CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

VIII. The Influence of the Acetyl Group on the Specificity of Hexoside-Protein Antigens

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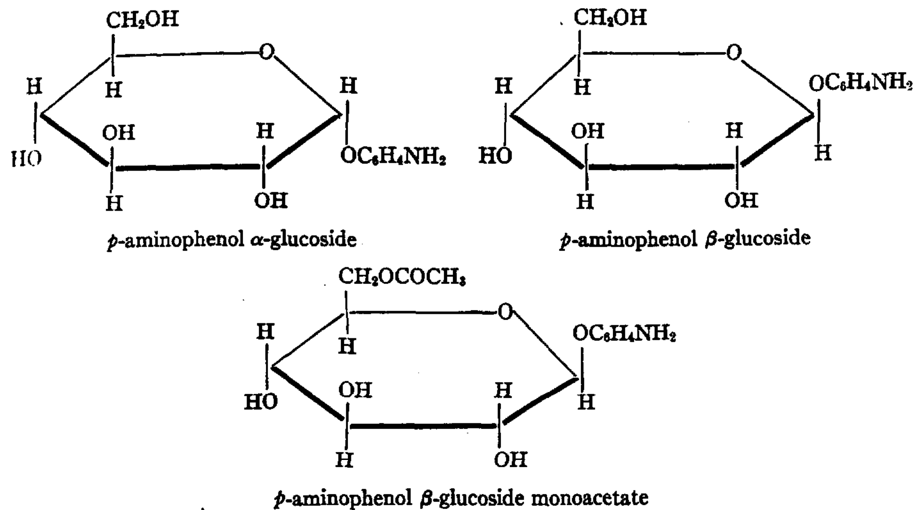
That the acetyl group (CH_3CO–) can exert a specific influence in determining the serological characteristics of a bacterial carbohydrate has recently been shown in this laboratory (1). The capsular polysaccharide of Pneumococcus Type I occurs in the intact cell as an acetyl derivative of a complex nitrogenous carbohydrate. The union of the acetyl groups with the polysaccharide itself is of a highly labile nature. When these groups are removed by mild alkaline hydrolysis, the resultant deacetylated carbohydrate is found to be deprived of certain immunological properties possessed only by the parent substance. Since the only demonstrable chemical change that occurs during alkaline hydrolysis is the removal of acetyl groups, the additional serological characteristics exhibited by the fully acetylated carbohydrate can be attributed only to the presence of the acetyl radical in the intact bacterial polysaccharide.

In the attempt to understand the immunological significance of the acetyl group in the more complex bacterial polysaccharides, a study has been made of the serological specificity of two related antigens each containing a simple carbohydrate radical in the acetylated and the unacetylated form. For this purpose, therefore, the *p*-aminophenol β-glucoside of glucose and its monoacetyl derivative have been synthesized. These two glucosides have been combined with the globulin of horse serum by means of the diazo reaction, and the resultant

"synthetic" carbohydrate azoproteins have been employed as antigens in the production of immune rabbit sera. The carbohydrate radicals of these two antigens are identical in their stereochemical relationships, yet they differ in that one of the derivatives contains an acetyl group. From the mode of synthesis it is probable that the acetyl radical is bound on the sixth carbon atom of the glucoside, although its exact allocation in the molecule is, for the purpose of this study, irrelevant.

It has previously been shown that conjugated carbohydrate-protein antigens containing either the azophenol α- or β-glucoside give rise in each instance to antibodies which are predominantly specific, yet capable of a secondary reaction with test antigens containing the heterologous hexoside (2). By means of the inhibition test it has been shown that the primary immune reaction is quite specific, since the union between homologous antigen and antibody is inhibited only by homologous glucoside, whereas the cross-reaction with the heterologous antigen is inhibited by both the homologous and the heterologous glucosides. Thus a difference in the stereochemical configuration of the carbon atom bearing the aglucon suffices to determine the immunological specificity of the α and β derivatives of glucose irrespective of the protein with which they are combined. The identity in structure of the remaining five carbon atoms of both these glucosides may account for the cross-reactions between the antisera and the heterologous hexoside-protein antigen. If the structural identity of the terminal portion of the hexoside molecule be altered through the introduction of a chemical grouping such as the acetyl radical, then one might anticipate that the immunological specificity of the altered glucoside might well be different from that of the same glucoside in its unacetylated form. That this phenomenon actually occurs will be seen from the following experimental data. For comparison the immunological properties of an antigen containing azophenol α-glucoside are included. The structural relationships of the three hexosides, α and β *p*-aminophenol glucosides and the acetyl derivative of the latter, are illustrated by the following graphic formulae:



p-aminophenol α-glucoside

p-aminophenol β-glucoside

p-aminophenol β-glucoside monoacetate

EXPERIMENTAL

I. CHEMICAL

1. p-Nitrophenol β-Glucoside Monoacetate.—10 gm. of *p*-nitrophenol β-glucoside were dissolved in 30 cc. of anhydrous pyridine. The solution was cooled to 0° and 2.61 cc. (1.1 mols) of acetyl chloride were slowly added. The mixture was kept cold for several hours and then allowed to stand at room temperature for 3 or 4 days. The pyridine was removed by distillation *in vacuo* and the residual syrup evaporated several times with absolute ethyl alcohol. The mixture was allowed to stand for 24 hours at ice box temperature to facilitate crystallization. Crystals of *p*-nitrophenol β-glucoside monoacetate were filtered from the mother liquors and washed with cold ethyl alcohol. 3.4 gm. were recovered. The compound was recrystallized several times from ethyl alcohol. The substance melted at 202–205°C. (uncorrected).

Analysis: 4.863 mg. substance gave 8.410 mg. CO_2 and 2.165 mg. H_2O.

$C_{14}H_{17}O_9N$ Calculated: C 49.00 per cent; H 5.00 per cent.
Found: C 49.19 per cent; H 5.19 per cent.

8.236 mg. substance when analyzed by Pregl's (3) method for the determination of acetyl groups, utilized 1.65 cc. N/70 NaOH.

CH_3CO- Calculated: 12.53 per cent.
Found: 12.32 per cent.

$$[\alpha]_D^{22°} = \frac{-2.33° \times 100}{2 \times 10 \times 0.1102} = -105.7° \text{ (in methyl alcohol)}$$

2. p-Aminophenol β-Glucoside Monoacetate.—2.0 gm. of *p*-nitrophenol β-gluco-

side monoacetate were dissolved in 100 cc. of warm methyl alcohol. The substance was reduced catalytically with 50 mg. of platinum oxide and hydrogen according to the method of Vorhees and Adams (4). From the alcoholic solution 1.2 gm. of *p*-aminophenol β-glucoside monoacetate were isolated. This compound crystallized as a snow-white product difficultly soluble in ethyl alcohol, more soluble in methyl alcohol, and much more easily soluble in water. It melted at 177–179°C. (uncorrected) and had a specific optical rotation of

$$[\alpha]_D^{21^\circ} = \frac{-1.69^\circ \times 100}{2 \times 10 \times 0.1204} = -70.2^\circ \text{ (in methyl alcohol)}$$

Analysis: 8.806 mg. substance when analyzed for acetyl groups, utilized 1.94 cc. N/70 NaOH.

CH_3CO– Calculated: 13.75 per cent.
Found: 13.55 per cent.

32.3 mg. sample when analyzed for nitrogen by the micro Kjeldahl method utilized 6.87 cc. N/70 HCl.

N Calculated: 4.47 per cent.
Found: 4.26 per cent.

The monoacetate of *p*-aminophenol β-glucoside is relatively stable to alkaline hydrolysis. Aqueous solutions must be warmed (40–50°) with N/10 alkali before the acetyl group begins to hydrolyze.

3. *p-Aminophenol α- and β-Glucosides.*—These compounds were prepared by methods previously described (2, 5).

4. *Preparation of Protein Azophenol α-Glucoside, β-Glucoside, and β-Glucoside Monoacetate Antigens.*—

(*a*) *Immunizing Antigens.*—The amino glucosides were combined with the globulin of normal horse serum in the manner previously described (2). The solutions of sugar-azo protein were sterilized by filtration through a Berkefeld candle and were used for immunization of rabbits.

(*b*) *Test Antigens.*—In order to avoid the possibility of antiprotein precipitins masking the specificity of the anticarbohydrate reactions, the test antigens used in the precipitin reactions were prepared by combining the respective glucosides to the proteins of chicken serum.

II. IMMUNOLOGICAL

Methods

Rabbits were immunized by the intravenous injection of solutions of the conjugated hexoside-protein antigens. The rabbits of one series received the antigen composed of horse serum globulin coupled to diazophenol α-glucoside, those of another series were treated with the same protein combined with diazophenol β-glucoside, while those of a third series received the protein in combination with the acetyl derivative of the β-glucoside. The rabbits of all three series were in-

jected intravenously with 1 cc. of the respective antigen, containing 10 mg. protein per cc., daily for six doses. The course of injections was repeated after a rest period of 1 week. 8 days following the last injection the rabbits were bled and the sera tested for precipitins against the homologous and heterologous test antigens. The technique of the precipitin and inhibition tests is the same as that described in the previous studies (6).

Carbohydrate Antibodies

1. Homologous Precipitin Reactions.—The sera of rabbits immunized with the carbohydrate-protein antigens were first tested for the presence of homologous precipitins against test antigens containing the homologous glucosides combined with the protein of chicken serum.

The precipitin reactions between the homologous antisera and the corresponding test antigens are summarized in Table I. The results

TABLE I

Homologous Precipitins in Sera of Rabbits Immunized with α-Gluco-Globulin, β-Gluco-Globulin, and Acetyl β-Gluco-Globulin

Antiserum	Test antigen	Dilution of test antigen		
		1:5,000	1:10,000	1:20,000
1. α-Gluco-globulin	α-Gluco-chicken serum	+++±	+++±	++±
2. β-Gluco-globulin	β-Gluco-chicken serum	++++	+++±	+++
3. Acetyl β-gluco-globulin	Acetyl β-gluco-chicken serum	+++	++±	++

++++ = Complete precipitation with disk-like precipitate.

of the homologous precipitin tests illustrate the property of the immune serum to react with an antigen containing the homologous glucoside, irrespective of the protein with which it is combined. It is further evident that the diazophenol glucosides when combined with protein function as excellent antigens.

2. Heterologous Precipitin Reactions.—In order to ascertain whether the antisera interact with the different carbohydrate antigens, the respective sera were tested against each of the heterologous test antigens. The results are summarized in Table II. From the results given in Table II, it may be seen that the immune serum obtained by immunization with an antigen containing the α-glucoside gives rise to

antibodies which cross-react with a test antigen containing the unacetylated β-glucoside, but not with one containing this glucoside in the acetylated form. On the other hand, an antiserum produced by immunization with β-gluco-globulin reacts not only with the homologous test antigen, but with α-gluco- and acetyl β-gluco-antigens as well. If one of the hydroxyl groups of the immunizing antigen containing the β-glucoside is covered with an acetyl group, then the homologous antiserum, although it still reacts with a β-gluco-antigen, fails to react with the α homologue. The chemical basis underlying

TABLE II

Heterologous Precipitins in Sera of Rabbits Immunized with α-Gluco-Globulin, β-Gluco-Globulin, and Acetyl β-Gluco-Globulin

Antiserum	Test antigen	Dilution of test antigen		
		1:5,000	1:10,000	1:20,000
1. α-Gluco-globulin	α-Gluco-chicken serum	+++±	++±	+±
	β-Gluco-chicken serum	+++	+±	+
	Acetyl β-gluco-chicken serum	0	0	0
2. β-Gluco-globulin	α-Gluco-chicken serum	++	+±	+
	β-Gluco-chicken serum	+++±	+++	++
	Acetyl β-gluco-chicken serum	++±	++	+±
3. Acetyl β-gluco-globulin	α-Gluco-chicken serum	0	0	0
	β-Gluco-chicken serum	+±	+	+
	Acetyl β-gluco-chicken serum	++±	++	++

++++ = Complete precipitation with disk-like precipitate.
0 = No precipitation.

the differences in the immunological properties of these glucosides will be discussed later.

3. Specific Inhibition Tests.—The selective specificity of each of the gluco-protein antigens is clearly demonstrated by the results of the inhibition tests which are given in Tables III, IV, and V. Analysis of the data presented in Tables III, IV, and V shows in each instance that upon addition of the corresponding glucoside to its homologous immune serum the specific precipitins are completely bound and are no longer capable of reacting with either the homologous or heterolo-

TABLE III

*Specific Inhibition of Precipitins in Acetyl β-Gluco-Globulin Antiserum by Homologous and Heterologous Glucosides**

Acetyl-β-gluco-globulin antiserum		*p*-aminophenol glucosides M/10			Salt solution to volume	Incubated 2 hrs. at 37°C.—no precipitation	Test antigen (1:5,000)*		Result
		α	β	Acetyl β			Acetyl β-gluco-chicken serum	β-Gluco-chicken serum	
Test No.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*		*cc.*	*cc.*	
1	0.2	—	—	—	0.3		0.5	—	++++
2	0.2	—	—	—	0.3		—	0.5	++
3	0.2	—	—	0.3	—		0.5	—	0
4	0.2	—	—	0.3	—		—	0.5	0
5	0.2	—	0.3	—	—		0.5	—	+++
6	0.2	—	0.3	—	—		—	0.5	0
7	0.2	0.3	—	—	—		0.5	—	+++±
8	0.2	0.3	—	—	—		—	0.5	+±

* α-Gluco-chicken serum antigen is not included in this table since it fails to react in acetyl β-gluco-globulin antiserum (*cf.* Table II).

TABLE IV

*Specific Inhibition of Precipitins in α-Gluco-Globulin Antiserum by Homologous and Heterologous Glucosides**

α-Gluco-globulin antiserum		*p*-aminophenol glucosides M/10			Salt solution to volume	Incubated 2 hrs. at 37°C.—no precipitation	Test antigen (1:5,000)*		Result
		α	β	Acetyl β			α-Gluco-chicken serum	β-Gluco-chicken serum	
Test No.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*		*cc.*	*cc.*	
1	0.2	—	—	—	0.3		0.5	—	++++
2	0.2	—	—	—	0.3		—	0.5	++±
3	0.2	0.3	—	—	—		0.5	—	0
4	0.2	0.3	—	—	—		—	0.5	0
5	0.2	—	0.3	—	—		0.5	—	+++
6	0.2	—	0.3	—	—		—	0.5	0
7	0.2	—	—	0.3	—		0.5	—	+++
8	0.2	—	—	0.3	—		—	0.5	++

* Acetyl β-gluco-chicken serum antigen is not included in this table since it fails to react in α-gluco-globulin antiserum (*cf.* Table II).

gous test antigens. The specificity of the inhibition reaction is demonstrated by the fact that a heterologous glucoside does not inhibit the homologous precipitin reaction. The acetylated β-glucoside antigen induces an immune response which is specifically distinct from that induced by an antigen containing this glucoside in its unacetylated form, for it is seen that the unacetylated β-glucoside fails to inhibit the reaction between the acetyl β-glucoside antigen and its homologous antibody (Test 5, Table III). This fact indicates clearly that the

TABLE V

Specific Inhibition of Precipitins in β-Gluco-Globulin Antiserum by Homologous and Heterologous Glucosides

β-Gluco-globulin antiserum		*p*-Aminophenol glucosides M/10			Salt solution to volume		Test antigens (1:5,000)			Result
		α	β	Acetyl β		Incubated 2 hrs. at 37°C.—no precipitation	β-Gluco-chicken serum	β-Acetyl-gluco-chicken serum	α-Gluco-chicken serum	
Test No.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*		*cc.*	*cc.*	*cc.*	
1	0.2	—	—	—	0.3		0.5	—	—	++++
2	0.2	—	—	—	0.3		—	0.5	—	+++
3	0.2	—	—	—	0.3		—	—	0.5	++
4	0.2	—	0.3	—	—		0.5	—	—	0
5	0.2	—	0.3	—	—		—	0.5	—	0
6	0.2	—	0.3	—	—		—	—	0.5	0
7	0.2	0.3	—	—	—		0.5	—	—	+++±
8	0.2	0.3	—	—	—		—	0.5	—	++
9	0.2	0.3	—	—	—		—	—	0.5	0
10	0.2	—	—	0.3	—		0.5	—	—	+++±
11	0.2	—	—	0.3	—		—	0.5	—	0
12	0.2	—	—	0.3	—		—	—	0.5	+±

acetyl group confers a distinct and additional specificity upon a simple carbohydrate the stereochemical structure of which remains unaltered. From the results in Table IV, it is seen that the acetyl β-glucoside does not inhibit the reaction of the unacetylated β-gluco-test antigen in α-gluco-globulin antiserum (Test 8). In Table V it may also be seen that the reaction between β-gluco-globulin antiserum and the homologous antigen, although inhibited by the β-glucoside (Test 4), is not inhibited by the same glucoside when one of the hydroxyl groups has been covered by an acetyl radical (Test 10).

DISCUSSION

The results of the present study not only confirm the view previously held that the immunological specificity of carbohydrates is determined by their stereochemical configuration, but they lend support to the further assumption that the introduction of a simple chemical group, such as the acetyl radical, endows a carbohydrate with a new and distinct specificity which is determined by the chemical nature of the group thus introduced. It has been previously pointed out that differences in the specific behavior of α- and β-glucosides of glucose may be accounted for by differences in the stereochemical configuration of the carbon atom bearing the aglucon, and that the basis for the immunological crossing may lie in the fact that the spatial configuration of the polar groups on the remaining five carbon atoms is identical. This explanation appears to be further supported by the results of the present study. For it can be seen from Table II that when β-gluco-test antigen, which normally reacts in α-gluco-globulin antiserum, is so altered that one of the polar groups (OH) of the five terminal carbon atoms of the carbohydrate radical is replaced by acetyl (CH_3CO), the resulting antigen fails to react in α-gluco-globulin antiserum.

Furthermore, α-gluco-test antigen, which normally reacts with β-gluco-globulin antiserum, fails to react in the immune serum produced by immunization with acetyl β-gluco-globulin. Similarly, the acetylated β-glucoside, due to the alteration in chemical constitution, fails to bind the antibody in α-gluco-globulin antiserum, and as a result permits both the α- and β-gluco-test antigens to react in their normal course. The β-glucoside likewise fails to inhibit the reaction between the acetyl β-gluco-test antigen, and homologous immune serum. This difference in the serological specificity of the antibodies induced by β-gluco-antigens in the acetylated and unacetylated form can be attributed only to the known differences in the chemical structure of these two glucosides. The latter differ from one another in that the acetyl β-glucoside is a derivative in which one of the polar groups (OH) of the carbohydrate has been altered by the introduction of an acetyl radical (CH_3CO).

A critical analysis of the results of these serological tests again emphasized the fact that the presence of an acetyl group in a carbohy-

drate exerts a determining influence on the specificity of an antigen of which it forms a part. In conclusion it may be pointed out that the differences in serological specificity exhibited by the acetylated and deacetylated polysaccharides of Type I Pneumococcus.are accurately paralleled by the purely synthetic system described.

SUMMARY

The chemical and immunological properties of the acetylated and unacetylated forms of the *p*-aminophenol β-glucoside of glucose have been described. The serological specificity of these β-glucosides in combination with protein has been correlated with known changes in chemical structure and has been compared with the immunological properties of the α-glucoside of the same hexose.

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