CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

XII. The Immunological Properties of an Artificial Antigen Containing Cellobiuronic Acid

By WALTHER F. GOEBEL, Ph.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

(Received for publication, June 24, 1938)

The problem of understanding the factors which govern the immunological specificity of bacterial polysaccharides is essentially biochemical in nature and can be approached in two ways. The chemical constitution of these complex substances may be elucidated by the classical methods of organic chemistry in the hope of correlating differences in structure with changes in specificity. On the other hand an approach may be made by rendering simple carbohydrates of known constitution antigenic through combination with protein, and correlating the specificity of the antibodies elicited with known changes in the chemical structure of the carbohydrate radicals in question. Although the latter method has certain obvious limitations, in our initial chemo-immunological studies on the specificity of carbohydrates we chose the second mode of approach, for it was our opinion that without a far reaching biological understanding, a purely chemical interpretation of this important immunological problem would be sterile indeed.

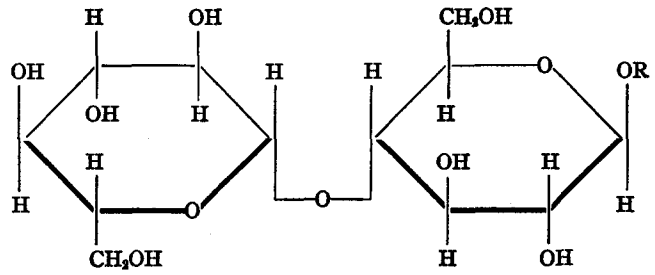
From initial attempts to understand the factors underlying the immunological specificity of carbohydrates certain fundamental facts have been revealed through the application of the immunochemical approach. It has been possible to demonstrate that intra- and intermolecular differences in the configuration of mono- and disaccharides are influential in determining specificity (1). Likewise it has been found that the introduction of a labile grouping, such as the acetyl group, will alter the immunological specificity of a monosaccharide (2). More recently it was demonstrated that the conversion of the

primary alcohol grouping occupying the sixth position in a hexose to the carboxyl group, conveys a new and distinct specificity upon the saccharide in question (3). Artificial antigens containing glucuronic and galacturonic acids, as opposed to those containing the corresponding monosaccharides, assume a new and important biological property, namely the capacity to precipitate in antipneumococcal sera (4). Although the hexose-uronic acid antigens possess serological properties which correlate them with the bacterial polysaccharides, it has not as yet been possible to induce immunity to pneumococcal infection in experimental animals by immunization with glucuronic or galacturonic acid antigens. Therefore, in order to understand more fully the rôle which the aldobionic acids, the fundamental building stones of certain bacterial polysaccharides, play in immunological phenomena we have found it advisable to study the immunological properties of antigens containing these acids.

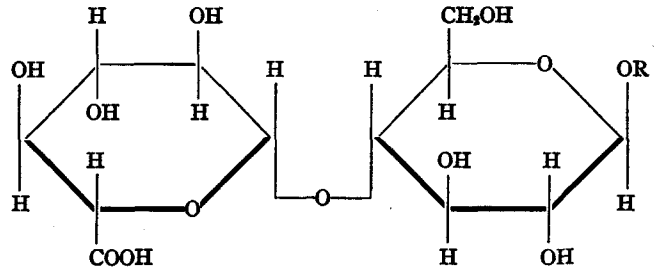
The aldobionic acid, cellobiuronic acid, is a disaccharide constituted from one molecule of glucuronic acid combined in glucuronosidic linkage with one molecule of glucose, through the hydroxyl group of the fourth carbon atom of the hexose. This linkage has the β configuration, and the ring structure of the two hexose constituents have been proven to be that of a pyranoside (5). Cellobiuronic acid can be obtained as an amorphous substance from the hydrolysis products of the capsular polysaccharides of either Types III or VIII Pneumococcus (6). The amorphous disaccharide can be converted to a crystalline alkaloidal salt, or to a characteristic crystalline heptaacetyl methyl ester. The latter derivative has served as the source material for the synthesis of the derivatives used in the present investigation.

Cellobiose is, of course, the disaccharide obtained from the hydrolysis of cellulose. It is constituted from one molecule of glucopyranose joined in glucosidic linkage to the hydroxyl group of a second glucopyranose molecule on carbon atom 4. The configurational relationship of all the asymmetric carbon atoms of cellobiose and cellobiuronic acid is identical, as is that of the intramolecular linkage. The *p*-aminobenzyl glycosides of cellobiose and cellobiuronic acid both have the β configuration. The only difference in these two substances, therefore, is the grouping occupying the twelfth position, which in the case of cellobiose is a primary alcohol group (CH_2OH) and in cellobiuronic acid a carboxyl group (COOH). Any differences in the immunological properties of antigens containing these two saccharides may therefore be attributed to this difference in chemical constitution. For the purpose of comparison the serological properties of two additional antigens, one containing the azobenzyl glycoside of glucose and the other that of glucuronic acid, have been included in this study. The structural relationship of these four

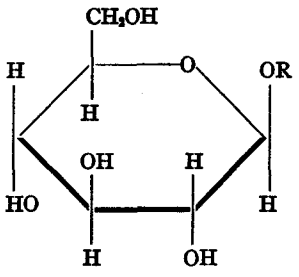
glycosides can be seen from the following graphic formulae in which R represents the aglucon $—CH_2C_6H_4NH_2$.



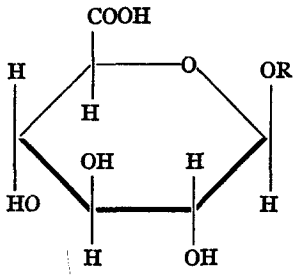
p-aminobenzyl β-cellobioside



p-aminobenzyl β-cellobiuronide



p-aminobenzyl β-glucoside



p-aminobenzyl β-glucuronide

Chemical Methods

Hexaacetyl p-Nitrobenzyl β-Cellobioside.—22 gm. of acetobromo cellobiose (7) were dissolved in 75 cc. of anhydrous chloroform. 6.6 gm. of dry *p*-nitrobenzyl alcohol and 7.2 gm. of dry silver oxide were added. The mixture was shaken until the supernatant liquid no longer gave a test for the bromo derivative. After filtering and removing the chloroform *in vacuo* the glycoside crystallized on the

addition of ethyl alcohol. 10 gm. of crude glycoside were recovered. The substance was recrystallized several times from alcohol yielding 7.6 gm. The pure substance was obtained as glistening needles melting at 178–180° (uncorrected).

Rotation.—$[\alpha]_D^{22°} = -34.8°$ in $CHCl_3$ (C = 1 per cent).

Analysis.—$C_{33}H_{41}O_{20}N$. Calculated. C 51.4, H 5.4.
Found. C 51.3, H 5.6.

p-Nitrobenzyl β-Cellobioside.—2.5 gm. of hexaacetyl *p*-nitrobenzyl cellobioside were suspended in 50 cc. of methyl alcohol and deacetylated at 0° with 1/30 mole of barium methylate (8). The barium was removed by adding one equivalent of N/1 sulfuric acid. After filtering off the precipitated barium sulfate, the crude deacetylated glycoside was obtained by concentrating the filtrate *in vacuo*, and recrystallizing the residue from the minimum quantity of 90 per cent ethyl alcohol; 1.2 gm. were recovered. The glycoside was repeatedly crystallized from this solvent until the optical rotation remained constant. The compound was obtained as rosettes of needles melting at 199–200° (uncorrected).

Rotation.—$[\alpha]_D^{20°} = -32.3°$ in H_2O (C = 1 per cent).

Analysis.—$C_{19}H_{27}O_{13}N$. Calculated. N 2.93.
Found. N 2.84.

p-Aminobenzyl β-Cellobioside.—1.0 gm. of *p*-nitrobenzyl cellobioside was dissolved in 50 cc. of methyl alcohol and reduced catalytically with hydrogen and platinum oxide as catalyst (9). On concentrating the alcoholic solution to a syrup *in vacuo*, and taking up in 95 per cent ethyl alcohol, 0.9 gm. of the glycoside was obtained as fine white needles melting at 188–190° (uncorrected) with decomposition.

Rotation.—$[\alpha]_D^{22°} = -35.2°$ in H_2O (C = 0.5 per cent).

Analysis.—$C_{19}H_{29}O_{11}N$. Calculated. N 3.13.
Found. N 3.03.

p-Nitrobenzyl β-Glycoside of Hexaacetyl Cellobiuronic Acid Methyl Ester.—11.9 gm. of acetobromo cellobiuronic acid methyl ester (10) were dissolved in 60 cc. of dry chloroform, and 5.2 gm. of *p*-nitrobenzyl alcohol and 6 gm. of dry silver oxide added. The mixture was shaken for 4 hours, and the chloroform solution finally separated from the residue of silver salts by filtration. The chloroform was removed *in vacuo*, and on dissolving the oily residue in ethyl alcohol, the crude glycoside crystallized. 6.2 gm. of an impure product melting between 160° and 180° were obtained. This substance was now dissolved in 300 cc. of hot ethyl alcohol and after standing 1 hour at room temperature 3.8 gm. of the hexaacetyl *p*-nitrobenzyl glycoside of cellobiuronic acid methyl ester were obtained as glistening pale yellow crystals melting at 192–193°.

Rotation.—$[\alpha]_D^{20°} = -41.7°$ in $CHCl_3$ (C = 0.6 per cent).

Analysis.—$C_{30}H_{36}O_{18}N(COOCH_3)$. Calculated. C 50.7, H 5.2, OCH_3 4.1.
Found. C 50.4, H 5.1, OCH_3 4.1.

p-Nitrobenzyl β-Glycoside of Cellobiuronic Acid Methyl Ester.—3.5 gm. of the acetylated glycoside were suspended in 150 cc. of methyl alcohol and deacetylated at 0° with barium methylate exactly as was the corresponding cellobioside. After

removal of the barium, 1.6 gm. of pure glycoside melting at 188–189° were obtained from the mother liquors. The compound crystallized as beautiful rosettes of needles from ethyl alcohol.

Rotation.—$[\alpha]_D^{22°} = -48.1°$ in CH_3OH (C = 1 per cent).

Analysis.—$C_{20}H_{27}O_{14}N$. Calculated. OCH_3 5.96.
Found. OCH_3 6.14.

Barium Salt of p-Aminobenzyl β-Glycoside of Cellobiuronic Acid.—1 gm. of the nitrobenzyl glycoside of cellobiuronic acid methyl ester was converted to the amino compound by catalytic reduction. The colorless alcoholic solution was evaporated *in vacuo*, the glycoside taken up in a few cubic centimeters of water, and one equivalent of 0.4 N barium hydroxide was slowly added. The mixture was warmed to 50° to effect complete hydrolysis of the methyl ester group. The clear very pale yellow solution of glycoside was concentrated to small volume *in vacuo*, and then poured into 20 volumes of chilled absolute ethyl alcohol. 0.9 gm. of the barium salt of the *p*-aminobenzyl glycoside of cellobiuronic acid was isolated as an amorphous powder, readily soluble in water and insoluble in the usual organic solvents.

Rotation.—$[\alpha]_D^{22°} = -44.0°$ in H_2O (C = 0.5 per cent).

Analysis.—$C_{19}H_{26}O_{12}N\frac{1}{2}Ba$. Calculated. N 2.66, Ba 13.04.
Found. N 2.65, Ba 12.50.

p-Aminobenzyl β-Glucoside and β-Glucuronide.—These derivatives were prepared by methods previously described (1,3).

Immunological Reactions

Methods

Immunizing antigens were prepared by combining the diazotized *p*-aminobenzyl β-glycoside of glucose, glucuronic acid, cellobiose, and cellobiuronic acid with the globulin fraction of normal horse serum. For each 600 mg. of globulin was used 1 millimole of glycoside. Before diazotization the barium salt of the cellobiuronide was first converted to the sodium salt by the addition of 1.2 equivalents of sodium sulfate, followed by removal of precipitated barium sulfate. The technique of preparing the antigens was the same as that described in earlier studies, as was the intravenous immunization of the rabbits. Two to three courses were given consisting of six daily intravenous injections of 5 mg. of antigen dissolved in sterile physiological salt solution. 7 days after the last injection the animals were bled from the ear, and the serum collected in the usual fashion.

In order to avoid protein cross reactions the test antigens used in the serological analysis were prepared by combining the diazonium derivatives of the various glycosides to the protein of chicken serum. The test antigen containing the azobenzyl ether of the capsular polysaccharide of Pneumococcus Type III was prepared by combining its diazonium derivative to a slight excess of crystalline egg albumin (11). Instead of precipitating the coupled derivative at the isoelectric point, the alkaline reaction product was dialyzed at 0° for 24 hours. The pH of

the solution was adjusted to 7.5, and the salt content made to 1 per cent by the addition of solid NaCl. In this way a soluble protein-polysaccharide antigen was obtained which appeared to be free from the uncombined polysaccharide derivative.

The immunizing antigens are referred to in the tables as C-globulin (cellobiose-globulin), Ca-globulin (cellobiuronic acid-globulin), G-globulin (glucose-globulin), and Ga-globulin (glucuronic acid-globulin). The test antigens prepared by combining the diazotized *p*-aminobenzyl glycosides to chicken serum, are referred to as C-chick, Ca-chick, etc. The S III test antigen is referred to as S III-egg, and the various glycosides are designated as C, Ca, G, and Ga for the cellobioside, cellobiuronide, glucoside, and glucuronide respectively. The technique of the specific inhibition tests is the same as that described in earlier studies.

1. Precipitin Reactions

Antisera of Rabbits Immunized with Antigens Containing the Azobenzyl Glycosides.—The sera of rabbits immunized with azoprotein (horse globulin) antigens containing the four saccharides, glucose (G), glucuronic acid (Ga), cellobiose (C), and cellobiuronic acid (Ca), yield a marked precipitate when the homologous carbohydrate derivative combined with a heterologous protein (chicken serum) is added. An analysis of Table I reveals, however, that the antibodies elicited by the glucose, glucuronic acid, and cellobiose antigens show sharper specificity than do those elicited by the antigen containing cellobiuronic acid. Cellobiose is a disaccharide constituted from one molecule of glucose combined in β-glucosidic union to the fourth carbon atom of a second glucose molecule. It is not surprising, therefore, that an antiserum elicited by an antigen containing this disaccharide would react with a test antigen containing the monosaccharide glucose and *vice versa*.

It has been shown that antigens containing glucose and glucuronic acid give rise to antibodies which are sharply specific and show no serological crossing (3). The fact that neither the glucuronic nor the cellobiuronic acid antigens react in C or Ca antisera lends additional support to the view that the conversion of the primary alcohol grouping (CH_2OH) of a saccharide to the carboxyl group (COOH) confers upon the newly formed uronic acid antigen a new and distinct specificity. Thus it is interesting to note that an antiserum to Ga-globulin, in addition to precipitating the homologous test antigen, likewise precipitates the test antigen containing cellobiuronic acid, but not the corresponding cellobiose antigen, and of course not the glucose antigen.

The lack of specificity exhibited by the cellobiuronic acid antiserum is difficult to explain. In a previous study on the specificity of artificial carbohydrate-protein antigens containing disaccharides (1 *b*) it was found that the specificity of antibodies elicited by such antigens was directed not only toward the disaccharide molecule as a whole, but toward the terminal hexose molecule as well, and but little, if at all, toward the hexose molecule bearing the aglucon. Thus an antiserum

TABLE I

Homologous and Heterologous Precipitin Reactions of Glucose, Glucuronic Acid, Cellobiose, and Cellobiuronic Acid Antisera

Antiserum prepared by immunization with	Test antigen used	Final dilution of test antigen			
		1:5,000	1:10,000	1:25,000	1:50,000
Ca-Globulin	Ca-chick	++	+++±	++±	++±
	C-chick	++±	++	++	+±
	Ga-chick	++	++	+±	+
	G-chick	+±	+	+	+
C-Globulin	Ca-chick	0	0	0	0
	C-chick	+++	+++	++±	++
	Ga-chick	0	0	0	0
	G-chick	+±	++	+±	+
Ga-Globulin	Ca-chick	++	++	++	+±
	C-chick	0	0	0	0
	Ga-chick	+++	+++	++±	++
	G-chick	0	0	0	0
G-Globulin	Ca-chick	0	0	0	0
	C-chick	++±	++±	++	+±
	Ga-chick	0	0	0	0
	G-chick	+++	+++	++±	+±

to an azoprotein containing lactose (4 β-glucosido-galactose) reacted with a test antigen containing galactose but not with one containing glucose. In the antiserum prepared by immunization with cellobiuronic acid antigen (4 β-glucuronosido-glucose) however, one finds antibodies capable of reacting not only with the glucuronic acid test antigen but with test antigens containing glucose and cellobiose as well. It appears, therefore, that in the serum to cellobiuronic acid there

is a reflection not only of the aldobionic acid molecule as a whole, but there are antibodies capable of reacting with both individual constituents, glucose and glucuronic acid, as well.

Reactions of a Test Antigen Containing the Capsular Polysaccharide of Pneumococcus Type III.—The capsular polysaccharide of the Type III Pneumococcus is constituted from molecules of cellobiuronic acid linked in glycosidic union to form a large non-diffusible molecule the precise magnitude of which has not been ascertained. On hydrolysis of the polysaccharide by dilute mineral acid, cellobiuronic acid is obtained. There is certain evidence in support of the view that units of aldobionic acid of sufficient molecular size (di- and tetra-aldobionides) still retain the property of precipitating in homologous Type III antipneumococcus horse serum (12). It has occurred to us, therefore, that

TABLE II

Precipitin Reactions of Pneumococcus Type III Polysaccharide Test Antigen in Glucose, Cellobiose, Glucuronic Acid, and Cellobiuronic Acid Antisera

Antisera prepared by immunization with	Final dilution of S III antigen				
	1:5,000	1:10,000	1:25,000	1:50,000	1:200,000
Ca-globulin	+++	+++	++±	++	+
C-globulin	0	0	0	0	0
G-globulin	0	0	0	0	0
Ga-globulin	0	0	0	0	0

the capsular polysaccharide of Type III Pneumococcus, or the monoaminobenzyl ether of this substance either free or in combination with a protein, might react in cellobiuronic acid antiserum. That this is the case and that the reaction takes place only in cellobiuronic acid antiserum, and not in cellobiose, glucose, and glucuronic acid antisera, can be seen from the results given in Table II. The significance of these findings will be discussed later.

2. Inhibition Reactions

1. Cellobiuronic Acid Antiserum.—The specificity of the cross precipitation reactions of Ca, C, Ga, G, and S III test antigens in cellobiuronic acid antiserum may best be studied by means of the specific inhibition tests, the results of which are given in Table III. From

the results presented in Table III it is seen that the reaction between cellobiuronic acid antiserum and homologous antigen is specific, for it can be inhibited only by the homologous glycoside, Ca. The heterologous reactions of the hexose and disaccharide antigens, C and G, on the other hand, are inhibited by the cellobiuronide, the glucoside, and cellobioside respectively. It is interesting to observe that the reaction of C antigen in Ca antiserum is inhibited only by Ca and C and not by G, whereas the reaction of the G antigen is inhibited not only by Ca and G, but by the cellobioside C as well. The fact that the cellobioside inhibits the reaction of the monosaccharide antigen G and that the reverse is not true, indicates that the precipitation of C antigen in Ca antiserum approaches more closely the homologous reac-

TABLE III

Inhibition of Precipitins in Cellobiuronic Acid Antiserum by Homologous and Heterologous Glycosides

Inhibiting glycoside	Cellobiuronic acid antiserum				
	Test antigen 1:10,000				
	Ca-chick	C-chick	Ga-chick	G-chick	S III chick
Ca	0	0	0	0	0
C	++	0	+±	0	++±
Ga	++	+±	0	+	0
G	+++	++	+±	0	++±
None	+++	++±	++	++	+++

tion than does the G reaction. It is of further interest to observe that the glucuronide Ga does not inhibit the reaction of C or G antigens in Ca antiserum. This fact would indicate that a uronic acid antibody *per se* is not involved in the precipitation reaction of the antigens in question. It likewise appears that the precipitation of both Ga and S III antigens in Ca antiserum involves specifically only a uronic acid antibody, for both reactions are inhibited not by the glucoside and cellobioside, but only by the glucuronide and cellobiuronide.

2. Glucose, Glucuronic Acid, and Cellobiose Antisera.—The specific inhibition tests of various antigens in G, Ga, and C antisera are given in Tables IV, V, and VI and the interpretation of the specificity of the

different tests presents no difficulty. In each instance the homologous reaction is inhibited only by the homologous glycoside, whereas the heterologous reaction is inhibited both by the glycoside homologous to the antiserum as well as by that homologous to the antigen. From these tests one important and new fact is brought to light, namely that the antibodies elicited by the uronic acid derivatives of glucose

TABLE IV

Inhibition of Precipitins in Cellobiose Antiserum by Homologous and Heterologous Glycosides

Inhibiting glycoside	Cellobiose antiserum	
	Test antigen 1:10,000	
	C-chick	G-chick
C	0	0
G	+++±	0
Ca	+++±	+++
Ga	+++±	+++
None	+++±	+++

TABLE V

Inhibition of Precipitins in Glucose Antiserum by Homologous and Heterologous Glycosides

Inhibiting glycoside	Glucose antiserum	
	Test antigen 1:10,000	
	G-chick	C-chick
G	0	0
C	++±	0
Ca	+++	++
Ga	+++	++
None	+++	++

TABLE VI

Inhibition of Precipitins in Glucuronic Acid Antiserum by Homologous and Heterologous Glycosides

Inhibiting glycoside	Glucuronic acid antiserum	
	Test antigen 1:10,000	
	Ga-chick	Ca-chick
Ga	0	0
Ca	++	0
G	++	+±
C	++±	+±
None	+++	++

and cellobiose, glucuronic and cellobiuronic acids, are distinct and specific. A similar relationship has been established in a previous study for antigens containing the azophenol glycosides of the monosaccharide glucose and the disaccharide cellobiose (1*b*) and is now reaffirmed for antigens containing the azobenzyl glycosides of these saccharides.

3. Precipitin Reactions of Antipneumococcus Sera with Test Antigens Containing the Azobenzyl Glycosides.—In a previous communication (3) it was shown that an azoprotein antigen containing glucuronic acid precipitates in antipneumococcus horse sera Types II, III, and VIII, whereas the corresponding glucose antigen is serologically inert. The precipitin reactions of Ga, C, and Ca test antigens in antipneumococcus horse and rabbit sera are presented in Table VII. It can be seen that the antigen containing the disaccharide cellobiose reacts not at

TABLE VII

Precipitin Reactions of Cellobiose, Cellobiuronic Acid, and S III Antigens in Antipneumococcus Sera

Antipneumococcus serum Type	Test antigen used	Antipneumococcus horse serum				Antipneumococcus rabbit serum			
		Final dilution of test antigen				Final dilution of test antigen			
		1:10,000	1:50,000	1:250,000	1:1,000,000	1:10,000	1:50,000	1:250,000	1:1,000,000
I	C-chick	0	0	0	0	−	−	−	−
II	"	+	0	0	0	0	0	0	0
III	"	+±	+	±	0	±	±	0	0
VIII	"	+++±	++	+	0	++	+	+	±
I	Ca-chick	0	0	0	0	−	−	−	−
II	"	++++	+++	++	+	+±	+	+	±
III	"	++++	+++±	++±	+	++±	++±	++	+
VIII	"	++±	++±	++	+	+±	+	±	0
I	S III-egg	0	0	0	0	−	−	−	−
II	"	++++	++±	+	0	0	0	0	0
III	"	++++	+++	++	+	++++	++++	++	+
VIII	"	+++	++±	+±	+	+	+	+±	±

all in Type I antipneumococcus horse serum, only feebly in Type II serum, and slightly more vigorously in Type III serum, whereas in Type VIII serum the reaction is still detectable in dilutions of one part in a quarter million. In rabbit sera of the corresponding types the reactions are negligible save in Type VIII serum. The chemical constitution of the Type VIII capsular polysaccharide is as yet unknown, but it appears to be constituted from molecules of glucose and glucuronic acid in the ratio of 7:2 (6*a*). On hydrolysis there is obtained glucose

and cellobiuronic acid. Whether the affinity of the cellobiose antigen for part of the Type VIII antibody is indicative of a cellobiose residue or configuration, in this polysaccharide molecule cannot be answered at the present time. The great avidity of the cellobiuronic acid antigen, on the other hand, for the antibodies of Types II, III, and VIII antipneumococcus sera, both of the rabbit and the horse, is evident from the results given in Table VII. It has been found that the capsular polysaccharides of both Types III and VIII contain cello-

TABLE VIII

Inhibition of Precipitin Reactions of Cellobiuronic Acid and S III Antigens in Antipneumococcus Horse Sera Types II, III, and VIII

Antipneumococcus horse serum Type	Inhibiting glycoside	Test antigen 1:10,000	
		Ca-chick	S III-egg
II	Ca	0	0
	C	+++±	+++
	Ga	±	0
	G	+++±	++±
	None	++++	+++
III	Ca	0	++++
	C	++++	++++
	Ga	+++±	++++
	G	++++	++++
	None	++++	++++
VIII	Ca	0	0
	C	++	+±
	Ga	+	+
	G	++	+±
	None	++	++±

biuronic acid as an integral part of the carbohydrate molecule. It is therefore not surprising that the uronic acid antigen reacts in these antisera. In the case of the test antigen containing the azobenzyl ether of the capsular polysaccharide of Type III Pneumococcus, the reaction in both Types III and VIII antisera is to be expected. However, the precipitation of this antigen in antipneumococcus serum Type II is surprising, particularly in view of the established specificity of the Type III pneumococcus polysaccharide itself. This remarkable

acquired property of the Type III capsular polysaccharide, when in combination with a large protein molecule, is difficult to understand. Control tests in which the *p*-aminobenzyl ether of the Type III polysaccharide itself was added to Type II antipneumococcus serum, showed no reaction whatsoever, nor did comparable dilutions of uncoupled egg albumin react in Type II serum. Qualitative tests indicate that the capsular polysaccharide of Type II Pneumococcus contains a uronic acid. That a reflection of this constituent is found in homologous antiserum is evident from the serological tests in which both glucuronic and cellobiuronic acid test antigens are found to react in high dilution. The explanation of the precipitation of the S III—egg antigen in Type II serum, and the failure of the uncombined capsular Type III polysaccharide to react may reside in the comparative molecular magnitude of the two substances in question. However, from the results of the specific inhibition tests given in Table VIII it is evident that precipitation of the S III antigen in antipneumococcus horse serum Type II is inhibited both by the glucuronide and the cellobiuronide, whereas in Type III serum the reaction is inhibited by neither glycoside. In Type VIII serum, on the other hand, inhibition is caused by the cellobiuronide, and precipitation is greatly diminished in the presence of glucuronide, though not completely prevented. It appears, therefore, that in Type II serum at least a portion of the antibody is directed toward the uronic acid, and when the heterologous S III test antigen is added to the immune serum union occurs between this antibody and the glucuronic acid portion of the test antigen. This reaction may be completely inhibited even by the simple glucuronide. In Type III antiserum the antibodies obviously combine with the polysaccharide molecule as a whole, for the reaction between the S III antigen and antiserum cannot be inhibited by glucuronide or cellobiuronide. In Type VIII serum, on the other hand, the reaction is inhibited by the cellobiuronide and not entirely by the glucuronide, a result which is to be anticipated on account of the common cellobiuronic acid nucleus present in the Type III and VIII polysaccharides.

The precipitation of the cellobiuronic acid antigen in Types II, III, and VIII sera is in each instance inhibited by cellobiuronide. But only in Type II serum is precipitation in these sera inhibited by

glucuronide, a fact which further substantiates the hypothesis that the reactive antibody in Type II serum is directed toward the hexuronic rather than the aldobionic acid. In conclusion it should be emphasized that the S III antigen (Table VII) does not react in Type II antipneumococcus rabbit serum. This fact indicates that an antibody directed against the uronic acid constituent is not present in antipneumococcus rabbit serum and confirms earlier observations (3,4).

DISCUSSION

The functional rôle of acid groups in determining certain of the serological characteristics of bacterial polysaccharides, a possibility which was foreseen some years ago by Landsteiner (13) from his work on azoprotein antigens, is affirmed by the results of the present study. The sharp specificity exhibited by antigens containing glucose and glucuronic acid is not shared by similar antigens containing the disaccharide cellobiose and its derivative cellobiuronic acid. The antiserum elicited by the cellobiuronic acid antigen reacts not only with the disaccharide antigen, but with simple glucose and glucuronic acid antigens as well. Thus it appears that the antibody to which cellobiuronic acid gives rise is directed toward both molecular constituents as well as toward the molecule as a whole. The hexose uronic acid constituents of the intact capsular polysaccharides in the form in which they occur in the bacterial cells are obviously not the sole determinant in orienting the specificity of the antipolysaccharide immune body. Nor can this property be attributed to the aldobionic acid molecule alone, for if it were the cellobiuronide should inhibit the reaction of Type III polysaccharide in homologous antiserum. That the uronic acids account for serological cross reactions, however, cannot be denied from the results of the foregoing experiments. The carbohydrate antibodies in antipneumococcus serum may be regarded as a mixture of different but closely related proteins certain of which are reactive with artificial antigens containing the simpler saccharide components of the capsular polysaccharide, but all of which are removed by absorption with the homologous type specific carbohydrate.

The capsular polysaccharide of Type III Pneumococcus appears to be a macro molecule constituted from units of cellobiuronic acid linked

in glycosidic union. The position of linkage, a problem in which we in this laboratory are at present engaged, is as yet undetermined. The molecular magnitude of the Type III carbohydrate is likewise uncertain (14). Yet in comparison to a protein molecule, the pneumococcus polysaccharide is a relatively simple chemical entity, for it is a molecule in which the same fundamental unit, cellobiuronic acid, is repeated periodically over and over again. One might anticipate, therefore, that an antibody elicited by cellobiuronic acid should bear some serological relationship to the parent polysaccharide itself. That this is the case is evident from the results of the foregoing serological analysis. A serum to the cellobiuronic acid antigen precipitates the capsular polysaccharide of Type III Pneumococcus when the latter is combined with egg albumin. Although it has not been indicated in the protocols, both the free capsular polysaccharide and the uncombined aminobenzyl ether of the latter likewise precipitate feebly in cellobiuronic acid antiserum in dilutions as high as one part in a quarter million. The antiserum to cellobiose, on the other hand, shows none of these precipitation reactions. The results of this study indicate, therefore, that the aldobionic acids have a unique biological function in determining the immunological characteristics of certain encapsulated microorganisms.

In conclusion it can be said that the antiserum to the artificial antigen containing cellobiuronic acid conveys passive protection on mice to infection with virulent pneumococci Types II, III, and VIII. The results of these experiments will be reported in a later communication.

SUMMARY

1. Artificial antigens containing the azobenzyl glycosides of the disaccharide cellobiose and the aldobionic acid, cellobiuronic acid, give rise in rabbits to antibodies which are specific and characteristic of the saccharide constituent. The antiserum to cellobiuronic acid shows broader serological cross reactions than does that to cellobiose.

2. An antiserum to the cellobiuronic acid antigen precipitates the capsular polysaccharide of Type III Pneumococcus when the latter is combined with a heterologous protein.

3. The cellobiuronic acid test antigen precipitates vigorously in antipneumococcus sera Types II, III, and VIII. The mechanism of these reactions is discussed.

BIBLIOGRAPHY

1. (*a*) Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1929, **50**, 521, 533. (*b*) Goebel, W. F., Avery, O. T., and Babers, F. H., *J. Exp. Med.*, 1934, **60**, 599.
2. Goebel, W. F., Babers, F. H., and Avery, O. T., *J. Exp. Med.*, 1934, **60**, 85.
3. Goebel, W. F., *J. Exp. Med.*, 1936, **64**, 29.
4. Goebel, W. F., and Hotchkiss, R. D., *J. Exp. Med.*, 1937, **66**, 191.
5. Hotchkiss, R. D., and Goebel, W. F., *J. Biol. Chem.*, 1937, **121**, 195.
6. (*a*) Goebel, W. F., *J. Biol. Chem.*, 1935, **110**, 391. (*b*) Heidelberger, M., and Goebel, W. F., *J. Biol. Chem.*, 1927, **74**, 613.
7. Zemplén, G., Csuros, Z., and Bruckner, Z., *Ber. Chem. Ges.*, 1928, **61**, 927.
8. Isbell, H. S., *Bureau Standards J. Research*, 1930, **5**, 1179.
9. Vorhees, V., and Adams, R., *J. Am. Chem. Soc.*, 1922, **40**, 1397.
10. Goebel, W. F., and Reeves, R. E., *J. Biol. Chem.*, 1938, **124**, 207.
11. Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1931, **54**, 431.
12. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1933, **57**, 373.
13. Landsteiner, K., Die Spezilizität der serologischen Reactionen, Berlin, Julius Springer, 1933, 75.
14. (*a*) Babers, F. H., and Goebel, W. F., *J. Biol. Chem.*, 1930, **89**, 387. (*b*) Heidelberger, M., and Kendall, F. E., *J. Biol. Chem.*, 1932, **96**, 541. (*c*) Heidelberger, M., *J. Am. Chem. Soc.*, 1938, **60**, 242.