TYPE-SPECIFIC POLYSACCHARIDE ANTIGENS OF GROUP B STREPTOCOCCI

II. The Chemical Basis for Serological Specificity of the Type II HCL Antigen*

By EARL H. FREIMER, M.D.

(*From The Rockefeller University, New York*)

Individual serological types occur among Group B streptococci, and in a recent report from this laboratory attention was directed to the type-specific substances of these microorganisms (1). The capsular antigens differentiating these bacteria are polysaccharides, chemically and serologically distinct from the group-specific antigen, a cell wall polysaccharide which provides the serological basis for their classification as Group B streptococci.

Antibodies directed against these type-specific polysaccharides of Group B streptococci not only give specific precipitin reactions, but also are capable of passively protecting mice against infection with mouse-virulent strains of the homologous type. The previous serological study had shown that each rabbit immunized with Group B streptococci of Type II produced two immunologically distinct type-specific antibodies (1). These two antibodies, which showed equal activity by weight in protecting mice against infection with a mouse-virulent strain of the same serological type, were easily distinguished by their precipitin reactions with Type II polysaccharide. This unusual finding of two distinct type-specific protective antibodies directed against different determinants in the capsule of the same Group B streptococcal strain was supported by the current studies of the chemical basis for the serological specificity of the Type II antigens.

Materials and Methods

Strains of Group B Streptococci.—Representative strains of the four specific types, Ia, Ib, II, and III were all from The Rockefeller University collection.

Preparation of Antigens and Antisera.—These were prepared as described in the previous report (1).

Precipitin Analysis.—Quantitative precipitin analyses were done by a spectrophotometric procedure (2).

Immunodiffusion.—Double diffusion was performed by a modification of the Ouchterlony

---

method (3). Slides for immunoelectrophoresis were prepared by the Scheidegger technique (4).

Analytical Methods.—Analyses for rhamnose, glucose, glucosamine, nitrogen, and phosphorus were performed as previously described (3). Total hexose was measured by the anthrone reaction using a glucose standard (5). Galactose was determined by a modified method employing galactose oxidase (Galactostat) available from the Worthington Biochemical Corporation, Freehold, N. J.

EXPERIMENTAL

Chemical Composition of the Group B Type II Polysaccharide.—Two serologically active type-specific polysaccharides were extracted from the Group B, Type II streptococcus. One was obtained by heating the bacteria at 100°C in dilute HCl, pH 2, while the other was removed by shaking the streptococci with glass beads in 2.5% trichloroacetic acid (TCA), pH 2 at 0°C. These extraction procedures as well as the methods for purification of the polysaccharides have been described in detail in the earlier report (1).

In that report, precipitin analyses and immunodiffusion studies showed that the type-specific polysaccharide removed by HCl extraction represented a partial antigen, which reacted with only one of the two type-specific antibodies. On the other hand, TCA extraction yielded the more complete polysaccharide, capable of reacting with both antibodies. Furthermore, heating the TCA polysaccharide in the presence of dilute HCl, altered this antigen, resulting in a polysaccharide identical in immunological reactivity to that of the HCl polysaccharide. These serological studies indicated that the "HCl antigen" represented a degraded form of the "TCA antigen," a relationship supported by the following chemical studies of these two purified polysaccharides.

In order to determine their constitution, acid hydrolysates of each polysaccharide were examined by ascending paper and by thin layer chromatography in N-butanol:pyridine:water, (6:4:3). Using these techniques, three monosaccharides, galactose, glucose, and glucosamine, were identified in each hydrolysate. No amino acids were detected. On the basis of this qualitative information, specific quantitative procedures were used to determine the amount of each sugar present in hydrolysates of the two polysaccharides. In addition, nitrogen, phosphorus, rhamnose, and anthrone determinations were performed.

Each preparation of Type II polysaccharide was analyzed in this manner, and the chemical composition of three lots of TCA antigen and two lots of HCl antigen is presented in Table I. These polysaccharides were alcohol-ether–dried powders, and each preparation contained from 3 to 5% water and from 1 to 2% of ash. They were free of other known cell antigens. The very low rhamnose content confirmed the serological studies which had shown that group-specific polysaccharide was not present. The absence of phosphorus indicated that these preparations did not contain significant amounts of polyglycerophosphate, and the nitrogen content could be accounted for by the glucosamine in the polysac-

charide. In addition, extraction with lipid solvents by the method of Folch (6) showed that these preparations were free from contamination with lipid components.

The analyses in Table I show that the two preparations of HCl antigen, 12B and 13B, are identical in chemical composition. Eight other lots of HCl antigen also were very similar in composition. From these results, it is apparent that the Type II HCl antigen of Group B streptococci is a polysaccharide essentially composed of galactose, glucose, and glucosamine. As the glucosamine is N-acetyl-

TABLE I

Chemical Composition of Type-Specific and Group-Specific Antigens from Group B Type II Streptococci

Lot Strain Preparation	Type-specific polysaccharide						Group-specific polysaccharide	
	12A V8 TCA	13A 18RS21 TCA	14A 18RS21 TCA	12B V8 HCl*	13B 18RS21 HCl*	13ARx 18RS21‡	7 V8 HCl	§090R Formamide
	%	%	%	%	%	%	%	%
Galactose	23.8	25.0	26.8	34.0	34.0	33.9	8.1	8.9
Glucose	15.8	18.5	21.1	27.4	27.1	25.6	<1.0	<1.0
Glucosamine	11.2	9.3	10.9	14.7	14.7	13.8	10.0	12.3
Rhamnose	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	49.1	50.2
Anthrone			62.9	64.5	65.0	64.0	76.6	
Nitrogen	1.70	1.70	1.70	1.50	1.55	1.67		
Phosphorus	0.0	0.0	0.0	0.0	0.0	0.0		

These preparations are alcohol-ether-dried powders with a water content of 3–5%.

* Average of 3 analyses.

‡ 13ARx is TCA preparation 13A after heating with dilute HCl.

§ From Curtis and Krause (7).

ated, these three monosaccharides comprise more than 85% of this molecule when calculated on a dry weight basis.

In contrast to the HCl antigen, there was considerable variation in the composition of the three TCA preparations, and the three monosaccharides, galactose, glucose, and N-acetylglucosamine, represented only 64% of the dry weight of TCA preparation, 14A. However, the mol ratio of galactose to glucose to N-acetyl glucosamine was 1.9:1.5:1.0 for HCl preparations 12B or 13B, and 2.0:1.5:1.0 for TCA preparation 14A. The similarity of the mol ratios shows that both the HCl- and the TCA-extracted polysaccharides contain essentially the same proportion of each of the three constituent monosaccharides. The difference in per cent composition reflects the presence of an additional component in the TCA molecule.

Previous studies had shown that TCA polysaccharide contained a component

easily hydrolyzed by brief heating with dilute mineral acid (1). Loss of this labile component converted the polysaccharide to an antigen serologically identical with HCl antigen. Lot 13ARx, TCA preparation 13A treated with dilute HCl, represents an example of this conversion. The relationships between these antigens were demonstrated by double diffusion in agar (1). A precipitin line formed between each preparation and an unabsorbed type-specific antiserum. The line of 13ARx, the converted TCA antigen, formed a spur with the line of 13A, the untreated TCA antigen, and merged to form a common band of identity with the precipitin lines of HCl antigens 12B and 13B. Examination of these preparations by immunoelectrophoresis using Veronal buffer (pH 9.3) revealed that TCA antigen 13A migrated toward the anode while the converted polysaccharide 13ARx behaved like the two HCl antigens and moved toward the cathode.[1]

The chemical composition of this converted polysaccharide 13ARx is recorded in Table I. It is distinctly different from that of TCA preparation 13A, and quite similar to the composition of the HCl antigens. Apparently the removal of the acid-labile component of the TCA antigen results in a polysaccharide, chemically as well as serologically, identical with the HCl-extracted antigen. This labile component probably represents the determinant which confers the additional serological specificity as well as the net negative charge to the TCA molecule.

Although the labile component has not as yet been chemically identified, the absence of both lipid and protein moieties in the TCA antigen suggests that it is likely to be a carbohydrate. Supporting evidence for this concept is based on the anthrone reaction which measures total hexose. The amount of anthrone-reacting material was essentially the same for both the HCl and the TCA antigens. In HCl preparations 12B and 13B, the per cent of galactose and the per cent of glucose add up to 61.4% as compared to an anthrone reactivity of 64.5%. On the other hand, in TCA preparation 14A, the sum of these two hexoses is only 47.9%. As this represents only 75% of the anthrone reactivity of 14A, the remainder may reflect the unidentified component of the TCA molecule. The anthrone reactivity of TCA preparations is variable, and in one recent lot which has not been completely evaluated, it was lower than that of 14A.

During extraction of the type-specific polysaccharide, some Group B cell wall carbohydrate also was extracted. After partial purification, this group-specific

---

[1] A similar change in electrophoretic mobility was observed during studies of the type-specific polysaccharide antigens extracted from Group B streptococci of Type 1a and Type 1b. Extraction with dilute HCl at 100°C resulted in antigens that moved toward the cathode, while antigens extracted either with the enzymes of *Streptomyces albus* or by shaking in the cold with 2 M NaCl, migrated toward the anode. Furthermore, heating these preparations with dilute mineral acid resulted in polysaccharides which then migrated toward the cathode.

antigen was separated from the type-specific antigens by fractional precipitation with ethanol. The chemical composition of this polysaccharide is included in Table I. The HCl-extracted Group B antigen contains about 50% of rhamnose, 10% of galactose, and 10% of glucosamine. This composition is essentially the same as that reported by Curtis and Krause for the group-specific antigen which they extracted from isolated Group B cell walls with hot formamide (7). For comparison, one of their analyses of Group B antigen is included in Table I.

Serological Properties of the TCA and the HCl Polysaccharides.—Investigations of the serological properties of each of the polysaccharide preparations were coupled with the chemical studies described above. Two distinct type-specific antibodies are produced by rabbits immunized with Type II streptococci. Although the relative proportions of these two antibodies vary from serum to serum, each serum contains HCl antibody which reacts with a determinant present in both the HCl and the TCA antigens, and TCA antibody reactive with a determinant present solely in TCA antigen (1). Using quantitative precipitin analyses, the amount of each antibody has been determined. The TCA reactivity of unabsorbed sera was compared to the same sera after absorption with HCl antigen.

The serological reactivity of several preparations of HCl antigen and several preparations of TCA antigen also have been compared by the quantitative precipitin technique. In general, sera with high titers of TCA antibody were used. In these experiments, increasing amounts of antigen were added to 0.1 ml aliquots of the serum, and the final volume of the mixture was adjusted to 1.0 ml with saline. The results of several experiments in which a number of different preparations of each antigen were tested with serum E4 are shown by the precipitin curves in Fig. 1. The three TCA precipitin curves were almost identical, and equivalence was reached with 60 μg of all three preparations of TCA antigen. At equivalence, more than twice as much antibody globulin was precipitated from this serum by the TCA antigen as by the HCl antigen. The precipitin curves representing the four preparations of HCl antigen also were essentially identical. These precipitin studies supported the chemical evidence which had demonstrated that all of the HCl preparations were quite similar in composition.

The chemical basis for the serological specificity of the Type II antigen was studied by using the technique of specific inhibition of the quantitative precipitin reaction. The HCl antigen was selected because it is chemically better defined and serologically less complex than the TCA antigen, and the initial inhibtion tests were carried out with the three constituent monosaccharides.

To evaluate the inhibitory effect of a sugar, 0.1 ml aliquots of a Type II antiserum were mixed with 20 mg of the monosaccharide or disaccharide to be tested, Type II HCl antigen was added, and the volume was adjusted to 1.0 ml with saline. In this experiment, four concentrations of HCl antigen 7S were used, and at each concentration, serum E5 was mixed

either with D-glucose, D-galactose, or N-acetyl-D-glucosamine. At each concentration of antigen, the antigen-antibody precipitate from a 0.1 ml sample of serum without any inhibitor served as the reference point.
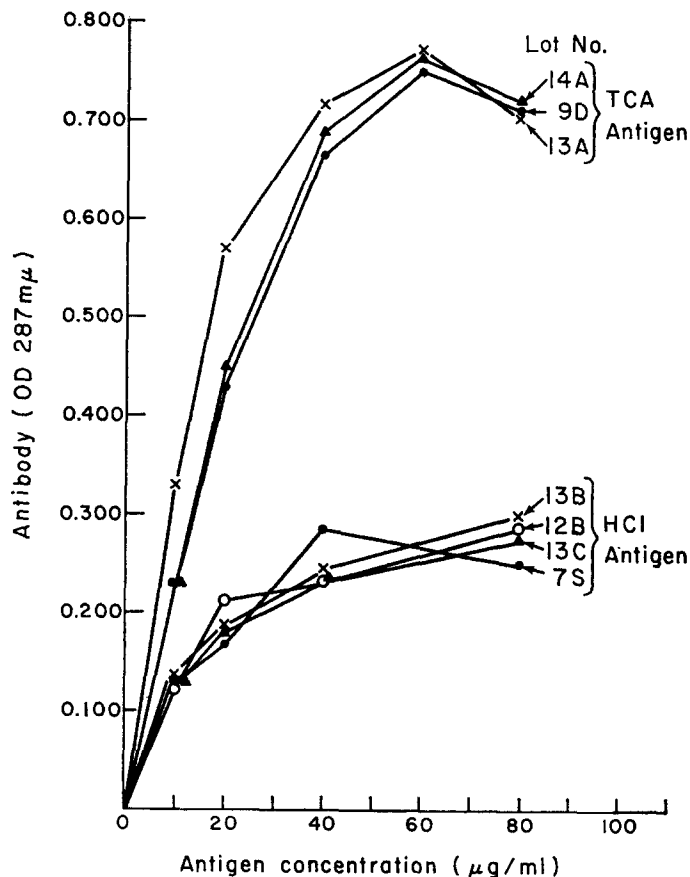

FIG. 1. Comparison of the serological reactivity of several preparations of TCA antigen and several preparations of HCl antigen by quantitative precipitin analysis. The three TCA precipitin curves are almost identical showing that similar amounts of antibody globulin are precipitated from Type II antiserum E_4 by each of the three TCA preparations. The precipitin curves representing the four preparations of HCl antigen also are essentially identical.

The results of one set of inhibition experiments have been recorded as precipitin curves in Fig. 2.

These curves show that 20 mg (110 μmoles) of D-galactose almost completely inhibited the precipitin reaction between Type II HCl antigen and its antibody while the same amount of either D-glucose or N-acetyl-D-glucosamine had essentially no inhibitory effect on this reaction. These results and those of many

similar precipitin inhibition experiments strongly suggest that D-galactose is the principal determinant of the Type II HCl-extracted polysaccharide. In contrast to these results, the three monosaccharides singly or in combination did not inhibit the precipitin reaction between TCA antigen and specific antibody.

To explore the possibility that the inhibitory effect of D-galactose might be nonspecific, a number of other monosaccharides including several galactose derivatives were examined. In this series of experiments, the inhibitory activity of each sugar was compared with that of galactose. Increasing concentrations of each sugar were added to 0.1 ml samples of serum, while the concentration of
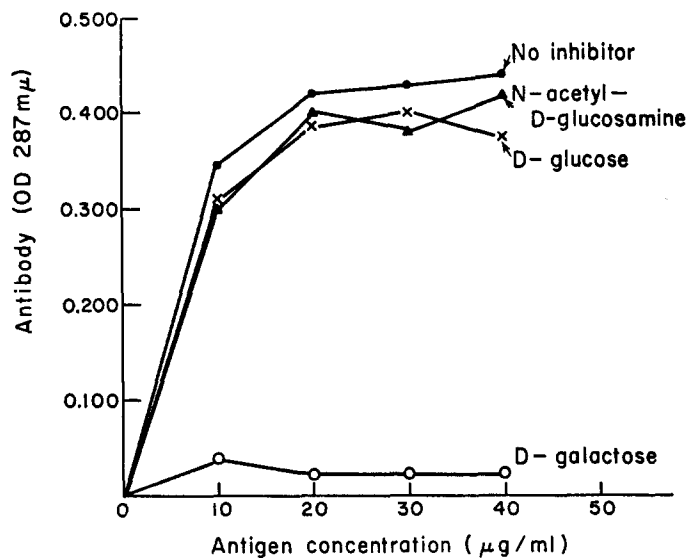

FIG. 2. Inhibition by D-galactose of the precipitin reaction between Type II HCl antigen and a Type II antiserum. No inhibition occurred in the presence of either D-glucose or N-acetyl-D-glucosamine. The final concentration of each monosaccharide was 20 mg per ml.

HCl antigen was held constant at a point close to antigen-antibody equivalence but still within the range of antibody excess.

By measuring the immune precipitate at different concentrations of the sugar, the relative ability of a monosaccharide to inhibit the Type II HCl antigen-antibody precipitin reaction could be determined. The results of some of these studies are shown in Table II. In order to compare inhibitory activity, the ratio of the decrease in precipitate in the presence of an inhibitor to the precipitate present in controls without any inhibitor has been calculated, and recorded as per cent inhibition. In these tests, only decreases of more than 10% have been considered to reflect inhibitory activity. 2 mg of D-galactose resulted in more than 50% inhibition and 20 mg produced 88% inhibition. On the other hand,

as much as 40 mg of either D-glucose or N-acetyl-D-glucosamine, the other monosaccharides present in the Type II HCl polysaccharide, had no inhibitory effect. In contrast to glucosamine, N-acetyl-D-galactosamine showed a significant inhibitory effect.

TABLE II

The Inhibitory Effect of Monosaccharides Related to Galactose on the Precipitin Reaction between the Type II HCl Antigen and a Type II Antiserum

Inhibitor	Concentration	Inhibition
	mg/ml	%
*D-Galactose	2	53
"	10	61
"	20	88
*D-Glucose	40	0
*N-acetyl-D-Glucosamine	40	0
N-acetyl-D-Galactosamine	20	32
D-Arabinose	20	0
D-Lyxose	20	0
D-Xylose	20	0
D-Mannose	20	0
D-Talose	2	25
D-Fructose	20	0
D-Tagatose	20	0
D-Fucose	20	33
L-Fucose	20	17
L-Rhamnose	20	0
2-Deoxy-D-Glucose	20	17
Inositol	20	0
Dulcitol (Galacitol)	20	21
D-Galacturonic acid	20	0
D-Galactono 1:4 Lactone	20	0

0, less than 10% inhibition.
* Monosaccharide present in the Group B Type II HCl polysaccharide.

When a variety of other monosaccharides including pentoses, aldohexoses, ketohexoses, and deoxyhexoses were tested, it was clear that only the close relatives of galactose were active inhibitors. For while concentrations of the other sugars as high as 20 mg/ml proved ineffective, D-fucose (6-deoxy-D-galactose), dulcitol (galacitol), and D-talose, the epimer of D-galactose, all showed significant inhibitory activity. Talose was the most active of these derivatives; as little as 2 mg producing 25% inhibition. These results served as additional evidence for the specificity of D-galactose in inhibiting the precipitin reaction between Type II HCl antigen and type-specific antiserum.

Having established D-galactose as the determinant of the Type II HCl anti-gen, attention was focused on the nature of the glycosidic linkage between the galactose and the remainder of the polysaccharide. In order to establish the configuration of the D-galactose, the inhibitory activity of the α- and the β-forms of methyl and phenyl galactopyranosides were compared by means of the precipitin inhibition technique. The results of this set of experiments which are shown in Table III demonstrated that the galactose was linked to the second sugar in a β-configuration. This conclusion is based on the following evidence. 2 mg of methyl-β-D-galactopyranoside produced 79% inhibition. 2 mg of the α-form were ineffective, and 20 mg only gave 49% inhibition. Thus the β-methyl galactopyranoside was more than 10 times as active as the α-form. The α- and β-forms of methyl glucopyranoside served as controls and proved inactive. Similar results also were obtained when the α- and β-phenyl galactopyranosides were tested. In this case, the β-form was four times as effective as the α-form.

Additional and even stronger confirmation of the β-linkage was obtained when a pair of disaccharides, the α- and β-forms of D-galactopyranosyl 1 → 6-D-glucose, melibiose and allolactose were compared. 75% inhibition was obtained with as little as 2 mg of allolactose, the β-form of this disaccharide, while as much as 20 mg of the α-form, melibiose, showed no significant inhibitory effect. Raffinose and stachyose, two of the higher oligosaccharides containing an α-galactoside linked to sucrose, also proved to be inactive.

Although these experiments indicated that the determinant of the Type II HCl antigen was a β-galactopyranoside, one exception should be noted. The α-form of the p-nitrophenyl derivative of galactose was found to be more active than the β-form. In contrast to this finding, the o-nitrophenyl-β-galactopyranoside showed more inhibition than either of the p-nitrophenyl compounds. Further comparisons were not possible because the α-form of the ortho compound could not be obtained.

Having identified the three constituent monosaccharide units, the nature of the terminal nonreducing unit, and the steric configuration of the glycoside linkage, an investigation into the nature of the second sugar as well as the position of the disaccharide bridge was begun. Although most of the galactose-containing disaccharides are not readily available, it was possible to obtain four representative β-galactopyranosides. The inhibitory activity of each disaccharide was measured by precipitin inhibition, and those results are included in Table III. The data show that the (1 → 6)-D-glucose and the (1 → 4)-D-galactose derivatives were essentially equal in inhibitory activity, and that both were more active than the β-D-galactopyranoside linked (1 → 4) to D-glucose. In addition, the (1 → 4) fructose-containing galactopyranoside also proved to be more active than the (1 → 4) glucose compound. Cellobiose, the β-glucopyranoside comparable to lactose was completely inactive.

The only direct conclusion that can be drawn from the inhibition studies

TABLE III

Inhibition by β-Galactopyranosides of the Precipitin Reaction between the Type II HCl Antigen and Type II Antiserum

Inhibitor	Concen-tration	Inhibi-tion
	mg/ml	*%*
D-Galactose	2	53
"	20	88
Methyl-α-D-galactopyranoside	2	0
"	20	49
Methyl-β-D-galactopyranoside	2	79
Methyl-α-D-glucopyranoside	20	0
Methyl-β-D-glucopyranoside	20	0
Phenyl-α-D-galactopyranoside	2	18
Phenyl-β-D-galactopyranoside	2	62
Phenyl-β-D-glucopyranoside	2	0
o-Nitrophenyl-β-D-galactopyranoside	2	58
p-Nitrophenyl-α-D-galactopyranoside	2	26
p-Nitrophenyl-β-D-galactopyranoside	2	0
p-Nitrophenyl-β-D-glucopyranoside	2	0
p-Nitrophenyl-β-*N*-acetyl-D-glucosamine	2	0
α-D-galactopyranosyl-(1 → 6)-D-glucose (melibiose)	20	0
β-D-galactopyranosyl-(1 → 6)-D-glucose (allolactose)	2	75
Sucrose	20	0
6-α-galactosyl sucrose (raffinose)	20	0
6-α-galactobiosyl sucrose (stachyose)	20	16
β-D-galactopyranosyl-(1 → 6)-D-glucose (allolactose)	2	75
β-D-galactopyranosyl-(1 → 4)-D-galactose (galactobiose)	2	80
β-D-galactopyranosyl-(1 → 4)-D-fructose (lactulose)	2	58
" "	20	95
β-D-galactopyranosyl-(1 → 4)-D-glucose (lactose)	2	34
" "	20	75
β-D-glucopyranosyl-(1 → 4)-D-glucose (cellobiose)	20	0

0, less than 10% inhibition.

with this limited series of disaccharides is that the terminal galactose determinant is not linked $(1 \rightarrow 4)$ to D-glucose in the Type II HCl-extracted polysaccharide. Additional β-galactopyranosides linked $(1 \rightarrow 3)$ or $(1 \rightarrow 6)$ to D-galactose as well as several derivatives linked to N-acetyl-D-glucosamine would be of great value in extending this study. However, these are not at present available. Finally, further chemical definition of the TCA-extracted Type II polysaccharide is essential to establish the serological nature of the second determinant.

DISCUSSION

The present report contains the results of further investigations of the type-specific polysaccharide antigens of Group B streptococci. In this study, the immunochemical relationships of the two Type II antigens have been explored, the three component sugars of the HCl-extracted antigen have been identified, and the chemical basis for serological specificity of this antigen has been clarified. A monosaccharide unit consisting of D-galactose has been established as the specific determinant of this antigen by means of precipitin inhibition studies. Furthermore, preliminary evidence obtained by treating the HCl polysaccharide with galactose oxidase suggests that most of the D-galactose is in a terminal position. Additional supporting evidence for this concept has been obtained by Dr. M. Heidelberger[2] who demonstrated that the Type II HCl antigen and the Type XIV capsular antigen of the pneumococcus cross-react. Terminal D-galactose is known to represent the determinant of the Type XIV pneumococcal polysaccharide.

The present study also provides evidence that the HCl antigen containing but a single antigenic determinant is less complex than the TCA-extracted polysaccharide. One of the determinants of TCA antigen is the same as that of HCl-extracted antigen. The other determinant of the TCA antigen has not been identified, but it is clear that loss of this fragment converts the TCA antigen to one chemically and serologically identical with HCl antigen.

Current studies are directed towards defining the immunochemical nature of the second determinant of the TCA antigen. Preliminary experiments indicate that this component is not a uronic acid or an O-acetyl group. However, further investigations have been hampered by the small yields of purified TCA polysaccharide.

SUMMARY

The type-specific antigen of Group B Type II streptococci has been prepared free of other known cell components. This antigen is a capsular polysaccharide containing D-galactose, D-glucose, N-acetyl-D-glucosamine, and a labile com-

---

[2] Personal communication.

ponent which has not been chemically characterized. Extraction of Type II streptococci with cold TCA yielded this antigen which contained two serological determinants. A partial antigen with only one of the determinants was obtained by extraction with dilute HCl at 100°C.

By means of the quantitative precipitin inhibition technique, a β-D-galacto-pyranoside has been established as the determinant of the HCl-extracted polysaccharide. The second determinant, present solely in the TCA antigen, has not been identified.

BIBLIOGRAPHY

1. Lancefield, R. C., and E. H. Freimer. 1966. Type-specific polysaccharide antigens of group B streptococci. *J. Hyg.* **64**:191.
2. McCarty, M., and R. C. Lancefield. 1955. Variation in the group-specific carbohydrate of group A streptococci. I. Immunochemical studies on the carbohydrates of variant strains. *J. Exptl. Med.* **102**:11.
3. Freimer, E. H. 1963. Studies of L forms and protoplasts of group A streptococci. II. Chemical and immunological properties of the cell membrane. *J. Exptl. Med.* **117**:377.
4. Scheidegger, J. J. 1955. Une micro-méthode de l'immuno-électrophorèse. *Intern. Arch. Allergy Appl. Immunol.* **7**:103.
5. Scott, T. A., and E. H. Melvin. 1953. Determination of Dextran with Anthrone. *Anal. Chem.* **25**:1656.
6. Folch, J., M. Lees, and G. H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**:497.
7. Curtis, S. N., and R. M. Krause. 1964. Antigenic relationships between groups B and G streptococci. *J. Exptl. Med.* **120**:629.