CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

II. Immunological Specificity of Synthetic Sugar-Protein Antigens

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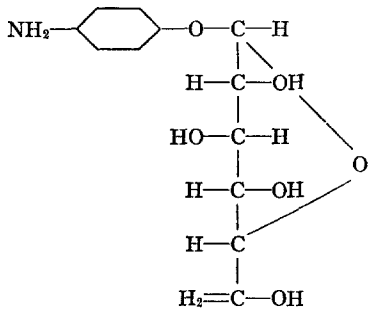
The function of carbohydrates as determinative substances in bacterial specificity is well illustrated in the immunological differentiation of the specific types of pneumococci and of Friedländer bacilli. Previous studies have demonstrated that the presence of type-specific polysaccharides in these encapsulated organisms determines the antigenic properties of the cell and the serological relationships of different strains.

In the course of investigations on the chemo-immunological nature of these complex bacterial antigens, the facts thus far ascertained lend support to the view that the specific polysaccharides function as true antigens only when combined with some other constituent of the cell. While the character of the substance which enters into combination with these complex sugars, and the nature of the linkage between them are still undetermined, it seems probable that the type-specific antigen consists of a protein or protein derivative conjugated with the specific carbohydrate; and that it is the latter component which orientates the specificity of the complex antigen thus formed. The possibility also exists that the polysaccharides which by themselves are non-antigenic, may, by reason of their acidic properties acquire antigenicity by combining to form salts with some basic constituent of the bacterial cell. This latter possibility, as well as the supposition that a mere change in the physical state of the polysaccharide as it exists in the intact cell may account for its antigenic behavior, seem in the light of present knowledge, to be

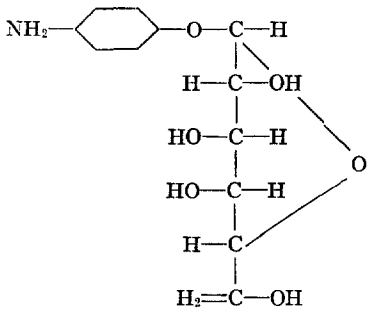
less likely than does the former view that the type-specific antigen consists of a conjugated carbohydrate-protein complex in which the carbohydrate radical determines the specificity of the whole.

With a view to the possible elucidation of the problems just stated, it appeared of considerable interest to determine the effect produced on the antigenic specificity of a protein by combining it with a relatively simple carbohydrate. This combination was effected, as described in the preceding paper (1), by synthesis of the p-aminophenol glucosides of glucose and galactose, and by coupling these diazotized glucosides with different proteins.

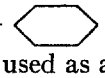
The experiment was made all the more exacting by the purposeful choice of two monosaccharides which have the same chemical formula and which differ from each other only in specific rotation and molecular configuration:—the groups on the fourth carbon atom in galactose forming the mirror image of the fourth carbon groups in glucose. The remainder of the molecule is the same in both sugars. The relationship of the p-aminophenol β-glucoside to p-aminophenol β-galactoside may be seen from the two structural formulae—



p-aminophenol β-glucoside



p-aminophenol β-galactoside

The diazonium derivatives of these two glucosides were attached to proteins by means of the linkage —N = N —  — and the two synthetic sugar proteins thus derived were used as antigens.

The specificity of these conjugated carbohydrate-proteins was further tested by linking the same diazotized glucoside to two chemically distinct proteins derived from widely remote biological species.

It will be shown that immune sera prepared with these complex

antigens contain two separate kinds of antibodies; one variety stimulated by the conjugated sugar-protein, and the other evoked by the protein itself, varying amounts of which coexist unbound in the same solution. The immunological specificity of the synthetic sugar-proteins described in the present paper will be discussed, therefore, with reference to these two kinds of antibodies; 1. the anticarbohydrate antibodies (anti-S) and 2. the antiprotein antibodies (anti-P.). It will be shown further that each variety of antibody is specifically related to the corresponding component of the antigen; that the anti-protein antibodies exhibit the species specificity of the original protein, and that the antibodies reactive with the conjugated sugar-proteins are specific for unrelated proteins containing the same diazotized carbohydrate group.

The results of cross precipitin reactions, precipitin absorption and inhibition tests with immune sera prepared with the synthetic sugar-proteins are presented in the following protocols.

EXPERIMENTAL

Methods

Rabbits were immunized by the intravenous injection of solutions of the conjugated carbohydrate-protein antigens. The animals of one series received the antigen composed of purified globulin from horse serum coupled to diazo phenol glucoside, and those of another series were treated with the same protein combined with the diazo phenol galactoside. The rabbits of both series were injected with 2 cc. of the respective antigen daily for six doses and the course of injections was repeated at weekly intervals until a total of 36 cc. of antigen was given. Eight days after the last injection the rabbits were bled and the serum tested for precipitins against the homologous and heterologous antigens.

The antigenic material was prepared by the method described in the preceding paper, and was sterilized by passage through a Berkefeld filter. The same stock solution was used throughout the course of immunization. None of the rabbits showed any evidence of toxic symptoms following repeated injections.

Preparation of proteins:—The two proteins to which the diazo phenol glucosides were linked were serum globulin (horse) and crystalline egg albumin. The globulin was prepared from horse serum by precipitation with dilute acetic acid as described in the preceding paper. The crystalline egg albumin was made from native egg white by the method of Soerensen (2).

Preparation of carbohydrate-proteins:—By the methods described in the preceding paper (1) the following synthetic sugar-proteins were prepared:

1. phenol β glucoside-azo-globulin.
2. phenol β galactoside-azo-globulin.
3. phenol β glucoside-azo-albumin.
4. phenol β galactoside-azo-albumin.

For the sake of convenience the above preparations will be referred to respectively as gluco-globulin, galacto-globulin, gluco-albumin, and galacto-albumin.

Precipitin reactions:—The immune sera were in all instances used in constant amounts of 0.2 cc. A dilution of serum in the proportion of 2 parts of serum to 3 parts of salt solution was prepared and 0.5 cc. of this dilution, containing 0.2 cc. of the original serum, was added to 0.5 cc. amounts of the varying dilutions of the antigens as shown in the protocols.

Standardization of antigens:—For purposes of comparison, the sugar protein antigens were standardized on the basis of nitrogen-content; this method, however, does not indicate the amount of bound carbohydrate and hence is not a measure of the effective antigen complex.

I. ANTICARBOHYDRATE ANTIBODIES: (ANTI-S)

1. Precipitin reactions with Gluco-globulin Anti-Serum

Serum prepared by immunization with gluco-globulin was tested for the presence of precipitins against the homologous antigen and two other sugar-proteins, gluco-albumin and galacto-albumin. Both of the latter test substances contain egg albumin, a protein foreign to the immunizing antigen. In the case of gluco-albumin, however, the carbohydrate radical is the same as that present in the sugar-protein used for immunization, while in the case of galacto-albumin both the protein fraction and the sugar derivative are heterologous with respect to the original antigen. The results of cross precipitin tests are given in Table I.

The data presented in Table I show that gluco-globulin anti-serum reacts not only with gluco-globulin but also with gluco-albumin. The fact that antibodies stimulated by gluco-globulin are specifically reactive with gluco-albumin, which contains the homologous glucose derivative bound to a protein unrelated to that present in the immunizing antigen, demonstrates that the carbohydrate radical and not the protein molecule orientates the specificity of these conjugated sugar-proteins. The specificity of the orienting carbohydrate is further emphasized by the fact that antibodies reactive with gluco-albumin show no reaction with galacto-albumin in which an isomeric carbo-

hydrate is conjugated with the same protein. This is further proof that the chemical constitution of the sugar radical, regardless of the nature of the protein to which it is attached, determines the serological specificity of the conjugated antigen.

TABLE I

Precipitin Reactions of Gluco-Globulin Antiserum

Showing the specificity of sugar-proteins when the same carbohydrate derivative is combined with two serologically distinct proteins.

Anti-Gluco-Globulin Serum

Dilutions of antigens†	Carbohydrate-Protein Antigens		
	Gluco-globulin (horse)	Gluco-Albumin (egg)	Galacto-Albumin (egg)
1:1000	++±	++	−
1:5000	++++	++++	−
1:10,000	++++	++++	−
1:20,000	+++±	+++	−
1:40,000	+++	+	−
1:80,000	+	±	−
1:100,000	±	−	−

++++ = Complete precipitation with clear supernatant and formation of a compact, disc-like precipitate not easily disrupted by shaking—characteristic of type specific polysaccharide reactions with anti-pneumonococcus serum.

± = Faint turbidity.

− = No reaction.

† The antigens were standardized on the basis of nitrogen content. This method does not measure the amount of glucoside bound to protein.

2. *Precipitin reactions with Galacto-Globulin Anti-serum*

The cross precipitin reactions with galacto-globulin antiserum and three synthetic sugar-proteins, galacto-globulin, galacto-albumin and gluco-albumin, are given in Table II.

Specific precipitins in the serum of rabbits immunized with galacto-globulin react with solutions of galacto-globulin and galacto-albumin in approximately equal titre. These antibodies, however, fail to precipitate gluco-albumin. (Table II.) The specificity of the orienting sugar radical is again revealed in the cross precipitin reac-

tions between galacto-globulin and galacto-albumin. The carbohydrate derivative alone is common to both of these compounds, while the protein fraction of each is wholly dissimilar. The specific relationships between these sugar-proteins, therefore, appear to depend upon the nature of the particular carbohydrate component rather than upon the kind of protein to which it is linked.

TABLE II

Precipitin Reactions of Galacto-Globulin Antiserum

Showing the specificity of anti-S antibodies by cross-reactions with two serologically distinct proteins containing the same sugar-radical.

Anti-Galacto-Globulin Serum

Dilution of Antigen	Antigens		
	Galacto-Globulin	Galacto-Egg-Albumin	Gluco-Egg-Albumin
1:5000	++++	+++±	−
1:10,000	++++	+++±	−
1:20,000	+++	+++	−
1:40,000	++±	++	−
1:80,000	++	+	−
1:100,000	+	±	−

++++ = Complete precipitation with compact, disc-like precipitate.
− = No reaction.

The data presented in Table I and II may be summarized briefly as follows:—

1. When two chemically different carbohydrate derivatives are bound to the same protein, the newly formed compounds are serologically distinct from one another. Simple differences in the molecular configuration of the two isomers, glucose and galactose, although confined to one single carbon atom in the molecule, suffice to orientate antigenic specificity when corresponding glucosides of these sugars are coupled to the same protein.

2. When the same carbohydrate radical is conjugated with two chemically and serologically distinct proteins, both of the sugar-proteins thus formed acquire a common serological specificity.

3. The newly acquired specificity of these sugar-proteins is determined by the chemical constitution of the carbohydrate attached to the protein molecule.

3. Specific Inhibition of Precipitin Reactions by Homologous Glucosides

In his studies on complex antigens Landsteiner (3) has shown that immune sera prepared with azo-proteins are markedly specific, precipitating unrelated proteins which contain the same azo groups. However, the simple azo compounds by themselves are not antigenic and are not precipitable in azo-protein antiserum. Nevertheless, when they are added to the immune serum prepared with protein coupled to the same diazotized compounds they specifically inhibit the antibodies from reacting subsequently with the homologous antigen.

Since in the present study the diazotized phenol glucosides of glucose and galactose exhibit immunological properties analogous to those of the diazotized amino compounds described by Landsteiner, the inhibiting action of these carbohydrate substances on the precipitins of homologous immune sera was studied.

Inhibition Test: Solutions of p-aminophenol β-glucoside and p-aminophenol β-galactoside in concentrations of 0.1 M. and 0.01 M. were added in amounts of 0.2 and 0.1 cc. to a constant unit of immune serum (0.2 cc.) and made up to volume by addition of salt solution. The mixtures were incubated at 37°C. for 2 hours, and the tubes examined for the presence of precipitate. To the test mixtures, 0.5 cc. of sugar protein antigen containing the homologous carbohydrate derivative was then added in optimal dilution, and the tubes again incubated for 2 hours. Readings were made at the end of the period of incubation and after 24 hours in the ice-box.

The results of the inhibition tests with homologous glucosides are given in Tables III and IV.

Since the inhibiting action of the homologous glucosides on specific precipitins is similar in each instance, the results will be discussed together. (Tables III and IV.) It is apparent that the glucosides by themselves are not precipitated in the presence of immune sera prepared with protein containing the homologous diazotized compounds.

It is also evident that although the carbohydrate substances alone fail to cause precipitation in immune serum, they, nevertheless, specifically inhibit the precipitating antibodies from reacting with the homologous sugar-protein when the latter is subsequently added to the serum mixture. The specificity of the reaction is shown by the fact that while the addition to homologous serum of 0.1 cc. of 0.01 M solution of the corresponding glucoside completely inhibits precipitation

when the specific carbohydrate-protein is added later, the addition of a ten to twenty-fold concentration of a heterologous glucoside, under the same conditions, exerts no inhibition on the precipitating antibodies.

TABLE III

Specific Inhibition by Homologous Glucoside of Precipitin Reactions with Gluco-Globulin Serum

	Gluco-Globulin Serum	Glucoside		Galactoside		Salt solution to volume	Incubation—2 hrs. at 37°C. No precipitate formed.	Antigen	Result	
		0.1 M.	0.01 M.	0.1 M.	0.01 M.			Gluco-Egg Albumin 1:10,000	2 hrs. 37°C.	24 hrs. ice-box
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*		*cc.*		
1	0.2	0.2	—	—	—	0.3		0.5	—	—
2	0.2	0.1	—	—	—	0.4		0.5	—	—
3	0.2	—	0.2	—	—	0.3		0.5	—	±
4	0.2	—	0.1	—	—	0.4		0.5	±	+
5	0.2	0.2	—	—	—	0.8		—	—	—
6	0.2	—	—	—	—	0.5		0.5	++	++++
7	0.2	—	—	0.2	—	0.3		0.5	++	++++
8	0.2	—	—	0.1	—	0.4		0.5	++	++++
9	0.2	—	—	—	0.2	0.3		0.5	++	++++
10	0.2	—	—	—	0.1	0.4		0.5	++	++++
11	0.2	—	—	0.2	—	0.8		—	—	—
12	0.2	—	—	—	—	0.7		—	—	—
13	—	—	—	—	—	0.7		0.5	—	—
14	—	0.2	—	—	—	1.0		—	—	—
15	—	—	—	0.2	—	1.0		—	—	—

+++ = Complete precipitation.
— = No reaction.

The simple monosaccharides,—glucose and galactose—from which the respective glucosides were synthesized, have been found to be inert in specific precipitin and inhibition reactions with immune sera prepared with protein united to the corresponding diazotized glucosides.

The data presented in Tables III and IV, may be summarized as follows:—

1. The unconjugated glucosides, although themselves not precipitable in immune serum, inhibit the reaction between the homologous sugar-protein and specific antibody.

2. This inhibition is specific, since heterologous glucosides exert no inhibiting action.

TABLE IV

Specific Inhibition by Homologous Galactoside of Precipitin Reactions with Galacto-Globulin Serum

	Galacto-Globulin Serum	Galactoside		Glucoside		Salt solution to volume	Incubation—2 hrs. 37°C. No precipitate formed.	Antigen	Result	
		0.1 M.	0.01 M.	0.1 M.	0.01 M.			Galacto-Egg Albumin 1:10,000	2 hrs. 37°C.	24 hrs. ice-box
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*		*cc.*		
1	0.2	0.2	—	—	—	0.3		0.5	—	—
2	0.2	0.1	—	—	—	0.4		0.5	—	—
3	0.2	—	0.2	—	—	0.3		0.5	—	±
4	0.2	—	0.1	—	—	0.4		0.5	±	±
5	0.2	0.2	—	—	—	0.8		—	—	—
6	0.2	—	—	—	—	0.5		0.5	++	+++
7	0.2	—	—	0.2	—	0.3		0.5	++	+++
8	0.2	—	—	0.1	—	0.4		0.5	++	+++
9	0.2	—	—	—	0.2	0.3		0.5	++	+++
10	0.2	—	—	—	0.1	0.4		0.5	++	+++
11	0.2	—	—	0.2	—	0.8		—	—	—
12	0.2	—	—	—	—	0.7		—	—	—
13	—	—	—	—	—	0.7		0.5	—	—
14	—	0.2	—	—	—	1.0		—	—	—
15	—	—	0.2	—	—	1.0		—	—	—

3. These glucosides exhibit the properties of haptens; they are non-antigenic substances which are specifically reactive, as shown by inhibition tests, with antibodies induced by proteins containing the homologous diazotized compounds.

II. ANTIPROTEIN ANTIBODIES (ANTI-P)

In the preceding experiments the immune sera prepared with conjugated sugar-proteins have been analyzed only with reference to

the presence of antibodies specifically related to the particular carbohydrate radical introduced into the protein molecule. These so-called anti-carbohydrate antibodies (Anti-S) are specifically reactive with unrelated proteins containing the same diazotized glucosides. However, in addition to the antibodies just described, the sera also contain

TABLE V

Precipitin Reactions of Gluco-Globulin Antiserum

Showing the species specificity of the antiprotein antibodies in cross reactions with antigens containing the same protein molecule.

Gluco-Globulin Serum

Dilution of Antigens	Carbohydrate-protein Antigens					
	Gluco-Globulin (horse)	Galacto-Globulin (horse)	Globulin (horse)	Gluco-Albumin (egg)	Galacto-Albumin (egg)	Albumin (egg)
1:5000	$\binom{++++}{xxxx}$	xxxx	xxxx	+++±	–	–
1:10,000	$\binom{++++}{xxxx}$	xxx	xxxx	+++	–	–
1:20,000	$\binom{+++}{xxx}$	xx	xx	++±	–	–
1:40,000	$\binom{+++}{xxx}$	x	$\frac{xx}{-}$	+±	–	–
1:80,000	$\binom{+}{x}$	$\frac{x}{-}$	x	±	–	–
1:100,000	$\binom{\pm}{x}$	–	$\frac{x}{-}$	–	–	–

x = Reactions with common species protein by Anti-P antibodies.
+ = Reactions with specific carbohydrate group by Anti-S antibodies.

anti-protein antibodies which are reactive only with the particular kind of protein present in the immunizing antigen. The following experiments deal with the occurrence, the species specificity and specific absorption of the anti-protein (Anti-P) antibodies.

1. Occurrence: In the conjugated antigens used for immunization

the total amount of protein present was estimated by determining the nitrogen content of the solution. However, this method of standardization is obviously inadequate, since it provides no measure of the relative amount of protein bound by the diazotized glucoside. As an excess of protein was always used for coupling with the carbohydrate, a greater or less amount of unbound protein remained free in solution depending upon the variable factors involved in the chemical reactions

TABLE VI

Precipitin Reactions of Galacto-Globulin Antiserum

Dilution of Antigen	Galacto-Globulin	Gluco-Globulin	Globulin	Gluco-Albumin	Galacto-Albumin	Albumin
1:5000	$\left(\frac{++++}{x}\right)$†	$\frac{x}{-}$	$\frac{x}{-}$	−	++++‡	−
1:10,000	$\left(\frac{++++}{x}\right)$	$\frac{x}{-}$	$\frac{x}{-}$	−	++++	−
1:20,000	$\left(\frac{+++}{x}\right)$	x	−	−	++±	−
1:40,000	$\left(\frac{++}{x}\right)$	$\frac{x}{-}$	−	−	++	−
1:80,000	$\left(\frac{+}{x}\right)$	−	−	−	+	−
1:100,000	$\left(\frac{x}{-}\right)$	−	−		−	

† = Bulky precipitate representing combined action of Anti-S and Anti-P.
‡ = Compact disk-like precipitate characteristic of Anti-S reactions.

associated with the process of diazotization. The occurrence of antiprotein antibodies in the serum of rabbits immunized with gluco-globulin and galacto-globulin is, therefore, presumably due to the presence in both antigens of an excess of common protein free in solution.

The differences observed in the titre of antiprotein precipitins in the two sera, (Tables V and VI) appear to be attributable to corresponding differences in the relative amount of free protein in each

antigen. This opinion is strengthened by the fact that the gluco-globulin antigen which yielded a high concentration of Anti-P precipitins was found on analysis to contain approximately 63 per cent more protein by weight than was present in the solution of galacto-globulin. This difference is reflected in the relative concentration of precipitins, since the serum prepared with the antigen having the greater amount of protein contains the higher concentration of anti-protein antibodies.

In this connection it is interesting to observe that, despite the differences in anti-protein response, both antigens gave rise to specific anticarbohydrate antibodies in approximately equal titre.

TABLE VII

Precipitin Reactions of Anti-horse Serum with Sugar-proteins containing horse serum Globulin

	Anti-horse Serum		
Dilution of Antigen	Gluco-Globulin	Galacto-Globulin	Original Globulin
1:1000	xx	xx	xxx
1:5000	xxxx	xxxx	xxxx
1:10,000	xxx	xxx	xxxx
1:20,000	xx	xx	xxx
1:40,000	x	x	xx
1:80,000	x	x	x

The antiprotein antibodies evoked by immunization with gluco-globulin and galacto-globulin, are presumably due to the presence in the antigens of free protein unbound by the diazotized glucosides. The supposition that, even in the absence of free protein, the sugar-protein compex alone may give rise to two qualitatively different antibodies, each specifically related to the corresponding constituent of the antigenic complex, affords an interesting alternative explanation of the occurrence of the two antibodies in the same immune serum. This concept involves the assumption that the binding of the diazo-glucoside to the protein does not entirely mask the groups essential to the specificity of the protein molecule, and that the carbohydrate radical through conjugation acquires specific antigenicity while the protein molecule retains in part its original antigenic properties.

2. *Specificity:* The antiprotein antibodies commonly found in these

immune sera exhibit only the species specificity of the particular kind of protein present. Since the same protein was used in preparing both test antigens, it is not surprising that a serum obtained with one shows cross precipitation with solutions of the other. The antiprotein precipitins in both sera react only in the presence of the homologous protein of the same species. They do not precipitate proteins unrelated to that present in the immunizing antigen. In this respect they are easily differentiated from the anticarbohydrate antibodies which, as shown in the present study, react with heterologous protein containing the same sugar radical.

TABLE VIII

Differentiation of Anti-S and Anti-P Antibodies by Specific Inhibition with Homologous Glucoside

Gluco-horse-Globulin Serum from rabbit*	Antigens							
	Gluco-Albumin (Egg)†				Globulin (Horse)‡			
	1:5000	1:10,000	1:20,000	1:40,000	1:5000	1:10,000	1:20,000	1:40,000
With the addition of Glucoside.....	−	−	−	−	xx	xx	xx	x
Without the addition of Glucoside.....	+++±	++++	+++	+±	xxx	xx	xx	x

* Precipitating serum prepared by immunization of rabbit with solution of glucoside coupled to globulin obtained from horse serum.

† Demonstrating anti-carbohydrate (anti-S) reactions.

‡ Demonstrating anti-protein (anti-P) reactions.

The species specificity of the antiprotein precipitins is demonstrated in Tables V and VI. The species relationship between homologous protein and its antibody is again illustrated in Table VII, in which it is evident that a precipitating serum prepared by immunization of rabbits with plain horse serum reacts with solutions of gluco- and galacto-globulin containing in common a native protein of the same animal origin. These protein-antiprotein reactions conform in all respects to the well known principles of species specificity. They are included here only to emphasize by contrast the preceding observations on the new specificity acquired by proteins of unrelated species when a simple sugar radical is attached to the protein molecule.

3. Specific Absorption: By fractional absorption with globulin alone, the antiprotein precipitins may be removed from an immune serum without appreciably diminishing the titre of the specific anti-carbohydrate antibodies. Thus, a distinct qualitative difference between these two antibodies had been demonstrated by specific absorption tests.

The specific inhibition of the anticarbohydrate antibody by the homologous glucoside has already been shown in Tables III and IV. This same technique has been employed in the differentiation of anti-S and anti-P. The anticarbohydrate antibodies contained in a serum prepared with gluco-globulin may be specifically inhibited by the addition of the homologous glucoside without impairing the titre of the antiprotein antibodies present in the same serum. This method is simpler than the absorption technique and is particularly applicable when dealing with antigens in the soluble form.

To summarize then, it may be stated that the occurrence of anti-protein precipitins in sera prepared with sugar-proteins appears to be related to the presence of free proteins in the solution. There is no evidence at present suggesting that this antibody arises as the result of dissociation of the sugar-protein complex either *in vitro* or *in vivo*. The antiprotein antibody exhibits the protein specificity of the species from which it is derived. It can be removed from immune sera by specific absorption, without loss in the titre of the coexisting anti-carbohydrate antibodies. Conversely, the anticarbohydrate anti-bodies can be specifically inhibited from reacting by the addition of the homologous glucoside without diminishing the activity of the antiprotein precipitins present in the same serum.

DISCUSSION

Obermeyer and Pick (4) studied the changes in specificity brought about by subjecting proteins to various chemical reactions, such as that brought about by iodine or nitric acid. They found that the chemical changes thus induced gave rise to new antigens with altered serological properties. Precipitins stimulated by the modified protein were specifically reactive with unrelated proteins similarly treated. The chemically altered proteins lost their species specificity but acquired a new specificity common to all proteins which had under-

gone the same chemical treatment as that to which the immunizing antigen had been subjected.

Landsteiner first prepared complex antigens, the chemo-specific groups of which consisted of known chemical substances attached to the protein molecule. The important and unexpected results of the investigations gave rise to the new concept that complex antigens may contain specifically reacting substances which by themselves are not antigenic but which acquire antigenicity when they are united to protein. Furthermore, Landsteiner showed that antibodies induced by these artificially prepared antigens react with the simple chemical compounds unattached to the antigenic protein. These specifically reactive but non-antigenic substances he called haptens,—substances which independently possess the specific antibody binding property, but lack the immunizing power of the whole antigen of which they form a part.

Complex antigens containing non-protein fractions are now known to occur in nature; for example, 1) the Forssman antigen of animal cells, the specificity of which is oriented by the presence of substances presumably lipoidal, and 2) the complex antigens of certain bacterial cells, in which the presence of specific polysaccharides determines antigenic specificity.

The significance of carbohydrates in the antigenic structure of bacteria lends added interest to the results of the present study in which it has been found that simple sugars have a determinative influence on the specificity of the proteins to which they have been chemically bound. As in the case of the type-specific antigen of Pneumococcus, the serological properties of these artificially prepared sugar-proteins appear to be determined by the chemical structure of the carbohydrate radical rather than by the character of the protein to which it is attached. So specific are these relationships that a serum prepared by immunization with gluco-globulin, the protein of which is derived from horse serum, reacts specifically with egg albumen to which the same sugar derivative has been coupled. Proteins of widely remote biological origin, therefore, when combined with the same carbohydrate radical have been shown to acquire in common a new and reciprocal specificity. Similarly, it has been found that when the same protein is combined separately with the corresponding

derivatives of two different sugars the resulting compounds are, in each instance, serologically distinct and specific.

In addition, the present work reveals the important fact that derivations of the two simple monosaccharides—glucose and galactose—which differ one from the other only in the spatial configuration of a single carbon atom, exhibit distinct immunological specificity when combined with the same or different proteins. Thus, for the first time, it has been shown by direct experimental evidence that asymmetry of the carbon atoms in the sugar radical alone suffices to determine differences in the specificity of sugar-protein antigens. Furthermore, these simple carbohydrate substances mask the species specificity of the protein molecule to which they are attached, and confer upon the combined antigen a new specificity which is dependent solely upon the chemical structure of the simple sugar radical.

The particular hexosides used in the present study were synthesized from glucose and galactose in form of the corresponding p-aminophenol β-glucoside and galactoside. These substances, when injected into the animal body, apparently do not stimulate the formation of demonstrable antibodies in the blood serum of the treated rabbits. At least it may be stated that attempts to induce antibody formation have failed despite the fact that both glucosides contain approximately five per cent of nitrogen. This lack of ability to stimulate the formation of antibodies is interesting in the light of Ford's original observation (5) that a nitrogenous glucoside isolated from a variety of poisonous mushroom produced antibodies which neutralized the hemolytic action of the glucoside. It may be mentioned in passing that the glucosides used in the present study are not hemolytic for rabbit blood cells. Moreover, both glucosides fail to cause the formation of a precipitate when added to an immune serum prepared with the homologous sugar-protein. The lack of specific precipitation in immune serum may be referable to the fact that the simple sugar derivatives are crystalloids and of relatively small molecular size when compared with the colloidal and highly complex sugars of Pneumococcus which react so readily in precipitin tests with specific antibacterial sera. However, despite the lack of specific precipitability, the homologous glucoside when added to the test mixture inhibits the precipitin reaction between the corresponding sugar-protein and its specific antibody.

The mechanism of the inhibition is not as yet clear. It is definite, however, that the inhibitory action of the sugar radical is specific. The precipitins for galacto-protein compounds, for example; are inhibited only in the presence of the homologous galactoside and are not affected by the addition of the heterologous glucoside (Tables III and IV). Moreover, the inhibition reaction, as Landsteiner has pointed out in the case of the azo-proteins, affords a specific method for the serological differentiation of simple chemical substances,—in this instance isomeric sugar derivatives—which by themselves are not antigenic and non-precipitable in immune sera. By reason of their serological specificity it appears justifiable to place these artificially prepared glucosides in the class of carbohydrate haptens, the most conspicuous examples of which are the specific polysaccharides naturally found in certain micro-organisms.

The results obtained with these synthetic sugar-proteins offer suggestive lines of approach to a further study of the chemo-immunological nature of those complex bacterial antigens in which carbohydrates are known to be the specific substances.

SUMMARY

1. When two chemically different carbohydrate derivatives are bound to the same protein, the newly formed antigens exhibit distinct immunological specificity.

2. When the same carbohydrate radical is conjugated with two chemically different and serologically distinct proteins both of the sugar-proteins thus formed acquire a common serological specificity.

3. The newly acquired specificity of the artificially prepared sugar-proteins is determined by the chemical constitution of the carbohydrate radical attached to the protein molecules. Simple differences in the molecular configuration of the two isomers,—glucose and galactose—suffice to orientate protein specificity when the corresponding glucosides of the two sugars are coupled to the same protein.

4. The unconjugated glucosides, although themselves not precipitable in immune serum, inhibit the reaction between the homologous sugar-protein and its specific antibody. The inhibition test is specific.

5. The sugar derivatives unattached to protein exhibit the properties of carbohydrate haptens; they are non-antigenic but specifically

reactive, as shown by inhibition tests, with antibodies induced by proteins containing the homologous diazotized glucoside.

6. The specificity of artificially prepared sugar-proteins is discussed with reference to the chemo-immunological nature of the bacterial antigens containing complex sugars.

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