CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

X. THE IMMUNOLOGICAL PROPERTIES OF AN ARTIFICIAL ANTIGEN CONTAINING GLUCURONIC ACID

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In previous studies it has been shown that differences in the specificity of artificially compounded carbohydrate-protein antigens can be directly correlated with changes in the stereochemical configuration of the carbohydrate radicals (1). Configurational differences alone, however, do not account for all variations in the immunological specificity of carbohydrates. It has been observed that structural changes, such as the presence of an acetyl group in the naturally occurring bacterial polysaccharides, or in the glycoside radical of artificially prepared carbohydrate-protein antigens, are likewise reflected in serological specificity (2).

The uronic acids have been found to be constituents of the specific polysaccharides of Pneumococcus (3) and of Friedländer’s bacillus (4). The occurrence of glucuronic acid and its isomers in these bacterial carbohydrates has led to the opinion that the highly polar carboxyl group of the uronic acids plays an important rôle in determining the specificity and serological reactivity of the soluble specific substances of encapsulated microorganisms. In support of this view it has been shown that the specific polysaccharide of Type I Pneumococcus may be rendered serologically inert merely by esterifying the carboxyl groups with diazomethane (5). The immunological activity of the carbohydrate may be restored, however, when the carboxyl groups are set free by saponifying the methyl ester with dilute alkali. It has been suggested, furthermore, that the immunological crossing exhibited by the specific polysaccharides of Type III and VIII Pneumococcus may be attributed to the configurational identity of the aldobionic acid nucleus common to both carbohydrates (6).
In order to study the immunological properties of uronic acids of known constitution, an artificial carbohydrate-protein antigen containing glucuronic acid has been prepared by combining the diazonium derivative of the \( \beta \)-aminobenzyl glucuronide with protein. The immunological properties of this antigen have been compared with those of a similar antigen containing the corresponding glycoside of glucose. Since the carbohydrate radicals of these two antigens have an identical stereochemical configuration, any differences in immunological properties must be directly attributable to constitutional changes brought about by differences in the chemical grouping occupying the sixth position in each glycoside molecule. From the accompanying structural formulae of the two \( \beta \)-aminobenzyl glycosides, it can be seen that in the glucoside this grouping is a primary alcohol (\( \text{CH}_2\text{OH} \)), whereas the highly polar carboxyl group (\( \text{COOH} \)) occupies the sixth position in the glucuronide.

\[
\begin{align*}
\text{p-Aminobenzyl } & \beta\text{-glucoside} \\
\text{p-Aminobenzyl } & \beta\text{-glucuronide}
\end{align*}
\]

**Chemical Methods**

*Tetracetyl p-Nitrobenzyl \( \beta \)-Glucoside.*—This glucoside was prepared by shaking an ethereal solution of 15 gm. of acetobromoglucose (7) with 1.2 mols of \( p \)-nitrobenzyl alcohol and 1.2 mols of silver oxide until the ethereal solution no longer showed the presence of free bromo compound. After filtering and concentrating the solution in vacuo, the glucoside crystallized on the addition of ethyl alcohol; 5.3 gm. of glucoside were recovered. The glucoside was recrystallized from alcohol. The substance separated as needles melting at 132–133° (uncorrected).

Rotation—\( \alpha \) = -40.9° in CHCl₃ (C = 1.0 per cent).

Analysis—C₁₉H₁₅O₉(COCH₃)₂N. Calculated. C 52.2, H 5.2. Found. " 52.4, " 5.3.

*p-Nitrobenzyl Glucoside*—10 gm. of tetracetyl \( p \)-nitrobenzyl \( \beta \)-glucoside were suspended in 200 cc. of absolute methyl alcohol at 0° and decetylated by treating with 1/30 mol of barium methylate, according to the method of Isbell (8). After
removing the barium by adding the equivalent quantity of N/1 sulfuric acid, the glucoside was recovered nearly quantitatively from the mother liquors. The compound crystallized as needles melting at 156-157° (uncorrected).

Rotation—\([\alpha]_{D}^{24} = -47.7^\circ\) in CH₃OH (C = 0.7 per cent).

Analysis—C₁₃H₁₇O₃N. Calculated. C 49.5, H 5.4.

Found. " 49.8, " 5.3.

\(p\)-Aminobenzyl Glucoside—5 gm. of \(p\)-nitrobenzyl glucoside were dissolved in 150 cc. of absolute methyl alcohol and reduced catalytically with hydrogen and platinum (9). On concentrating the mother liquors the glycoside crystallized as needles melting at 142-143° (uncorrected).

Rotation—\([\alpha]_{D}^{100} = -61.8^\circ\) in H₂O (C = 0.8 per cent). Further recrystallization failed to change the melting point or rotation.

Analysis—C₁₃H₁₉O₄N. Calculated. C 54.7, H 6.7, N 4.9.

Found. " 54.9, " 7.1, " 4.7.

\(p\)-Aminobenzyl Glucuronide—2.0 gm. of the \(p\)-nitrobenzyl glycoside of glucuronic acid methyl ester (10) were dissolved in 100 cc. of absolute methyl alcohol and reduced catalytically with hydrogen and platinum oxide. After removing the platinum by filtration and concentrating the mother liquors, the \(p\)-aminobenzyl glycoside of glucuronic acid methyl ester separated from the solution as an amorphous snow white flocculent precipitate. The glycoside was dissolved in water and treated with exactly one equivalent of 0.4 N barium hydroxide at 60°, or sufficient base to hydrolyze the methyl ester grouping and to form the barium salt of the \(p\)-aminobenzyl glucuronide. The latter was isolated by concentrating the solution to small volume in vacuo, and precipitating the barium salt with 10 volumes of methyl alcohol. The salt was filtered and dried to constant weight.

Rotation—\([\alpha]_{D}^{3} = -71.2^\circ\) in H₂O (C = 1.0 per cent).

Analysis—(C₁₂H₁₄O₃NCOO)₂Ba. Calculated. Ba 18.7, C 42.5, H 4.4, N 3.8.

Found. " 18.5, " 41.9, " 4.6, " 3.6.

**Immunological Reactions**

Methods.—The immunizing antigens were prepared by combining the diazonium derivative of the \(p\)-aminobenzyl glycoside of glucose and the sodium salt of the corresponding glycoside of glucuronic acid with the globulin of horse serum in alkaline solution. The resulting azoprotein antigens were purified in the usual way, and were finally diluted with isotonic salt solution to a concentration of 0.5 percent. Rabbits were immunized by the intravenous injection of 1 cc. of these solutions daily for six doses. After a rest period of 1 week, the course of injections was repeated. 8 days after the last injection the rabbits were bled and the sera tested for homologous and heterologous precipitins. The technique of the precipitin and inhibition tests is the same as that described in earlier papers. In the specific inhibition tests, the \(p\)-aminobenzyl glycoside of glucuronic acid was used in the form of its sodium salt, prepared by adding to a solution of the barium salt the equivalent quantity of solid sodium sulfate. The barium sulfate was removed by centrifugation.
Specific Precipitin and Inhibition Tests.—The sera of rabbits im-
munized respectively with the azoprotein antigens containing the
benzyl glycosides of glucose and glucuronic acid were tested for the
presence of homologous and heterologous precipitins. In order to
eliminate any interference from common protein antibodies, the test
antigens were prepared by combining the same glycosides with the
proteins of chicken serum in the usual manner. The results of the
precipitin tests are given in Table I. From the results given in Table
I it can be seen that the two carbohydrate-protein antigens give rise
in rabbits to antibodies which are specific and show no serological

crossing. The specificity of these serological reactions is further em-
phasized by the results of the specific inhibition tests given in Table
II, in which it may be seen that the precipitation of the glucose test
antigen in homologous antiserum is inhibited only by the glucoside,
whereas the specific reaction of the glucuronic acid antigen in homol-
ogous antiserum is inhibited only by the glucuronide.

It has previously been pointed out that the carbohydrate radicals
of the artificial antigens differ from each other only in the nature of the
chemical grouping on the sixth carbon atom of each glycoside. Al-
though the stereochemical configuration of the asymmetric carbon
atoms of the two glycosides is in each instance identical, yet this

| Antiserum prepared by immunization with | Test antigen used | Final dilution of test antigen |
|----------------------------------------|-------------------|------------------------------|
|                                        |                   | 1:1000  | 1:10,000 | 1:50,000 | 1:100,000 |
| Glucose-globulin*                       | Glucose-chick     | ++      | ++++     | +++      | ++        |
|                                        | Glucuronic acid-chick | 0   | 0        | 0        | 0         |
| Glucuronic acid-globulin                | Glucose-chick     | 0       | 0        | 0        | 0         |
|                                        | Glucuronic acid-chick | ++++ | ++++±    | +++      | ++        |

* For the sake of brevity the immunizing antigens, prepared by combining the p-aminobenzyl glycosides of glucose and glucuronic acid with normal horse serum globulin, are referred to as glucose-globulin, etc. The test antigens, prepared by combining the same glycosides to chicken serum proteins, are referred to as glucose-chick, etc.
configurational identity is not reflected in the specificity of the antibodies to which the conjugated protein-glycosides give rise. The distinct and sharply defined specificity of these two antigens appear, therefore, to be directly attributable to differences in polarity of the primary alcohol group in the glucoside molecule, and the carboxyl group in the glucuronide molecule.

Precipitin Reactions of Glucuronic Acid and Glucose Antigens in Antipneumococcus Sera Types II, III, and VIII.—The opinion has frequently been expressed in communications from this laboratory that the uronic acid constituents of the specific polysaccharides of encapsulated bacteria are important in determining the specificity of the latter, and that the carboxyl groups of the polysaccharide may actually enter into chemical combination with reactive groups in the homologous antibody molecule. The specificity of these serological reactions is believed to be governed by the configurational relationship of the specifically reacting groups in the homologous antibody and carbohydrate molecules.

The important function of the uronic acids of bacterial polysaccharides in determining immunological specificity is strikingly emphasized by the results given in Table III. It may be seen that the glu-
curonic acid-protein antigen reacts in high dilutions with Types II, III, and VIII antipneumococcus horse sera, whereas the corresponding glucose-protein antigen is serologically inert. It has been found in this laboratory that the specific carbohydrates of Types III and VIII (and probably Type II) Pneumococcus contain glucuronic acid as an important constituent of the polysaccharide molecule. The Type I

| Antipneumococcus horse serum | Test antigen used | Final dilution of test antigen |
|-----------------------------|-----------------|-------------------------------|
| Type                        |                 | 1:10,000                      |
| I                           | Glucose-chick   | 0                             |
| II                          | "              | 0                             |
| III                         | "              | +                             |
| VIII                        | "              | 0                             |
| I                           | Glucuronic acid-chick | 0 |
| II                          | "              | ++                            |
| III                         | "              | ++++                          |
| VIII                        | "              | ++                            |

TABLE IV
Precipitin Reaction of Glucuronic Acid Test Antigen in Unabsorbed and Absorbed Type III Antipneumococcus Serum

| Antipneumococcus horse serum Type III | Final dilution of glucuronic acid test antigen |
|---------------------------------------|-----------------------------------------------|
|                                       | 1:2000 | 1:10,000 | 1:100,000 |
| Unabsorbed                            | ++     | +++      | ++++      |
| Absorbed with SSS III                  | 0      | 0        | 0         |

pneumococcus specific carbohydrate, on the other hand, apparently contains galacturonic acid. The precipitation of the artificial antigen in Types II, III, and VIII antipneumococcus horse serum may be attributed to the interaction of the glucuronic acid radical of the azo-protein with antibodies elicited by the uronic acid groupings of the bacterial polysaccharides. It may be seen in Table IV that if the
type-specific antibodies in pneumococcus serum Type III are first
removed by absorption with the soluble specific substance, the ab-
sorbed serum fails to react with the artificial glucuronic acid-protein
antigen. It is evident, therefore, that the precipitation of the arti-
ficial antigen in antipneumococcus serum Type III (and probably in
Types II and VIII as well) represents a reaction between the type-
specific polysaccharide antibodies and the uronic acid radical of the
glucuronic acid-protein antigen. This fact is emphasized by the

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\text{TABLE V}
\]
\textbf{Inhibition of Precipitin Reaction of Glucuronic Acid Antigen in Antipneumococcus Sera Types II, III, and VIII, by p-Aminobenzyl Glycosides of Glucose and Glucuronic Acid}

| Antipneumococcus horse serum | 0.9 per cent NaCl solution | Inhibiting glycoside | Glucuronic acid-chick test antigen (1:5000) | Result |
|-----------------------------|---------------------------|---------------------|--------------------------------------------|-------|
| Type                        |                           |                     |                                            |       |
| II                          | 0.2                       | 0.3                 | 0.5                                        | ++++  |
|                             | 0.2                       | 0.3                 | 0.5                                        | ++   |
|                             | 0.2                       | 0.3                 | 0.5                                        | 0    |
| III                         | 0.2                       | 0.3                 | 0.5                                        | ++++ |
|                             | 0.2                       | 0.3                 | 0.5                                        | ++++ |
|                             | 0.2                       | 0.3                 | 0.5                                        | 0    |
| VIII                        | 0.2                       | 0.3                 | 0.5                                        | +++  |
|                             | 0.2                       | 0.3                 | 0.5                                        | +++  |
|                             | 0.2                       | 0.3                 | 0.5                                        | 0    |

results given in Table V, in which it may be seen that the serological
activity of the glucuronic acid-protein antigen in antipneumococcus sera Types II, III, and VIII is completely and specifically inhibited
by the addition of the p-aminobenzyl glycoside of glucuronic acid,
but not by the corresponding glycoside of glucose.

In contrast to these observations, it has been found (Table VI) that
when Type III antipneumococcus serum is absorbed with the glu-
curonic acid antigen, the antibodies reactive with the homologous capsular polysaccharide are not completely removed. It has been
found, furthermore, that the absorbed serum still reacts with the heterologous Type VIII pneumococcus polysaccharide.

Although the glucuronic acid-protein antigen reacts in antipneumococcus horse sera Types II, III, and VIII, no serological reactions occur in the corresponding antipneumococcus rabbit sera. The sera of rabbits which have been immunized with the glucuronic acid-protein antigen do not agglutinate Types II, III, or VIII pneumococci, nor do they precipitate the corresponding specific capsular polysaccharides.

**TABLE VI**

**Precipitin Reactions of Specific Polysaccharides of Types III and VIII Pneumococcus in Type III Antipneumococcus Serum Absorbed with Glucuronic Acid Antigen**

| Antipneumococcus serum Type III | Specific polysaccharide used as test antigen | Final dilution of specific polysaccharide |
|-------------------------------|---------------------------------------------|----------------------------------------|
|                               |                                             | 1:200,000 | 1:1,000,000 | 1:2,000,000 | 1:4,000,000 | 1:6,000,000 |
| Unabsorbed                    |                                             |           |             |             |             |             |
| Absorbed with glucuronic acid-chick |                               |           |             |             |             |             |
|                               | **Type**                                   | III       | III         | IIA         | IIB         | IIC         |
|                               |                                             | ++++      | +++±        | +++±        | ++±         | +±          |
|                               |                                             | +±±±      | +±±±        | +±±±        | +±±±±       | +±±±±±±±    |
| Unabsorbed                    |                                             |           |             |             |             |             |
| Absorbed with glucuronic acid-chick |                               |           |             |             |             |             |
|                               | **Type**                                   | VIII      | VIII        | VIII        | VIII        | VIII        |
|                               |                                             | +++±      |+++±         |+++±         |+++±         |+++±         |
|                               |                                             | ++±±±     | ++±±±±±±±    |++±±±±±±±    |++±±±±±±±±±±±|
|                               |                                             | +±±±±±±±  | +±±±±±±±±±±± | +±±±±±±±±±±±| +±±±±±±±±±±±|

**DISCUSSION**

The important rôle played by acid groups in determining the specificity of certain azoproteins has been emphasized and extensively investigated by Landsteiner and his coworkers (11). In the case of azoproteins containing substituted aromatic nuclei, it has been especially well demonstrated that the nature of the acid groups and their relative position in the benzene nucleus are important factors in determining serological specificity. It is not surprising, therefore, to find that artificial antigens containing the azobenzyl glycosides of glucose and glucuronic acid show separate and distinct specificities. Each glycoside contains four asymmetric carbon atoms. The p-aminobenzyl glycosides of glucose and glucuronic acid have an identical stereochemical configuration, however, and differ from one another
only in the nature of the chemical grouping occupying the sixth position. In the case of the glucoside this grouping is a primary alcohol (CH₂OH), whereas in the glucuronide a highly polar carboxyl (COOH) group occupies this position. This difference in molecular structure suffices to confer distinct and specific serological characteristics upon antigens containing these two carbohydrate radicals. It is remarkable that the antisera to these two antigens show no serological crossing despite the identity in configuration of the asymmetric carbon atoms of each carbohydrate. The immunological specificity exhibited by both antigens appears referable, therefore, to a distinct individuality conferred upon each by changes in the chemical grouping occupying the sixth position in each hexoside radical.

The important rôle played by acid groups in determining the serological specificity of certain bacterial polysaccharides is likewise emphasized by the precipitin reaction of the glucuronic acid antigen in antipneumococcus horse sera Types II, III, and VIII. In this respect the artificial glucuronic acid-protein antigen bears a striking immunological relationship to the specific bacterial polysaccharides, though the chemical relationship resides solely in the common uronic acid constituent. In preliminary reports of this investigation (12), it was pointed out that the serological activity of the artificial glucuronic acid-protein antigen in antipneumococcus sera might be attributed to the interaction of the antigen with antibodies related to the uronic acid constituents of the bacterial polysaccharides. Further evidence in support of this point of view has since been presented by Marrack (13) and his associates, who have found that an artificial azoprotein antigen containing the naturally occurring glycoside of glucuronic acid, euxanthic acid, reacts in high dilutions in Type II antipneumococcus serum.

That the serological activity of the glucuronic acid antigen in antipneumococcus sera Types II, III, and VIII represents a reaction between the artificial antigen and the specific carbohydrate antibodies in these sera is clearly substantiated by the results of the specific absorption and inhibition tests. Despite the intimate serological relationship which the glucuronic acid antigen bears to the capsular polysaccharides of Types II, III, and VIII pneumococcus, yet it is not possible to remove from antipneumococcus serum Type III the carbohydrate
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antibodies by absorption with the artificial antigen. This lack of complete reciprocal absorption is not fully understood, but it is possible that quantitative precipitin studies may throw light on this perplexing question.

SUMMARY

1. Artificial carbohydrate-protein antigens containing the azobenzyl-glycosides of glucose and glucuronic acid give rise in rabbits to antibodies which are distinct and immunologically specific.

2. The artificial antigen containing glucuronic acid reacts in high dilutions in antipneumococcus horse sera Types II, III, and VIII. The chemical basis for this serological activity is discussed.

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