provided by C. E. de Moor,

Central Public Health Laboratory, Utrecht, The Netherlands. The cells were grown for 17 hr at 37 C in Todd-Hewitt broth (Difco) supplemented with glucose and salts (12), removed from the medium by centrifugation, and, in some cases, washed with distilled water and lyophilized. Growth was estimated by optical density measurements at 550 nm.

Lyophilized cells were extracted with either 0.01, 0.05, or 0.2 N HCl (2 mg/ml) at 100 C for 10 min and adjusted to pH 7 with NaOH, and the volume was brought to 2 ml. The buffer and saline extracts of whole cells were prepared by stirring the lyophilized cells for 10 min in 0.1 M sodium acetate buffer (pH 5.0) or in 0.85% NaCl. The extracts were filtered through a membrane filter (Millipore Corp.; 0.45  $\mu$ m), and the filtrate was tested for the presence of the antigen by the precipitin test.

Cell walls were prepared as previously described (14) and then extracted either at 90 C with 5% trichloroacetic acid (12) or with 0.05 N HCl at 100 C for 10 min (16). The trichloroacetic acid extracts were freed of the acid by extensive dialysis against water and then were lyophilized. The HCl extracts were neutralized with NaOH. Both extracts were tested for the presence of antigen by the capillary precipitin test (16).

Sonic extracts were prepared by suspending lyophilized cells (1 g/50 ml) in 0.02 M sodium acetate

<sup>1</sup> Present address: LaRabida—University of Chicago Institute, Chicago, Ill. 60649.

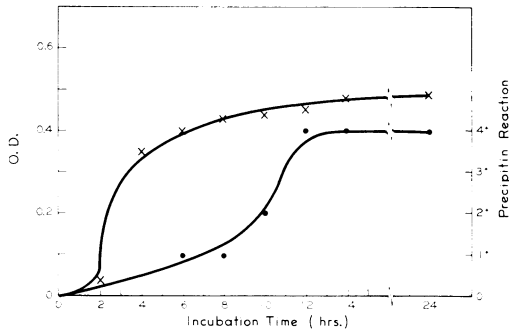


FIG. 1. Growth characteristics and antigen production by strain 734. Upper curve represents OD at 550 nm, and the lower curve indicates capillary precipitin reaction.

buffer (pH 5.0), with subsequent sonic treatment for 10 min at 8 amp (Branson Sonifier, Branson Instruments, Inc., Stanford, Conn.) During sonic treatment, the cell suspension was maintained at about 5 C by immersing the container in an ice bath. The mixture was centrifuged at 15,000  $\times g$  for 20 min. The supernatant fluid, containing the antigen and contaminating proteins and nucleoproteins, was deproteinized by shaking with chloroform-amyl alcohol (9:1). The aqueous phase was deproteinized until there was no precipitate at the interface, concentrated by flash evaporation at 45 C, dialyzed against distilled water, and lyophilized. The lyophilized antigen was then treated with proteolytic enzymes and with ribonuclease as previously described (14).

**Immunological procedures.** The qualitative and quantitative precipitin determinations were performed as previously described (12). To determine the ease of removal of the antigen from the cells, portions of the culture were removed at various intervals and centrifuged, and the cells were suspended in 0.85% saline to the original volume and mechanically mixed (Cyclo-Mixer, Clay-Adams Co.) for 2 min. The suspension was filtered through a membrane filter (Millipore; 0.45  $\mu$ m), and the filtrate was tested by the qualitative procedure. Dilutions of the filtrate were made with 0.85% saline.

Antiserum was prepared in rabbits by injections of lyophilized whole cells as previously described (14). Strains 734, 735, and 12652 were employed. Three milliliters of serum was adsorbed with 1.0 ml of lyophilized group A cells for 3 hr at 37 C and 15 hr at 4 C with occasional mixing. After adsorption, these sera showed no precipitin reaction when tested against extracts of cells from stock strains belonging to serological groups A, B, C, D, E, F, G, H, K, L, M, N, O, P, Q, S, and T. In each case, 1 ml of cell extract contained antigenic material released from 1 mg of lyophilized cells with 0.05 N HCl at 100 C for 10 min.

Double diffusion analysis was performed in agarose gel (17), and immunoelectrophoresis was performed in agarose containing high-resolution buffer (Gelman Instrument Co., Ann Arbor, Mich.).

**Ion-exchange chromatography.** Ion-exchange chromatography was performed on diethylaminoethyl

(DEAE)-Sephadex A-25 (Pharmacia, Uppsala, Sweden). The Sephadex resin was treated with 0.5 M NaOH: $\text{NH}_4\text{CO}_3$  aqueous mixture until it was free of  $\text{Cl}^-$  ions and then washed with distilled water. The resin was then equilibrated with 0.01 M  $\text{NH}_4\text{CO}_3$ : $\text{NH}_4\text{HCO}_3$  buffer (pH 8.2). Elution was accomplished with a series of ammonium carbonate-bicarbonate buffers which were of the indicated molarity. About 400 mg of partially purified antigen in 0.02 M buffer was applied to the column (2.1 by 30 cm). The column was eluted with 0.01 M buffer with a flow rate of 20 ml/hr. The eluate was collected in 5.0-ml fractions, and the eluted antigen was detected in the fractions by capillary precipitin test. Also, elution of ultraviolet light-absorbing material was monitored at 257 nm by an ISCO analyzer (ultraviolet analyzer, model UA-2, Instrumentation Specialities Co., Lincoln, Neb.) Successive elutions were made at 0.02, 0.04, 0.06, 0.08, and 0.1 M buffer. The fractions were concentrated by flash evaporation at 45 C and lyophilized.

Thin-layer chromatography was carried out as previously described (15).

**Acid hydrolysis of the antigen.** The antigen was hydrolyzed in 6 N HCl (2 mg/ml) for 12 hr at 100 C for hexose analysis or in 1 N  $\text{H}_2\text{SO}_4$  (2 mg/ml) at 100 C for 4 hr for pentose analysis. The hydrolysates containing HCl were dried over  $\text{P}_2\text{O}_5$  and NaOH under vacuum, and those containing  $\text{H}_2\text{SO}_4$  were neutralized by the addition of Amberlite beads ( $\text{CO}_3^{2-}$  form). In the latter case, the solution was withdrawn by pipette, the beads were washed several times with water, and the solution was made to a known volume. Any insoluble material was removed by centrifugation.

**Analytical methods.** Determinations of glucose, glucosamine, glycerol, phosphorus, and rhamnose were performed as previously described (8). Acetylated hexosamines were determined by a modification of the method of Reissig et al. (5) and total hexoses by the procedure of Dubois et al. (3). An equimolar mixture of glucose and galactose served as standard in the latter case. The release of glucose from the antigen by

TABLE 1. Precipitin titers of antigen extracts obtained by various procedures from cell walls and whole cells<sup>a</sup>

Strain	Cell walls extracted with		Whole cells extracted with		
	0.05 N HCl	5% Trichloroacetic acid	0.85% Saline	0.1 M Acetate (pH 5)	Sonic treatment <sup>b</sup>
734	1:128 <sup>c</sup>	1:8	1:32	1:32	1:64
735	1:64	1:8	1:16	1:16	1:32

<sup>a</sup> The cell wall extracts were dissolved in saline and tested with antiserum by capillary precipitin test. See Materials and Methods for treatment of whole cell extracts before testing.

<sup>b</sup> In 0.02 M sodium acetate (pH 5.0).

<sup>c</sup> Maximum dilution giving a positive precipitin reaction.

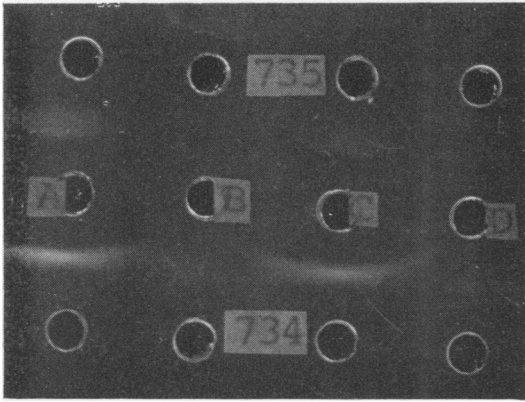


FIG. 2. Immunodiffusion reaction of acid extracts with 734 and 735 antisera. The top well contains 735 antiserum and the lower well contains 734 antiserum. The center wells contain the acid extracts. A and C contain 0.01 N HCl extracts of strains 734 and 735 respectively; B and D contain 0.05 N HCl extracts of the same strains.

$\beta$ -glucosidase was performed as previously reported (15).

## RESULTS

To determine at what stage during growth of the culture the quantity of antigen synthesized was at maximum levels, portions of the culture were withdrawn, and the cells were tested for their antigen content. Figure 1 shows that antigen synthesis began in the late exponential phase and reached maximum in the stationary phase. Strains 734 and 735 exhibited the same properties.

Cell walls prepared by Mickle disintegration (14) were found to contain the antigen. Dilute HCl (0.05 N, pH 1.65) at 100 C was considerably more effective than 5% trichloroacetic acid (pH 1.60) at 90 C (Table 1). Experiments with whole cells then showed that the antigen could be removed easily by stirring the cells at room temperature in physiological saline or 0.1 M acetate buffer (pH 5.0). Both procedures were equally effective. In an effort to obtain a larger yield of antigen, physical disruption of the cell at pH 5.0 was examined. Table 1 shows that more antigen was released by this method than by either of the former methods. This procedure was employed for large-scale preparation of the antigen.

It has been shown (2) that, at 100 C, extraction of group R cells with 0.2 N HCl does not yield an active extract, whereas 0.066 N HCl does extract the serologically active antigen. We have likewise found that ethanol precipitates from 0.2 N HCl extracts do not react with antiserum. Consequently, extracts were made with lower concentrations of HCl and tested by immunodiffusion to determine the sensitivity of the antigen to hydrogen ion and to determine whether more than one antigen was present in the extracts. Figure 2 shows that at least two antigens were extracted at 100 C in 0.01 N HCl, whereas very little antigen was present in the 0.05 N HCl extract. It would appear that the antigens of the R streptococci are sensitive to 0.05 N HCl. This concentration is routinely employed in the capillary precipitin test for the detection of grouping and typing antigens.

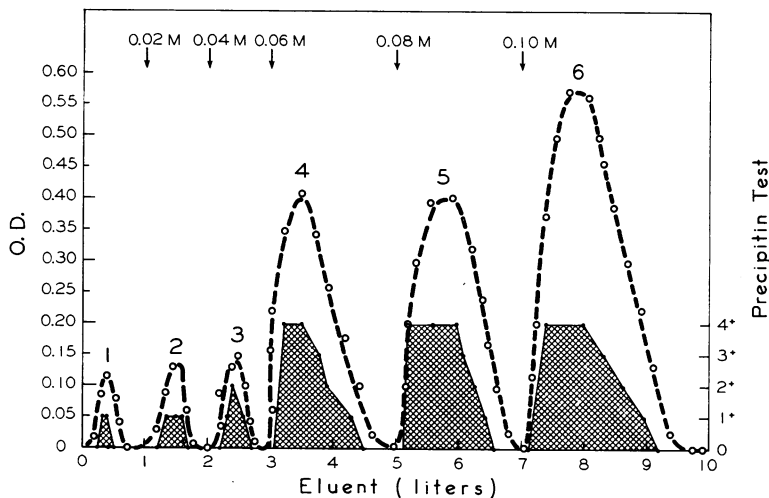


FIG. 3. Fractionation of the antigen from strain 734 cells on a diethylaminoethyl-Sephadex ( $\text{CO}_3^-$ ) column. The numbers at the top of the diagram indicate the various buffers used to elute the column. Shaded areas represent intensity of precipitin test against specific antiserum, and unshaded areas represent the adsorption at 220 nm.

TABLE 2. Yield of antigen fractions from diethylaminoethyl-Sephadex column

Fraction	Molarity of eluant	Yield	
		Amt (mg) <sup>a</sup>	Per cent
1	0.01	5.0	0.05
2	0.02	4.0	0.04
3	0.04	5.0	0.15
4	0.06	41.7	0.42
5	0.08	105.2	1.05
6	0.10	78.7	0.78

<sup>a</sup> From 10.0 g of lyophilized whole cells.

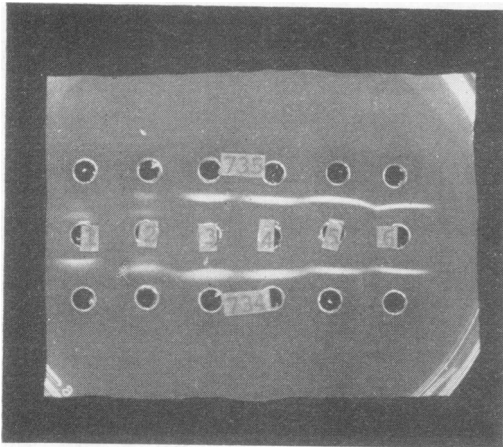


FIG. 4. Immunodiffusion reaction of the antigenic fractions obtained by ion-exchange chromatography. Upper and lower wells contain the antisera. The center wells contain the antigenic fractions 1 to 6.

A large-scale extract was prepared from whole cells of strain 734 by sonic oscillation in 0.02 M acetate, partially purified, and then fractionated on a DEAE-Sephadex A25 ( $\text{CO}_3^{2-}$ ) column. Figure 3 shows the elution pattern of the extract. Each of the six fractions contained antigen; however, it is evident that fractions 4, 5, and 6 contained the bulk of the active material. The yield of each from 10 g of dry cells is given in Table 2. Fractions 4, 5, and 6 contained 90% of the material eluted from the column.

The immunodiffusion characteristics of each fraction are shown in Fig. 4. It is evident that at least two antigens are present in fractions 1 and 2; however, only one of these antigens is apparent in the remaining four fractions. The sharpness of the precipitin band between fractions 3 and 6 indicates considerable purification of the antigen during passage through the column. It is clear from Fig. 5 that the lines of identity in

strains 734 and 735 identify a single antigen present in these strains. Fractions 1, 2, and 3 were not investigated further.

Fractions 4, 5, and 6 were examined by immunoelectrophoresis (Fig. 6). Each reacted with 734 antiserum, migrated to the positive pole, and appeared homogeneous. The migration of the fractions was about the same.

The qualitative composition of fractions 4, 5, and 6 was determined by thin-layer chromatography after acid hydrolysis at 100 C in 1 N  $\text{H}_2\text{SO}_4$  for 6 hr. Figure 7 shows that fractions 4, 5, and 6 are composed principally of glucose, galactose, glucosamine, and lesser quantities of rhamnose

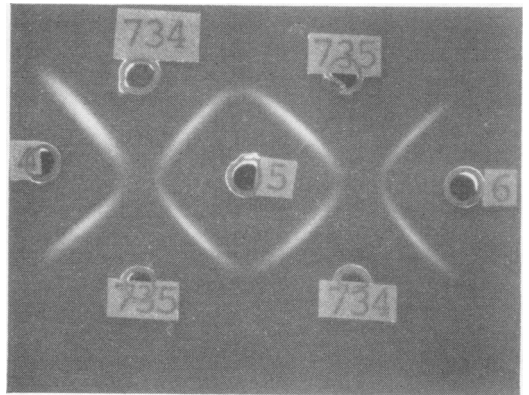


FIG. 5. Immunodiffusion of fractions 4, 5, and 6 against 734 and 735 antiserum.

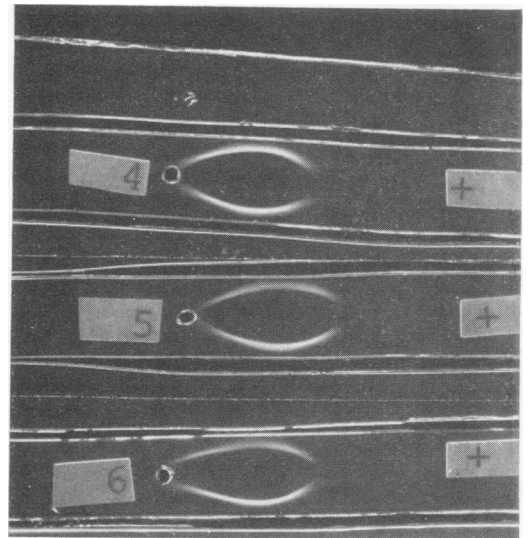


FIG. 6. Immuno-electrophoresis of antigen fractions 4, 5, and 6 against 734 antiserum.

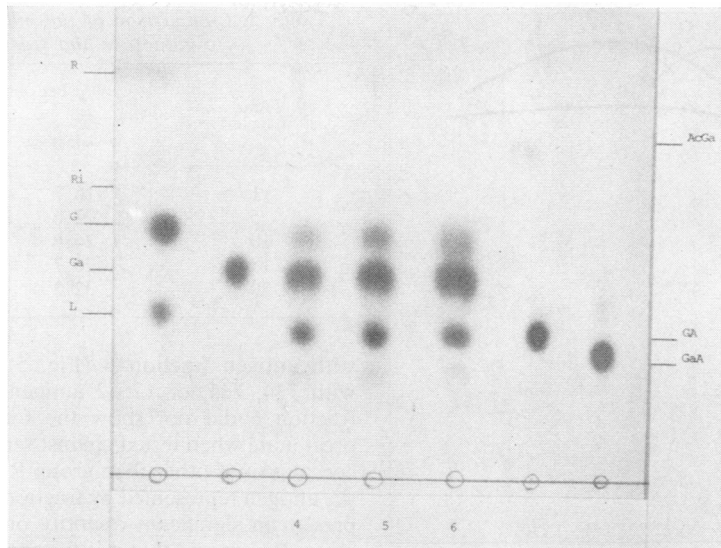


FIG. 7. A thin-layer chromatogram of the antigenic fractions. The chromatogram was developed in pyridine-ethylacetate-acetic acid-water (5:5:3:1 by volume) and stained with alkaline silver nitrate. Numbers 4, 5, and 6 represent the acid hydrolysates of the antigenic fractions 4, 5, and 6. The reference sugars used are to the right and to the left side: R, rhamnose; G, glucose; Ga, galactose; GA, glucosamine; L, lactose; AcGa, N-acetylglucosamine; Ri, ribitol; GaA, galactosamine.

TABLE 3. Composition of antigen fractions separated by chromatography

Fraction	Protein	Phosphorous	D-Glucose	Rhamnose	D-Galactose	Glucosamine	Muramic acid
4 <sup>a</sup>	2.9 <sup>b</sup>	0.11	10.45	8.84	43.83	7.81	0.18
5 <sup>a</sup>	0.9	0.13	12.57	7.85	40.76	10.44	0
6 <sup>a</sup>	1.1	0.07	13.53	11.53	43.77	11.11	0

<sup>a</sup> Less than 1% galactosamine.

<sup>b</sup> All values expressed as percentage.

and galactosamine. The complete quantitative analysis is given in Table 3. Rhamnose, although showing only traces on thin-layer chromatography, was present in significant quantity. The four six-carbon compounds make up 70 to 80% of the material analyzed. The protein content, based on the sum of the amino acids separated by chromatography, is less than 3% in each fraction, and glycerol, based on gas chromatographic analysis, is absent. The absence of ribitol (Fig. 7) and glycerol and the lack of appreciable quantities of phosphorus show that the polysaccharide antigen fractions do not contain significant quantities of teichoic acids or phosphorylated sugars. Muramic acid is absent in fractions 5 and 6; thus, it is unlikely that cell wall peptidoglycan is present.

Figure 8 illustrates the precipitin curves of fractions 4, 5, and 6 against 734 antiserum. The equivalence points are 5  $\mu$ g for fraction 4, 3.5

$\mu$ g for 5, and 2.5  $\mu$ g for 6. Table 4 shows the inhibitory effect on the precipitin reaction of a number of mono-, di-, and tetrasaccharides when tested at the equivalence point. Of the four constituent hexoses, galactose possesses a significant effect, especially for a single hexose. Stachyose, a tetrasaccharide containing  $\alpha$ -D-galactose- $\alpha$ -D-galactose in terminal position, was approximately twice as effective an inhibitor as D-galactose over the range of 1 to 20  $\mu$ moles. (Table 5). Consequently, an alpha-linked terminal location for immunodominant D-galactose sugar is indicated. Lactose, which contains galactose in beta form, was a weak inhibitor. The effects of glucose and glucosamine indicate that these hexoses also participate in the specificity of the antigen, although not as extensively as galactose. Rhamnose and N-acetylglucosamine, on the other hand, do not appear to contribute to this property of the antigen.

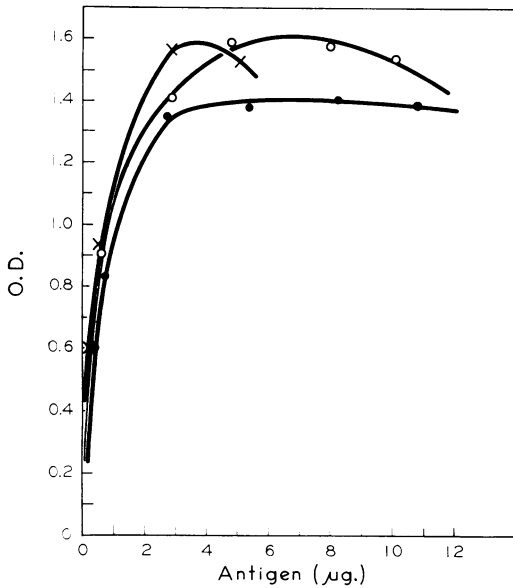


FIG. 8. Quantitative precipitin curves of group antigen preparations extracted by sonic treatment from strain 734 whole cells. Symbols: fraction 4 (O—O), fraction 5 (●—●), fraction 6 (X—X). OD readings made at 750 nm.

TABLE 4. Inhibition of quantitative precipitin reaction between fraction 6 and specific antiserum

Inhibitor	Per cent inhibition <sup>a</sup>
Lactose [O-β-D-galactopyranosyl-(1,4)-β-D-glucopyranose]	5.24
D-Glucose	12.48
D-Glucosamine	16.48
N-acetyl-D-glucosamine	4.49
Stachyose {[O-α-D-galactopyranosyl-(1,6)] <sub>2</sub> -α-D-glucopyranoside-(1,2)-D-fructose}	33.8
β-Cellobiose [O-β-D-glucopyranosyl-(1,4)-β-D-glucopyranosyl]	15.40
L-Rhamnose	1.75
D-Galactose	24.14
D-Glucuronic acid <sup>b</sup>	5.2
D-Galacturonic acid <sup>b</sup>	2.41

<sup>a</sup> A 5.0-μmole amount of inhibitor was used in each case.

<sup>b</sup> Uronic acids were neutralized before testing.

## DISCUSSION

The polysaccharide antigen reported here is most likely the group antigen of the R streptococci. Cross-adsorption tests show that stock strains 734, 735, and 12652 possess a common antigen. Strain 734 antiserum, after adsorption

TABLE 5. Comparison of the inhibitory action of D-galactose and stachyose

Amt (μmoles)	Per cent inhibition	
	D-Galactose	Stachyose
1	16.7	25.9
5	22.6	47.3
10	24.6	52.2
15	26.7	64.2
20	32.2	72.1

with antigen fraction 6 (Fig. 5), did not react with 734, 735, or 12652 antigen extracts. Also, fraction 6 did not show the formation of any precipitate when tested against sera to all streptococcal groups other than group R. It is clear that the antigen represented by fraction 6 is the antigen present in significant quantity on the surface of group R cells and that it is the antigen responsible for their identification as a serological group.

Evidence for the presence of a minor antigen in sonic extracts of 734 and 735 has been found (Fig. 4). This antigen, however, is sensitive to acid and will cause no interference in the identification of the R streptococci if antigen extracts are prepared with 0.01 N HCl. In the present work, this antigen has been separated from the group antigen by column chromatography.

The ease of removal of the antigen from the whole cell (Table 1) indicates a surface location on the cell wall. India ink preparations of cells in culture fluid did not indicate a capsular structure; however, when antiserum was added, a modest swelling of a capsule-like structure occurred. The location of the antigens at the cell surface may be related to their sensitivity to acid. Acetate (0.1 M, pH 5.0) is recommended for the routine removal of the R antigen.

The data in Tables 4 and 5 indicate that galactose in terminal alpha linkage plays a significant role in the immunological specificity of the antigen. No other streptococci are known to depend on α-galactose for specificity in either group or type polysaccharide antigens. However, the type 4 antigen of the F streptococci possesses specificity principally attributable to galactose in beta linkage (18), as do a pH 2-resistant fraction of the group B type II antigen (4, 6), and a type antigen of *Streptococcus salivarius* (G. C. Kothari, Ph.D. thesis, University of Utrecht, 1968). α-Galactose specificity is known to function in several of the pneumococcal capsular polysaccharides (10) and somatic and capsular polysaccharides of the Enterobacteriaceae (7). The inhibitory effects of β-cellobiose and D-glucose (Table 4) indicate that D-glucose in beta linkage

also participates in the specificity, although to a lesser extent than galactose. However, no glucose was released by the action of  $\beta$ -glucosidase; hence a subterminal location for this hexose appears likely. Further studies are required to provide details of the structure of the R antigen. The release of antigenic fractions of lower molecular weight than fractions 4, 5, and 6 by sonic oscillation, as well as by mild acid hydrolysis, may be suitable for this purpose.

#### ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant HE-03709 from the National Heart Institute and by grants from the Office of Naval Research (NR 103-608), the Grainger Fund, and the Hemac Fund. H.D.S. is the recipient of Public Health Service Research Career Award K6-GM-16284 from the National Institute of General Medical Sciences.

#### LITERATURE CITED

- Colman, G., and R. E. O. Williams. 1965. The cell walls of streptococci. *J. Gen. Microbiol.* **41**:375-387.
- de Moor, C. E. 1963. Septicaemic infections in pigs, caused by haemolytic streptococci of new Lancefield groups designated R, S and T. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **29**:272-280.
- Dubois, M. K., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **28**:350-356.
- Freimer, E. H. 1967. Typespecific polysaccharide antigens of group B streptococci II. The chemical basis for serological specificity of the type II HCl antigen. *J. Exp. Med.* **125**:381-392.
- Kabat, E. A., and M. M. Mayer. 1961. *Immunochemistry*, p. 510-511. Charles C Thomas, Publisher, Springfield, Ill.
- Lancefield, R. C., and E. H. Freimer. 1966. Type-specific polysaccharide antigens of group B streptococci. *J. Hyg.* **64**:191-203.
- Luderitz, O., K. Jann, and R. Wheat. 1968. Somatic and capsular antigens of gram-negative bacteria. In M. Flarkin and E. Stotz (ed.), *Comprehensive biochemistry*. Elsevier Publishing Co., Amsterdam.
- Matsuno, T., and H. D. Slade. 1970. Composition and properties of a group A streptococcal teichoic acid. *J. Bacteriol.* **102**:747-752.
- Perch, B., P. Kristjansen, and K. Skadhauge. 1968. Group R streptococci pathogenic for man. *Acta Pathol. Microbiol. Scand.* **74**:69-76.
- Rebers, P. A., E. Hurwitz, and M. Heidelberger. 1961. Immunochimistry of pneumococcal types II, V, and VI. II. Inhibition tests in the type VI precipitating system. *J. Bacteriol.* **82**:920-926.
- Roberts, W. S. L., and F. S. Stewart. 1961. The sugar composition of streptococcal cell walls and its relation to hemagglutination pattern. *J. Gen. Microbiol.* **24**:253-260.
- Slade, H. D. 1965. Extraction of cell-wall polysaccharide antigen from streptococci. *J. Bacteriol.* **90**:667-672.
- Slade, H. D., and W. C. Slamp. 1960. Studies on *Streptococcus pyogenes*. V. Biochemical and microscopic aspects of cell lysis and digestion by enzymes from *Streptomyces albus*. *J. Bacteriol.* **79**:103-112.
- Slade, H. D., and W. C. Slamp. 1962. Cell-wall composition and grouping antigens of streptococci. *J. Bacteriol.* **84**:345-351.
- Soprey, P. R., and H. D. Slade. 1971. Chemical structure and immunological specificity of the streptococcal group E cell wall polysaccharide antigen. *Infect. Immun.* **3**:653-658.
- Swift, H. F., D. T. Wilson, and R. C. Lancefield. 1943. Typing group A hemolytic streptococci by M precipitin reactions in capillary pipettes. *J. Exp. Med.* **78**:127-133.
- Tan, E. M., and H. G. Kunkel. 1966. Characteristics of a soluble nuclear antigen precipitating with sera of patients with lupus erythematosus. *J. Immunol.* **96**:464-471.
- Willers, J. M. N., M. F. Michel, M. J. Sysma, and K. C. Winkler. 1964. Chemical analysis and inhibition reactions of the group and type antigens of group F streptococci. *J. Gen. Microbiol.* **36**:95-105.