CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

VII. Immunological Specificity of Antigens Prepared by Combining α- and β-Glucosides of Glucose with Proteins

By OSWALD T. AVERY, M.D., WALTHER F. GOEBEL, Ph.D., and FRANK H. BABERS

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

(Received for publication, February 24, 1932)

The study of antigens prepared by chemically combining sugar derivatives with protein has revealed the fact that carbohydrates exert a determining influence on the immunological specificity of the compounds of which they form a part. In preceding papers evidence has been presented showing that mere differences in the structural configuration of a single carbon atom may determine the serological specificity of two sugar derivatives identical save in this one respect.

As previously pointed out, the *p*-aminophenol β-glucosides of glucose and galactose, differing from each other only in the interchange of the H and OH groups on a single carbon atom, exhibit an individual specificity irrespective of the protein to which they are attached (1, 2). Moreover, the specific rôle of the sugar radical in the reactive part of the antigen is revealed by the fact that the glucosides alone, unattached to protein, specifically inhibit the precipitin reaction between the corresponding antiserum and homologous antigen.

The inhibition of the antigen-antibody reaction by the intervention of the specific sugar hapten was found to occur both *in vitro* and *in vivo*. In guinea pigs passively sensitized with the precipitating serum of immune rabbits, the fatal anaphylactic shock which invariably follows the intravenous administration of the homologous sugar-protein was completely prevented by a single prophylactic injection of the specific glucoside alone. The unconjugated glucosides, although themselves not capable of inducing shock, specifically inhibited the anaphylactic reaction in sensitive animals when given immediately before the introduction of the shock-producing antigen (3). The specificity of the β-glucosides of glucose and galactose was demonstrated by the fact that the inhibition of the anaphylactic reaction, like the inhibition of the precipitin test *in vitro*, occurred only when the glucoside used was the same as that present in the specifically reacting antigen.

These earlier studies concerned themselves with the specificity of the β-glucosides of two different hexoses, glucose and galactose. The present paper deals with the specificity of two different glucosides of the same sugar, namely, the *p*-aminophenol α- and β-glucosides of glucose.

The "α and β antigens" formed by the union of the diazonium derivatives of these glucosides with protein differ from each other only in the spatial relations of the terminal carbon atom in each sugar component. Since the ultimate composition of both antigens is chemically the same, any observable differences in their immunological specificity can be referable only to known differences in the molecular structure of each. The following experiments were carried out to determine to what extent two isomeric glucosides of the same sugar, in combination with a single protein, would influence the specificity of two antigens otherwise identical. The present paper deals with the question of the specificity of the precipitins present in the serum of rabbits immunized with α- and β-gluco-globulin.

EXPERIMENTAL

Methods

The synthesis of the α- and β-*p*-aminophenol glucosides of glucose and the method of coupling the diazonium derivatives to protein are given in detail in the preceding paper (4). The method of the intravenous immunization of rabbits and the technique of the precipitin and inhibition tests differ in no essential respect from those described in the earlier studies (2).

The *immunizing antigens* were prepared by combining each of the glucose derivatives to globulin derived from normal horse serum; on the other hand the *test antigens* used in the precipitin reactions, contained the respective glucosides bound to the proteins of chicken serum. The use of proteins of remote biological origin in the two sets of antigens excludes the possibility of common antiprotein precipitins masking the specificity of the carbohydrate reactions.

I. Anticarbohydrate Antibodies

(*1*) *Specific Precipitin Reactions.*—The sera prepared by immunization of rabbits with α- and β-gluco-globulin were first tested for the presence of precipitins specifically reactive with an antigen containing the same carbohydrate radical. The test antigens employed in the precipitin reactions were in each instance prepared by combining the

respective glucoside with a protein unrelated to that used in the immunizing antigen. Under these conditions, the results of the tests are not confused by the presence of a common protein and the specificity of the reactions are directly interpretable in terms of known differences in the chemical structure of sugar radical in the reactive part of each antigen.

TABLE I

*Specific Precipitins in Sera of Rabbits Immunized with α-Gluco-Globulin**

Immune rabbit sera	Test antigen: α-gluco-chicken serum*			
	1:5,000	1:10,000	1:20,000	1:40,000
1	++++	+++	++	+±
2	++	++	+	±
3	+++	+++	++	+

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

* In order to avoid the reactions of a common protein, the *immunizing antigen* was prepared by combining α-glucoside with the globulin from horse serum, while the *test antigen* was similarly prepared by coupling the same glucoside to the proteins of chicken serum.

TABLE II

Specific Precipitins in the Sera of Rabbits Immunized with β-Gluco-Globulin

Immune rabbit sera	Test antigen: β-gluco-chicken serum*			
	1:5,000	1:10,000	1:20,000	1:40,000
1	+++	++	+±	+
2	++++	++++	+++	+++
3	++++	++++	+++	+++

++++ = Complete precipitation with compact disk formation.

* Unrelated proteins in immunizing and test antigens (see footnote, Table I).

The precipitin reactions between the homologous test antigens and the antisera of rabbits immunized with α- and β-gluco-globulin are given in Tables I and II.

The results of the precipitin tests illustrate the capacity of the immune sera to react with an antigen containing the homologous glucoside irrespective of the protein to which it is attached. They confirm the earlier observations concerning the orienting influence of the

sugar radical on the specificity of the protein with which it is combined.

(2) *Specific Inhibition Tests.*—The selective specificity of the antibodies reactive with the two isomeric derivatives of glucose is clearly demonstrated by the results of the inhibition tests given in Tables III and IV.

Analysis of the data presented in Table III shows that the α-glucoside alone, when added in excess to the homologous antiserum specifically binds the precipitins and thus renders them unable to react subse-

TABLE III

Specific Inhibition of Precipitins in α-Gluco-Globulin Antiserum by Homologous Glucoside

α-gluco-globulin antiserum		Glucosides M/15		Salt solution to volume		Test antigen: α-gluco-chicken serum*			Result
		α	β			1:2,000	1:5,000	1:10,000	
	cc.	cc.	cc.	cc.		cc.	cc.	cc.	
1	0.2	—	—	0.3	2 hrs. at 37°C. No precipitation	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	+++

+++ = Disk-like precipitate

0 = No precipitation, showing complete inhibition.

* See footnote, Table I.

quently with the test antigen containing the same sugar derivative. The specificity of this inhibition is shown by the fact that the addition of an equal concentration of the β-glucoside to α immune serum has little or no appreciable effect on the precipitin reaction with the α antigen. Similar relationships are shown to exist in the specific inhibition of the precipitins in β antiserum by the β-glucoside (Table IV).

The results of the inhibition tests reveal the specificity of the anti-carbohydrate reactions, since in the case of both α and β antisera the precipitins for the homologous antigen are inhibited only by the corre-

sponding glucoside. Although the glucosides by themselves, unattached to protein, fail to produce precipitation in immune sera, each by binding the specific antibodies in its homologous antiserum prevents them from again reacting with a test antigen containing the same glucoside. In this sense the glucosides unattached to protein function as haptens, the chemo-specific groups of which unite with the corresponding antibodies without causing visible change in the reaction mixture. Marrack and Smith (5) using a direct method have

TABLE IV

Specific Inhibition by Homologous Glucoside of Precipitins in β-Gluco-Globulin Antiserum

β-gluco-globulin antiserum		Glucosides M/15		Salt solution to volume	2 hrs. at 37°C. No precipitation	Test antigen: β-gluco-chicken serum*			Result
		α	β			1:2,000	1:5,000	1:10,000	
	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	++++

++++ = Complete precipitation with disk-like precipitate.
0 = No precipitation, showing complete inhibition.
* See footnote, Table I.

recently brought experimental proof of Landsteiner's original view that the union between hapten and antibody is a specific combination between the chemo-specific groups of the reacting agents. As Landsteiner has pointed out in the case of non-protein radicals which function as haptens, the union of these glucosides with antibody does not lead to precipitation unless the reacting groups are attached to protein.

The preceding experiments have dealt with the homologous immune reactions of two antigens of known chemical constitution which differ one from the other only in the α and β type of glucosidic union. The results show that mere differences in the spatial arrangement of the

groups on the terminal carbon atom in these two derivatives of glucose suffice to confer on each antigen a marked degree of differential specificity. For, as shown by the specific inhibition reactions, it is possible to differentiate selectively between the two isomeric glucosides of the same sugar by the use of immune sera.

Despite the reflection in serological specificity of differences in the stereochemical relationships of the first carbon atom bearing the non-sugar constituent of each glucoside, it must be borne in mind that in both the α and β derivatives the spatial arrangement of the polar groups on the remaining five carbon atoms is identical. Quite different, however, are the structural relations existing in the case of the β-glucosides of glucose and galactose, the immunological specificity of which has previously been shown to be absolute (2). In these latter glucosides the stereochemical arrangement of the groupings on the terminal carbon atom are both in the β position, but the symmetry of the polar groups on the remaining five carbon atoms is not identical, for on the fourth carbon atom of galactose the H and OH groups are rotated through an angle of 180°. This difference in molecular configuration, with its consequent change in the spatial relationship of the polar groups, is the important if not the sole factor determining the individual specificity of the β-glucosides of glucose and galactose. On the basis of these facts, one might anticipate an absolute specificity in the case of the similar glucosides of two different hexoses, but an overlapping specificity in isomeric glucosides of the same hexose.

(*3*) *Cross-Precipitin Reactions.*—It is therefore not surprising to find that this partial similarity in the chemical structure of the α and β derivatives of glucose is reflected in the serological relationships of both substances. The results of the cross-precipitin tests presented in Table V show that antibodies present in α and β antisera cross-react in each instance with the heterologous test antigen.

The cross-precipitin reactions are sharply defined and are quantitatively only slightly less than the reactions between each antiserum and its homologous antigen.

Interesting relationships in the overlapping specificity of both sugar derivatives are brought out in the reciprocal inhibition tests, the results of which are given in Table VI.

Analysis of the data presented in Table VI shows that the addition of α-glucoside to α antiserum inhibits the precipitins from reacting

with both α and β test antigens. However, the addition of an equal concentration of β-glucoside to α antiserum inhibits the precipitin

TABLE V

Cross-Precipitin Reactions of α- and β-Gluco-Globulin Antisera with Heterologous Antigens

Immune sera	Test antigens*					
	α-gluco-chicken serum			β-gluco-chicken serum		
	1:5,000	1:10,000	1:20,000	1:5,000	1:10,000	1:20,000
α-gluco-globulin	++++	+++	++	++±	++	+
β-gluco-globulin	++	++	+	++++	++++	+++

++++ = Complete precipitation with compact disk-like precipitate.
* See footnote, Table I.

TABLE VI

Inhibition of Cross-Precipitin Reactions by Homologous and Heterologous Glucoside

Gluco-globulin antisera	Glucosides M/15		Salt solution to volume		Test antigen* 1:10,000		Results
	α	β			α	β	
cc.	cc.	cc.	cc.		cc.	cc.	
α 0.2	—	—	0.3	2 hrs. at 37°C.	0.5	—	+++
α 0.2	0.3	—	—		0.5	—	0
α 0.2	—	0.3	—		0.5	—	++±
α 0.2	—	—	0.3		—	0.5	++
α 0.2	0.3	—	—		—	0.5	0
α 0.2	—	0.3	—		—	0.5	0
β 0.2	—	—	0.3	2 hrs. at 37°C.	—	0.5	++++
β 0.2	—	0.3	—		—	0.5	0
β 0.2	0.3	—	—		—	0.5	+++±
β 0.2	—	—	0.3		0.5	—	++
β 0.2	—	0.3	—		0.5	—	0
β 0.2	0.3	—	—		0.5	—	0

Plus signs indicate positive reaction with gradation in amount of precipitate.
0 = no precipitation, showing complete inhibition.
— = reagent not used.
* See footnote, Table I.

reaction with the β test antigen without appreciably diminishing its capacity to precipitate the α antigen. Similar relationships hold in

the case of the β antiserum with respect to the inhibition of precipitins by the homologous and heterologous glucosides.

It is evident from these results, that the addition of the homologous glucoside to its antiserum completely inhibits the precipitins for both the homologous and heterologous test antigens. On the other hand, the addition of the heterologous glucoside to immune serum inhibits only the precipitins for the heterologous test antigen and has but slight effect upon the antibodies reactive with the homologous antigen. The results of the cross-inhibition tests with the α- and β-glucosides show that the reaction of an immune serum with its homologous antigen is specifically inhibited only by the homologous glucoside; while the cross-reaction between this serum and the heterologous antigen is

TABLE VII

*Precipitins for Globulin in Sera of Rabbits Immunized with α- and β-Gluco-Globulin**

Immune sera	Globulin from horse serum			
	1:5,000	1:10,000	1:20,000	1:40,000
α-gluco-globulin	+	+±	++	++
β-gluco-globulin	+	++	++	+++

+++ = flocculent precipitate, not compact.

* Both immunizing antigens were prepared by combining each glucoside with globulin from horse serum.

completely inhibited by either glucoside. This lack of reciprocal inhibition of the precipitins in α and β antisera may be interpreted as further evidence of the lack of the immunological identity of the two isomeric glucosides.

II. Antiprotein Antibodies

As previously described the immunizing antigens were prepared by combining the α and β derivatives of glucose with globulin obtained from normal horse serum. The sugar-protein antigens were standardized on the basis of their nitrogen content so as to contain 5 mg. of protein per cc. Each rabbit received during the course of immunization a total of approximately 160 mg. of protein antigen.

In addition to the antibodies reactive with the specific glucosides, there are also present in both immune sera precipitins for the globulin used in preparing the immunizing antigens (Table VII).

The precipitating antibodies for horse serum globulin may arise in response to the presence in both antigens of free protein unbound by the diazotized glucosides. However, it is also possible that even in the absence of free protein, the sugar-protein antigen as a whole may stimulate the formation of two qualitatively different antibodies, each specifically related to the corresponding component of the antigenic complex. This concept of the dual antigenicity of a single complex antigen involves the assumption that the coupling of the glucoside to the protein has not masked the groups essential to the specificity of the protein, and that while the sugar radical through conjugation acquires specific antigenicity, the protein molecule retains, in part at least, its original antigenic properties.

DISCUSSION

Landsteiner and Lampl (6), working with ortho, meta, and para substituted aromatic compounds, have pointed out the significance of the spatial arrangement of the groupings upon which the specificity of these compounds depends. In more recent studies on the serological differentiation of steric isomers of *p*-aminobenzoylamino acetic acid and tartaric acid, Landsteiner and van der Scheer (7) have further emphasized the dependence of immunological specificity upon chemical structure.

The results of the present study add further evidence in support of the view that the immunological specificity of carbohydrates is determined by their chemical constitution. Differences in the specific behavior of the α- and β-glucosides of glucose may be accounted for by known differences in the spatial position of the groups on the first carbon atom of each glucoside. These structural changes are so sharply reflected in serological specificity that it is possible by means of immune sera to differentiate selectively between the two isomeric glucosides of the same sugar. However, granting the difference in the chemical structure of the two glucosides it becomes necessary to account for their overlapping specificity. The chemical basis for this immunological crossing may lie in the fact that the spatial arrangement of the polar groups on the remaining five carbon atoms is identical in both glucosides. This partial similarity of molecular grouping might then account for the degree of immunological likeness exhibited

by the two substances. Although in a portion of the molecule of both glucosides the structural relationship is identical, nevertheless they behave chemically as separate entities and possess serologically a separate and distinct specificity. If complete reciprocal inhibition of precipitins is accepted as the criterion of serological identity then the failure of both glucosides to exhibit this capacity may be taken as further evidence of differences in the immunological specificity of each.

The lack of reciprocal absorption of agglutinins by two organisms mutually agglutinable in the immune serum of each is generally conceded to indicate a lack of immunological identity. Relationships of this order are known to exist between Pneumococcus Type II and Friedländer bacillus Type B. In both instances the reactive substance has been identified chemically as the specific polysaccharide peculiar to the capsule of each organism (8). While the structural constitution of these complex sugars is not as yet fully known, considerable knowledge has been gained concerning their chemical properties. For example, it is known that the Pneumococcus Type II polysaccharide is built up of glucose units and that chemically it bears a close resemblance to the polysaccharide recovered from the Type B Friedländer bacillus. However, the two substances are not chemically identical, although the similarity between them is sufficient to result in a certain likeness in immunological specificity (9). In the absence of precise knowledge of the structural relations of the two polysaccharides, it seems reasonable to assume that both contain in a portion of the complex molecule the same or a closely similar configuration of atoms. This similarity of molecular grouping might then account for the immunological similarity of the two substances.

Considerable evidence for this point of view is found in the results of the present study concerning the specificity of the α- and β-glucosides of glucose. A comparison of the serological relationships between the two isomeric derivatives of glucose and the capsular polysaccharides of the two organisms in question is presented in Table VIII.

Analogous relationships extending even to the cross-immunity reactions and the lack of reciprocal absorption and inhibition of antibodies are evident in both the groups of simple and complex carbohydrates. While the comparison is instructive it is not necessarily valid in the case of the capsular polysaccharides, for the final interpretation of

these relationships must await further knowledge of the structural relations of the more complex bacterial sugars.

TABLE VIII

Comparison of the Serological Relationship between Isomeric Derivatives of Glucose and the Capsular Polysaccharides of Two Unrelated Species of Bacteria

α-gluco-globulin antiserum			Antipneumococcus serum Type II		
By addition of	Precipitins are		Absorbed with	Agglutinins are	
	inhibited for	not inhibited for		removed for	not removed for
α-glucoside	α and β test antigens		Pneumococcus Type II	Pneumococcus Type II *B. friedlaenderi* Type B	
β-glucoside	β test antigen	α test antigen	*B. friedlaenderi* Type B	*B. friedlaenderi* Type B	Pneumococcus Type II
β-gluco-globulin antiserum			**Anti-*friedlaenderi* serum Type B**		
By addition of	Precipitins are		Absorbed with	Agglutinins are	
	inhibited for	not inhibited for		removed for	not removed for
β-glucoside	α and β test antigens		*B. friedlaenderi* Type B	Pneumococcus Type II *B. friedlaenderi* Type B	
α-glucoside	α test antigen	β test antigen	Pneumococcus Type II	Pneumococcus Type II	*B. friedlaenderi* Type B

The test antigens in all instances were prepared by combining the respective glucoside with a protein biologically unrelated to that in the immunizing antigen; see footnote, Table I.

Enders (10) has recently suggested that cross-relationships between Type II Pneumococcus and Type B Friedländer bacillus may be

due to the presence in pneumococci of a type-specific agglutinogen unrelated to the specific carbohydrate.

In the case of the synthetic antigens containing the α and β compounds of glucose alone, the evidence indicates that the immunological relationships of the reactive glucosides are determined by known variations in their chemical constitution and are independent of the protein to which they are attached. In view of these findings it seems not unlikely that in the case of the polysaccharides, because of their more complicated structure and the greater possibility for variation in molecular configuration, there may be found many examples of a similar overlapping specificity among carbohydrates of unrelated origin.

SUMMARY

The chemical and immunological properties of the *p*-aminophenol α- and β-glucosides of glucose are described and correlated. The results are discussed with reference to their possible bearing on the chemo-immunological nature of the specific polysaccharides of bacterial origin.

BIBLIOGRAPHY

1. Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1929, **50**, 521.
2. Avery, O. T., and Goebel, W. F., *J. Exp. Med.*, 1929, **50**, 533.
3. Tillett, W. S., Avery, O. T., and Goebel, W. F., *J. Exp. Med.*, 1929, **50**, 551.
4. Goebel, W. F., Babers, F. H., and Avery, O. T., *J. Exp. Med.*, 1932, **55**, 761.
5. Marrack, J. R., and Smith, F. C., *Nature*, 1931, **128**, 1077.
6. Landsteiner, K., and Lampl, H., *Biochem. Z.*, 1918, **86**, 388.
7. Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1928, **48**, 315; 1929, **50**, 407.
8. Heidelberger, M., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1925, **42**, 701.
9. Avery, O. T., Heidelberger, M., and Goebel, W. F., *J. Exp. Med.*, 1925, **42**, 709.
10. Enders, J. F., *J. Exp. Med.*, 1932, **55**, 191.