FURTHER STUDIES ON THE CHEMICAL BASIS FOR SEROLOGICAL SPECIFICITY OF GROUP A STREPTOCOCCAL CARBOHYDRATE

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It was reported previously that the dominant factor in the serological specificity of the group-specific carbohydrate of Group A streptococci appears to depend upon side chains of *N*-acetyl-glucosamine (1). The evidence for this concept was obtained by the use of an induced enzyme, derived from a soil bacillus, which destroys the capacity of the carbohydrate to react with Group A antisera. It was shown that the enzyme acts by removing one-half to two-thirds of the total hexosamine of the carbohydrate in the form of free *N*-acetyl-glucosamine. The residual carbohydrate remains as a non-dialyzable unit after enzymatic treatment and assumes a new serological specificity which closely simulates that of certain variant strains of Group A streptococci and is dependent on a rhamnose-rhamnose linkage. Thus, removal of side chains of *N*-acetyl-glucosamine destroys the Group A specificity of the carbohydrate and simultaneously uncovers an antigenic determinant which is masked in the intact carbohydrate.

The present paper deals with additional evidence in support of the view that the dominant specificity of Group A carbohydrate is dependent upon *N*-acetyl-glucosamine side chains. The azo antigen technique has been applied to the problem in an attempt to obtain artificial antigens in which *N*-acetyl-glucosamine is the primary determinant of specificity. In addition, *N*-acetyl-glucosaminides have been employed in studies of specific inhibition of the precipitin reaction and in testing the specificity of the induced enzyme.

Materials and Methods

The procedures for preparing Group A carbohydrate, homologous rabbit antisera, and the enzyme which attacks the carbohydrate (A enzyme) are described in previous publications (1, 2). Quantitative precipitin determinations were again carried out by spectrophotometric analysis of washed precipitates after solution in 0.1 N NaOH.

N-acetyl-glucosaminides.—β-Pentacetyl-glucosamine obtained from free β-glucosamine by the method of Westphal and Holzmann (3) was used as starting material for preparation of β-glucosaminides. The procedure of Westphal and Schmidt (4) was employed in the synthesis of *p*-aminophenyl-β-*N*-acetyl-glucosaminide. The recrystallized product had an $[\alpha]_D^{25}$ of +12.0° in aqueous solution. Phenyl-β-*N*-acetyl-glucosaminide ($[\alpha]_D^{25} = -10°$) was prepared

by a modification of this method. A sample of phenyl-α-*N*-acetyl-glucosaminide ($[\alpha]_D^{23}$ = +208°) was generously supplied by Dr. Karl Meyer.

Azo Antigens.—The following proteins were coupled with *p*-aminophenyl-β-*N*-acetyl-glucosaminide as described by Westphal and Schmidt (4): crystalline egg albumin, crystalline bovine serum albumin, horse serum, and chicken serum. In some instances the alkalinity of the reaction system was controlled so that the pH did not exceed 9.0, but the antigens so prepared did not show significantly greater reactivity or antigenicity. Chicken serum azo antigens with certain other mono- and disaccharides were supplied by Dr. W. F. Goebel.

Various methods were employed in an attempt to prepare satisfactory rabbit antisera to the azo antigens. The best results were obtained by repeated intravenous injection of 0.5 to 1.0 ml. of 1 per cent solution by the same schedule used in preparing streptococcal antisera (2).

Chemical Analyses.—Glucosamine was determined by the modified procedure described by Rondle and Morgan (5), and *N*-acetyl-glucosamine by the method of Aminoff, Morgan, and Watkins (6).

EXPERIMENTAL

Additional Evidence on the Identity of the N-acetyl-glucosamine Side Chains.—A 3-substituted hexosamine, first described by Strange and Dark (7), has been shown to occur in the cell walls of a variety of Gram-positive microorganisms, including Groups A to G streptococci (8). This substance, currently designated as muramic acid, was not detected in our original studies on the composition of Group A streptococcal carbohydrate. However, reexamination of hydrolysates of the carbohydrate by the two dimensional paper chromatographic system employed by Cummins and Harris (8) revealed the presence of the second hexosamine component in addition to glucosamine. This finding raises the question of whether muramic acid might be included among the *N*-acetyl-hexosamine side chains and thus play a role in the serological activity of the carbohydrate. Further experiments were carried out to test this possibility.

N-acetyl-hexosamine removed enzymatically from Group A carbohydrate by the induced enzyme was concentrated and compared with known *N*-acetyl-glucosamine. The two substances showed identical mobilities on two dimensional paper chromatography, and no separation occurred when the two were mixed and applied in a single spot. The color developed by the enzymatic split product in the *N*-acetyl-hexosamine test was identical quantitatively and in its adsorption spectrum with that of *N*-acetyl-glucosamine. Similarly, after mild hydrolysis to remove the *N*-acetyl group, the behavior of the material was identical with authentic glucosamine in the Elson-Morgan reaction. In addition, it was shown that enzymatic treatment of the carbohydrate to remove the *N*-acetyl-hexosamine side chains caused no diminution in the intensity of the muramic acid spot on paper chromatography of hydrolysates of the residual carbohydrate. These findings taken together are consistent with the original view that the antigenic determinant of Group A carbohydrate is *N*-acetyl-glucosamine and indicate that muramic acid is not involved in its serological specificity.

Reactivity of Group A Streptococcal Antisera with Azophenyl-N-acetyl-glucosaminide Antigens.—The fact that *N*-acetyl-glucosamine residues occurring as side chains appeared to represent the major antigenic determinant of Group A carbohydrate suggested that the azo antigen technique might be usefully applied to obtain further confirmation of this concept. Thus, the coupling of *p*-aminophenyl-β-*N*-acetyl-glucosaminide to proteins through the diazo linkage should result in the formation of antigens in which the antigenic determinants

TABLE I

Precipitin Reactions of Streptococcal Grouping Sera with Azophenyl-N-acetyl-glucosaminide Antigens

Serum		Coupled antigens									
Group	No.	Crystalline egg albumin, *mg./ml.*					Crystalline bovine serum albumin, *mg./ml.*				
		5.0	1.0	0.2	0.04	0.008	5.0	1.0	0.2	0.04	0.008
A	R13	+	++	+	±	−	±	++	+	±	−
	R458	+++	++++	++±	+	+	++	++±	+±	+	+
	R48	+++	+++	++±	+	±	++±	++±	++	+	±
	Pool 6	+	+±	+	±	tr	±	+±	+	±	tr
A var.	R496	−	−	−	−	−	−	−	−	−	−
	R156	−	−	−	−	−	−	−	−	−	−
B	R99	−	−	−	−	−	−	−	−	−	−
C	R275	−	−	−	−	−	−	−	−	−	−
D	R236	−	−	−	−	−	−	−	−	−	−
E	R93	−	−	−	−	−	−	−	−	−	−
F	R281	−	−	−	−	−	−	−	−	−	−
G	R285	−	−	−	−	−	−	−	−	−	−
H	R249	−	−	−	−	−	−	−	−	−	−
L	R468	−	−	−	−	−	−	−	−	−	−
M	R377	−	−	−	−	−	−	−	−	−	−

Amount of precipitate indicated by tr(ace) to ++++.
The absence of visible precipitate indicated by −.

bear at least a superficial resemblance to those of the carbohydrate. This prediction was experimentally confirmed by the finding that antigens prepared by coupling this glucosaminide with a variety of proteins all gave strong reactions in precipitin tests with Group A antisera. Qualitative precipitin reactions with two of these azo antigens are presented in Table I. The four Group A rabbit antisera illustrated are representative of a large group which were tested, all of which reacted to some degree with the azo antigens. In contrast to these results, antisera to variant strains of Group A streptococci which lack the *N*-acetyl-glucosamine determinant and antisera to other groups of streptococci show no reaction. In no case did the carrier proteins

alone form precipitates with Group A antisera. It is apparent, therefore, that the synthetic antigens react as predicted with Group A antisera and appear to be specific for this group of streptococcus.

The *p*-aminophenyl glycosides of closely related substances, such as unacetylated glucosamine and *N*-acetyl-galactosamine, were not available for closer examination of the stereospecificity of this cross-reaction with Group A antisera. However, the possibility of non-specific reactivity with this general type of antigen was eliminated by tests with azo antigens prepared by coupling *p*-aminophenyl-glycosides of several mono- and disaccharides with chicken serum. Ring tests were used to increase the sensitivity of the reaction. The

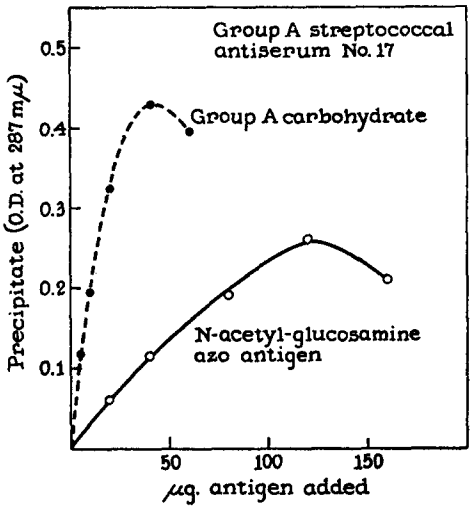

FIG. 1. Quantitative precipitin curves using the homologous carbohydrate and egg albumin-azophenyl-β-*N*-acetyl-glucosaminide with Group A antiserum. Reaction system: 0.2 ml. antiserum in final volume of 1 ml.

results recorded in Table II demonstrate that none of these additional antigens gave detectable precipitates with the Group A antiserum used, and that chicken serum provides a satisfactory antigen when coupled with the *N*-acetyl-glucosaminide.

Quantitative precipitin tests were carried out to determine the extent of cross-reactivity between the azo antigens and Group A antisera. Typical precipitin curves in which Group A carbohydrate and *p*-aminophenyl-*N*-acetyl-β-glucosaminide coupled to crystalline egg albumin were used as precipitating antigens are illustrated in Fig. 1. It is evident that maximum precipitation with azo antigen required larger quantities than with the homologous carbohydrate. However, the curve shows a relatively sharp maximum with definite inhibition in the range of excess antigen. The data presented in

Fig. 1 exaggerate the degree of cross-reactivity, since the azo antigen contributes to the spectrophotometric reading of the redissolved precipitate at 287 mμ while the Group A carbohydrate has no adsorption at this wave length. Thus, the optical density readings of the precipitate obtained with azo antigen represent antibody plus antigen. Attempts were made to apply corrections both on the basis of the amount of antigen added and by quantitating the red color of the redissolved precipitates, but the results of absorption experiments indicated that these calculations also resulted in high estimates of cross-reactivity. The most reliable information was obtained by complete absorption of antisera with the azo antigen and determination of the amount

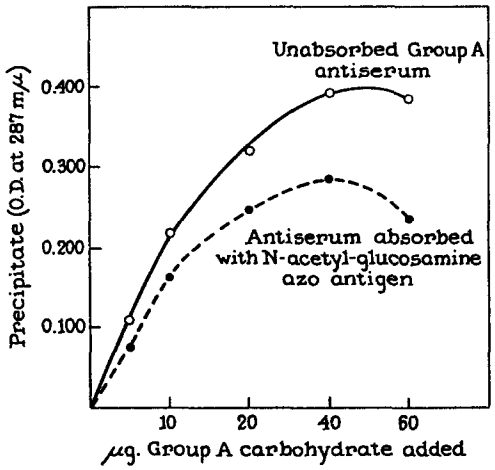

FIG. 2. Absorption of Group A antiserum with egg albumin–azophenyl-β-*N*-acetyl-glucosaminide. The difference between the two curves represents the amount of Group A antibody absorbed by the synthetic antigen.

of residual antibody precipitable by Group A carbohydrate. Data from this type of experiment with one antiserum are presented in Fig. 2. The results indicate that about one-quarter of the homologous Group A antibody was removed by absorption with the azo antigen. The amount of antibody removed in similar absorption experiments with various specimens of antisera ranged from 15 to 30 per cent of the total. It is clear, therefore, that only part of Group A antibody is capable of reacting with the synthetic model antigen.

Lack of Reverse Cross-Reaction between Group A Carbohydrate and Antisera to Azo Antigens.—Some difficulty was encountered in obtaining strong precipitating rabbit antisera to the azophenyl-*N*-acetyl-glucosaminide antigens. However, reasonably satisfactory antisera were obtained with antigens prepared by coupling the glucosaminide to crystalline bovine serum albumin and to horse serum. These antisera reacted well in precipitin tests with *N*-acetyl-

glucosaminide azo antigens prepared with heterologous proteins, and like the Group A antisera they did not react with the other glycoside azo antigens represented in Table II. On the other hand, despite numerous attempts it was not possible to demonstrate any precipitation when Group A carbohydrate was used as antigen. Even the use of larger volumes of serum and prolonged

TABLE II

Reactivity of Azophenyl Glycoside—Chicken Serum Antigens with Group A Antiserum

Glycoside determinant of azo antigen	Approximate concentration of antigen, *mg./ml.*		
	1.0	0.3	0.1
β-Glucose	−	−	−
Galactose	−	−	−
Maltose	−	−	−
Gentiobiose	−	−	−
Lactose	−	−	−
N-acetyl-glucosamine	+++	++	+
None (uncoupled chicken serum)	−	−	−

TABLE III

Failure of Group A Carbohydrate to Inhibit Reaction of Azophenyl-N-acetyl-glucosaminide Antigen with Antiserum

Amount antigen added	Antigen-antibody precipitate (O.D. at 287 mμ) in presence of Group A carbohydrate at final concentration:		
	5 mg./ml.	2.5 mg./ml.	0
μg.			
10	0.091	0.113	0.107
25	0.236	0.250	0.260
50	0.353	0.358	0.366
100	0.414	0.412	0.402

Antiserum prepared by immunizing with crystalline bovine serum albumin–azophenyl-*N*-acetyl-glucosaminide.

Test antigen–crystalline egg albumin–azophenyl-*N*-acetyl-glucosaminide.

Test system–0.2 ml. antiserum. Final volume 1.0 ml.

refrigeration with a wide range of concentrations of the carbohydrate did not result in the appearance of detectable precipitate. Furthermore, the presence of excess carbohydrate failed to inhibit the formation of precipitate by these antisera on the addition of test azo antigen. This is illustrated by an experiment in which quantitative precipitin reactions were carried out with rabbit antiserum to the bovine serum albumin azo antigen using an egg albumin azo antigen as the test substance (Table III). It is apparent that Group A carbohydrate in concentrations up to 5 mg./ml. does not significantly depress

the amount of precipitate formed. These findings indicate not only that the carbohydrate is unable to precipitate with antibody to the azo antigen but that it does not even combine with the specific sites to form soluble complexes.

It must be concluded that the cross-reaction between Group A antisera and the azophenyl-*N*-acetyl-glucosaminide antigens is non-reciprocal. The implications of this fact with respect to the chemical configuration of the antigenic sites are not clear on the basis of present knowledge. The precise nature of the linkage of *N*-acetyl-glucosamine to the skeleton of the Group A carbohydrate is unknown, although it is obviously totally unlike that present

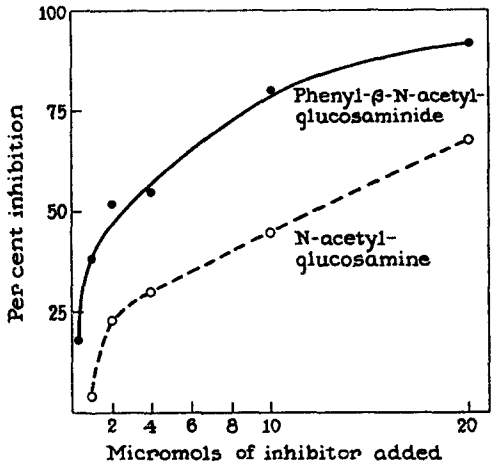

FIG. 3. Inhibition of the precipitin reaction between Group A carbohydrate and homologous rabbit antiserum by *N*-acetyl-glucosamine and phenyl-β-*N*-acetyl-glucosaminide. Reaction system: 0.2 ml. antiserum in final volume of 1 ml.

in the synthetic glucosaminide antigens. This structural difference, together with probable differences in the spacing and periodicity of the *N*-acetyl-glucosaminide side chains, may account for the lack of reciprocal cross-reactivity.

Precipitin Inhibition by Glucosaminides.—It was shown previously that *N*-acetyl-glucosamine inhibits the reaction between Group A carbohydrate and its homologous antibody (1). In the present studies glucosaminides, such as phenyl-β-*N*-acetyl-glucosaminide, proved to be much more effective inhibitors on a weight basis. This is illustrated in Fig. 3 in which the inhibitory potency of *N*-acetyl-glucosamine and phenyl-β-*N*-acetyl-glucosaminide in the Group A system are compared. The glucosaminide at 2 μmols per ml. gives greater reduction in the specific precipitate formed than *N*-acetyl-glucosamine at 10 μmols per ml. The effect of glucosaminides on each of the several antigen-antibody systems described above is summarized in Table

IV. In each case, the precipitin reaction was carried out at or near the equivalence point; and the final concentration of inhibitor was 4 μmols per ml.

It is apparent from Table IV that the phenyl-β-*N*-acetyl-glucosaminide at this concentration again shows about a 50 per cent reduction in the precipitate formed by the Group A carbohydrate and its antibody. The reaction of the egg albumin azo antigen both with Group A antiserum and with antiserum to the bovine albumin azo antigen is much more sensitive to the phenyl-β-*N*-acetyl-glucosaminide, and in each case inhibition is almost complete. The cross-reaction (Group A antiserum and egg albumin–azo antigen) is more

TABLE IV

Specific Inhibition of Precipitin Reactions by Phenyl-N-acetyl-glucosaminides

Antiserum prepared by immunization with	Test antigen	Inhibitor	Ag-Ab precipitate per ml. serum	Inhibition
			μg. N	*per cent*
Group A streptococci	Group A carbohydrate	α-phenyl-N-Ac-G	210	23
		β-phenyl-N-Ac-G	140	48
		None	270	—
Group A streptococci	Egg-albumin-azo-phenyl-N-Ac-G	α-phenyl-N-Ac-G	11	96
		β-phenyl-N-Ac-G	0	100
		None	262	—
Bovine albumin-azo-phenyl N-Ac-G	Egg-albumin-azo-phenyl-N-Ac-G	α-phenyl-N-Ac-G	250	37
		β-phenyl-N-Ac-G	2	99
		None	398	—
Group A variant streptococci	Variant carbohydrate	β-phenyl-N-Ac-G	750	—
		None	730	—

Reactions carried out near equivalence point in each system. 4 micromols inhibitor (1.2 mg./ml.) added. Final volume 1 ml.

profoundly affected than either of the homologous reactions, and definite inhibition can be detected at a concentration of 0.2 μmols per ml. of the β-phenyl compound. Unrelated antigen-antibody systems, exemplified by the Group A variant carbohydrate and its antibody, are unaffected by the glucosaminide.

Data indicating that phenyl-α-*N*-acetyl-glucosaminide also acts as inhibitor are included in Table IV. In each case, the α-compound is less inhibitory than the β-compound at equivalent concentration. Again the cross-reacting system appears to have the highest sensitivity. Since the azo antigens were prepared with *p*-aminophenyl-β-*N*-acetyl-glucosaminide, the superiority of the β- over the α-phenyl compound is to be expected in those

systems in which the azo antigens are used. The fact that the β-phenyl compound is also more effective as an inhibitor of the reaction between Group A carbohydrate and its antibody suggests that the *N*-acetyl-glucosamine side chains are attached to the remainder of the carbohydrate through β-glucosaminide linkages. This is more clearly indicated by studies, to be described below, on the specificity of the enzyme which removes the side chains from the intact carbohydrate molecule.

Specificity of the Enzyme Which Attacks Group A Carbohydrate.—In the original studies it was found that production of A enzyme by the soil bacillus is induced by the presence of Group A carbohydrate and that only traces of enzyme can be detected in the absence of substrate (1). After the nature of the action of the enzyme on the carbohydrate was determined, it was observed that *N*-acetyl-glucosamine can also serve as inducing agent although the yield of enzyme is consistently lower than with the carbohydrate. When the β-glucosaminides used in the present study became available, each of them (phenyl-, *p*-nitrophenyl-, and *p*-aminophenyl-) proved to be active in inducing formation of the enzyme. In fact, phenyl-β-*N*-acetyl-glucosaminide is now used as the inducing agent of choice in preparing the enzyme, since the potency of the product is at least as great as that prepared with carbohydrate and there is no problem of removing residual carbohydrate from the material. The enzyme induced by phenyl-β-*N*-acetyl-glucosaminide appears to be identical with that induced by carbohydrate in that it removes *N*-acetyl-glucosamine side chains from Group A carbohydrate with concurrent loss of serological activity. In contrast to these findings, the phenyl-α-*N*-acetyl-glucosaminide does not cause the production of detectable enzyme by the soil organism, and significant hydrolysis of the compound into phenol and *N*-acetyl-glucosamine does not occur during prolonged incubation with the bacteria. The reaction thus appears to be specific for the β-anomer.

Further information on specificity was obtained by experiments on the action of enzyme concentrates on synthetic substrates. Carbohydrate-induced enzyme which had been concentrated by ammonium sulfate and dialyzed was used for this purpose and tested in comparison with emulsin known to contain β-glucosaminidase. The reaction was carried out with the phenyl-*N*-acetyl-glucosaminides in 0.2 M acetate buffer pH 6, and hydrolysis detected by determination of either the phenol or *N*-acetyl-glucosamine released. The type of results obtained are recorded in Table V. Both emulsin and the A enzyme split the β-compound extensively but are without effect on the α-compound, confirming the view that the A enzyme is a β-glucosaminidase. However, it differs from emulsin in its ability to act on the side chains of intact Group A carbohydrate. Even on prolonged incubation of emulsin with Group A carbohydrate there is no detectable release of *N*-acetyl-glucosamine and no loss of serological activity.

Action of Enzymes on Synthetic Azo Antigens.—In view of the marked difference in the action of the β-glucosaminidases of emulsin and of the soil bacillus on Group A carbohydrate, it was of interest to test these enzymes for their effect on the synthetic azo antigens. The following type of experiment was used for this purpose.

TABLE V

Specificity of Induced Enzyme for β-N-acetyl-glucosaminide

Substrate	Enzyme	Total *N*-acetyl-glucosamine released	Fraction hydrolyzed
		μg.	*per cent*
Phenyl-α-*N*-acetyl-glucosaminide	Emulsin	0	0
"	A enzyme	0	0
"	None	0	0
Phenyl-β-*N*-acetyl-glucosaminide	Emulsin	320	85
"	A enzyme	387	103
"	None	0	0

System: 0.5 mg. substrate in final volume of 1.0 ml. in 0.2 M acetate buffer, pH 6. Emulsin (General Biochemicals, Inc.)—1 mg./ml. Incubation 6 hrs./37°C.

TABLE VI

Effect of Enzymes on Azophenyl-β-N-acetyl-glucosaminide Antigen

Enzyme	Total N-acetyl-glucosamine released	Quantitative precipitin determination	
		Group A antiserum	Antiserum to azo-antigen
	μg.	*O.D. at 287 mμ.*	*O.D. at 287 mμ*
A enzyme	98	0.363	0.355
Emulsin	46	0.436	0.335
None	3	0.512	0.364

Substrate: horseserum–azophenyl-β-*N*-acetyl-glucosaminide.

Precipitin figures: maximum values obtained in titration of enzyme-treated antigens. Antiserum to azoantigen prepared with bovine albumin–azophenyl-β-*N*-acetyl-glucosaminide.

Horse serum–azophenyl-β-*N*-acetyl-glucosaminide at a final concentration of approximately 2 mg./ml. was mixed with each of the enzymes in small dialysis sacs. The reaction mixture at a volume of 5 ml. was dialyzed at 25°C. against 5 ml. 0.85 per cent NaCl for 48 hours. Under these conditions, the action of the A enzyme on Group A carbohydrate is complete within a few hours. The total amount of *N*-acetyl-glucosamine released was estimated on the basis of analysis of the dialysate. The treated antigens were tested at various concentrations in quantitative precipitin reactions with Group A antiserum and antiserum to bovine albumin-azo antigen. The results are recorded in Table VI.

The same type of result was obtained in similar experiments in which antigens with other protein components were used as substrate. It is apparent from Table VI that both enzymes release some *N*-acetyl-glucosamine from the antigen, but that the A enzyme removes about twice as much as emulsin. This difference between the two enzymes was consistently observed. If one assumes complete coupling of the *p*-aminophenyl-β-*N*-acetyl-glucosamine with protein, the amount of substrate used in these experiments would represent more than 1 mg. of available *N*-acetyl-glucosamine. Consequently, it would appear that the enzymes are capable of hydrolyzing only a small portion of the glucosaminide linkages in the azo antigen. This conclusion is supported by the results of the precipitin analysis of treated antigen (Table VI). The A enzyme and emulsin result in slight reduction in precipitate formation (30 and 15 per cent respectively) in the cross-reaction with Group A antiserum, but cause no significant change in reactivity of the antigen with antiserum to bovine albumin—azophenyl-β-*N*-acetyl-glucosaminide.

Thus, although the enzymes are capable of hydrolyzing the low molecular β-*N*-acetyl-glucosaminides, their action is to a large degree blocked when the glucosaminide is coupled to protein. This behavior of the azo antigens is in contrast to the apparent accessibility of Group A carbohydrate to the A enzyme, and provides further evidence for the dissimilarity of the model antigens to the carbohydrate. This is consistent with the finding that the cross-reaction between carbohydrate and azo antigen is not reciprocal.

DISCUSSION

The azo antigen technique of Landsteiner has proved useful in confirming the evidence previously obtained that *N*-acetyl-glucosamine represents the primary antigenic determinant of Group A streptococcal carbohydrate. From the inhibition tests and enzymatic studies it seems clear that this determinant is attached to the remainder of the carbohydrate molecule through β-glucosaminidic linkages. Although it seems most likely that this linkage is through one of the hydroxyls of rhamnose, this relationship has not yet been established by the isolation and identification of oligosaccharide split products containing the two monosaccharides.

The possibility that certain of mammalian polysaccharides which contain *N*-acetyl-glucosamine may, under appropriate conditions, cross-react with antibody to the Group A carbohydrate has still not been thoroughly explored. It is of interest that Westphal and Schmidt (4) originally undertook the synthesis of *p*-aminophenyl-β-*N*-acetyl-glucosaminide to evaluate the role of *N*-acetyl-glucosamine as a determinant group in certain polysaccharide antigens, particularly the blood group substances. They showed that although native blood Group A substance isolated from hog gastric mucin did not appear

to react with antisera to the azo antigen, A substance treated with papain and cold acid strongly inhibited the reaction between the azo antigen and rabbit antibody. The finding that *N*-acetyl-glucosamine does not act as an antigenic determinant in untreated blood group substance is in accord with the detailed studies from the laboratories of Morgan and of Kabat on the specificity of these substances. The papain and acid treatment, which apparently produces endgroup *N*-acetyl-glucosaminides in the molecule, alters but does not completely destroy the blood group activity of the substance. Because of the widespread occurrence of *N*-acetyl-glucosamine in mammalian polysaccharides and mucoproteins a more extensive search is indicated for substances in which it may serve as an antigenic determinant.

SUMMARY

Azoproteins prepared with *p*-aminophenyl-β-*N*-acetyl-glucosaminide react in precipitin tests with Group A streptococcal antisera. The reaction is nonreciprocal, and antisera to the azoprotein do not react with Group A carbohydrate.

Phenyl-*N*-acetyl-glucosaminides inhibit the reaction of Group A carbohydrate with homologous antisera and with antisera to the azoprotein. The β-anomer is more effective as an inhibitor than the α-anomer.

Formation by a soil bacillus of the enzyme which removes *N*-acetyl-glucosamine from Group A carbohydrate is induced by phenyl-β-*N*-acetyl-glucosaminide but not by the α-compound. The enzyme, like the glucosaminidase of emulsin, appears to be specific for β-glucosaminides.

Neither the induced enzyme nor emulsin effectively remove all of the *N*-acetyl-glucosamine from the azoprotein antigens.

The findings support the view that β-*N*-acetyl-glucosaminide side chains represent the major antigenic determinant of Group A streptococcal carbohydrate.

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