GLYCOGEN, AN IMMUNOLOGICALLY SPECIFIC POLYSACCHARIDE*

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(Received for publication, December 26, 1953)

It was shown recently (1) that certain synthetic polyglucoses (2) yielded precipitates with the antibodies in a number of antipneumococcal horse sera, notably those of Types II, VII, IX, XII, XVIII, XX, and XXII. Since the constituent sugars and the structure of the type-specific polysaccharide of the first of these pneumococcal types are known, a partial interpretation of the results was attempted in terms of Stacey's finding that the glucose in this specific polysaccharide forms branching points with linkages in the 1,4 and 6 positions (3). Such an interpretation appeared tentatively justified because the 1,4 and 1,6 linkages are those of most frequent occurrence in the synthetic polyglucoses (2). It was further inferred that glycogens and amylopectins, which both contain branching points of glucose with α-linkages in the 1,4 and 6 positions, should also react with the same series of antisera. A description is given in the present communication of experiments designed to test this inference. Preliminary qualitative tests at 0–5° are shown in Table I. The remainder of the paper is taken up mainly with quantitative data obtained with various glycogens derived from maize, the oyster, and from animal organs. Quantitative experiments with amylopectins showed irregularities which have not yet been traced to their source, so that a report on these substances will be made later.

EXPERIMENTAL

Specific Polysaccharides, Antisera, and Estimation of Antibody Nitrogen.—Pneumococcal specific polysaccharides were either prepared as described in reference 4 or were obtained through the courtesy of E. R. Squibb and Sons, New Brunswick, New Jersey.

The antisera used were generously supplied by the Bureau of Laboratories, New York City Department of Health, and by the Division of Laboratories and Research, Department of Health, State of New York.

*This work was carried out under the Harkness Research Fund of the Presbyterian Hospital, New York, and a grant from The Rockefeller Foundation.

TABLE I

Qualitative Tests with Glycogens and Antipneumococcal Horse Sera

Serum type	II	III C*	VII	IX	XI	XII	XVIII	XX	XXII	XXVII	XXVIII	Normal horse sera			
												a	*b*	*c*	*d*
Source of glycogen															
Cat liver	++		+±	+++	±	+++	−	++±	+++			−			
Dog liver	+±		−	+++±	+	+++		++±	+++±	−		+	++	++	+
Human, v.G.1 (9)	+±		+±	+++	±	++±	±	++±	+++±	−	±	−	++	++±	+
Human, v.G.10 (9)	++		++	++	+±	+++	−	++±	+++			+			
Rabbit liver	++		+	++±		+++		++±	+++						
Horse muscle	+	−	−	+++	±	+++	−	++±	+++				++		+±
Horse liver acid-prepared	++±	−	+	+++	+±	+++±	+	+++	+++						++
Horse liver alkali-prepared	+++	−	++	+++	++	+++±	++	+++	+++				+++		++±
Rat liver	+±*	−		++±		+++		++±	+++						
Ascaris	++*		+±	++	+±	++±	±	++	++			±			
Oyster	++±*			++±		++±		++±	+++	−	+		++	++±	+
Maize	++		++	++±		++±	+	++	+++						
Phosphorylase limit dextrin	++		+±	+++	+	+++	±	+++	++±			±			
β-amylase limit dextrin	++±		+±	++±	±	+++±	±	+++	++±			−			
Maize amylopectins															
Alkali-prepared	+*		+	+±	±	+, +++‡	±	+±	++±			+			
Acid-prepared	±*		+	+	+	±, ±‡	±	+	+			+			
β-amylase limit dextrin	+		±			++±, ++‡	−	++	++						

"Normal" horse serum *b* gave ++ reactions with the specific polysaccharides of Types II and XII pneumococcus; serum *c* reacted only with the latter. A bleeding of serum *b*, taken about 3 months later after injections of tetanus toxoid, showed weaker reactions with Types II and XII polysaccharides and with horse liver glycogen.

* Serum absorbed with pneumococcal C substance, the somatic, group specific polysaccharide.

‡ Two different antisera.

Determinations of antibody nitrogen were carried out in duplicate in 8 ml. centrifuge tubes, usually at or near 0° according to the method in reference 5, except that the washed specific precipitates were analyzed according to the Markham method (6). Except for reactions with the homologous pneumococcal polysaccharides, the mixtures were allowed to stand in the refrigerator for 4 to 10 days, with occasional twirling, before analysis. In many instances in sera other than Type IX the supernatants deposited additional small amounts of precipitate on longer standing and these are included in most of the analyses.

As in many cross-reactions precipitation of the antisera by glycogen has a large temperature coefficient. It became apparent toward the end of the present studies that this effect was unusually large in the glycogen reaction (Fig. 1) so that closer control of temperature would seem desirable and will be undertaken in extensions of the work now under way.

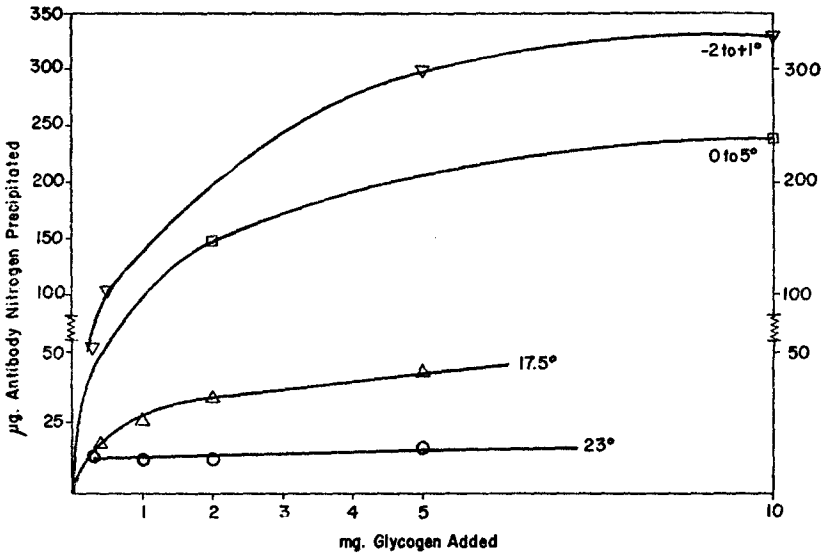

FIG. 1. Precipitation of antipneumococcus Type XII horse serum by glycogen at various temperatures.

Precipitation of Apparently Normal Sera by Glycogen.—As noted in Table I four presumably normal horse sera showed negative to marked precipitation with different samples of glycogen and amylopectin. Quantitative analyses with serum *a* gave 1 to 5 μg. of precipitate nitrogen per ml.; sera *b* and *c* gave maxima of 10 and 15 μg. per ml., respectively.

In the meantime Dr. Pierre Grabar of the Pasteur Institute, Paris, has observed interfacial precipitation with glycogen in many supposedly normal sera. Chicken sera reacted strongly, horse sera definitely, human sera occasionally, and rabbit sera weakly or not at all. Quantitative estimations on the three most reactive horse sera showed 4.5, 6.5, and 20 μg. of precipitate nitrogen per ml. Thanks are due Dr. Grabar for permission to mention these results. Two of the three sera, therefore, showed roughly the same precipitation as did serum *a* in the present studies; the third serum was about as reactive with glycogen as serum *c* and precipitated as much nitrogen with it as did a potent antipneumococcus Type II serum (Table II). Since the broad polyglucose specificity (1) is by no means confined to the pneumococci (7) it would appear possible that the horses in question had been exposed to microorganisms showing this specificity and had produced antibodies to them.

TABLE II

Antibody Nitrogen Precipitated by Fractions of Oyster Glycogen from 1.0 Ml. Portions of Antipneumococcal Horse Sera

Fraction		Antiserum, type				
		II	IX	XII	XX	XXII, No. 243
	mg.	*μg.*	*μg.*	*μg.*	*μg.*	*μg.*
A	0.2	17	21	28	25	36
	0.5	19	62	68	50	83
	1.0	18	114	122	65	119
	2.0		156	180	57*	137
	5		204	250		
	6					140‡
	8		234			
	9			290		
	12		236			
	15			246		
B	0.2			65	42§	57
	0.5			137	51§	94
	1.0		32	178	52§	113
	2		36			
	2.4			230		
	4		56			
	7.2			236		
D	0.2		2	97	22	20
	0.5		2	162	24	31
A_1	0.2					30
	0.3	17‖	32			
	0.5					58
	1	21‖	107			104
	2		177	118		130
	5		259			142
	10		271	141		
A_2	0.3	19‖	36			
	1.0	20‖	120		45	
	2		164	148	48¶	121
	5		214			128
	10		232	239		

With Type VII and Type XVIII antisera, fraction A_2 precipitated 7 and 18 μg. of nitrogen, respectively.

* The supernatant gave an additional 33 μg. N with synthetic polyglucose fraction B(1). This precipitated 107 μg. N from the unabsorbed serum.

‡ The supernatant yielded 24 μg. of precipitate N with polyglucose B, which precipitated 164 μg. N from the unabsorbed serum.

§ Analyses after 5 days in the cold. Small amounts of precipitate formed in the supernatants on longer standing but are not included in these instances.

‖ 2.0 ml. of C-absorbed serum actually used; calculated to 1 ml.

¶ One determination only.

TABLE II—*Concluded*

Fraction	Antiserum, type				
	II	IX	XII	XX	XXII, No. 242
mg.	*μg.*	*μg.*	*μg.*	*μg.*	*μg.*
A_3 0.3	18‖	13			
1	18‖	51			
2		74			
5		52			
Homologous pneumococcal specific polysaccharide	1061	1655	1820	468	847
Maximum per cent antibody precipitated by glycogen	2	16	16	14	17

It is also to be noted from Table I that four of the antipneumococcus horse sera gave tests as weak as those of the least reactive of the so called normal sera. Precipitation of the more reactive normal sera is being studied in greater detail.

Glycogens Used.—Many of the samples of liver glycogens and limit dextrins were generously supplied by Dr. Gerty T. Cori, to whom the authors are most grateful. Rat liver glycogen was kindly furnished by Dr. Z. Dische. *Ascaris* glycogen was prepared by Dr. Dan H. Campbell and obtained from Dr. Elvin A. Kabat. The glycogens of dog and rabbit livers were prepared according to the method outlined in reference 8. The samples of human liver glycogen from cases of von Gierke's disease are described in reference 9, and the glycogens from cat and rabbit livers and from horse muscle in reference 10. Maize glycogen, sample *a*, was prepared according to the description in reference 11, while sample *b* was furnished by Dr. T. J. Schoch. Oyster glycogen was purchased from the Nutritional Biochemicals Corporation. Both limit dextrins in the glycogen series were prepared from rabbit liver glycogen. The suggestion to use glycogen from the horse in further tests of the antipneumococcal horse sera was made by Dr. N. W. Pirie at the International Microbiological Congress in Rome. The glycogens from horse liver were kindly supplied by Dr. Pierre Grabar and Miss Janine Courçon.

Fractionation of Glycogen.—3 gm. of air-dry oyster glycogen was dissolved in 60 ml. of 5 per cent sodium acetate solution. The mixture was chilled, centrifuged to remove traces of insoluble material, and treated with chilled isopropyl alcohol (4) in an ice bath until a definite precipitate (fraction A) formed. This occurred with 21.5 ml. of the alcohol. The mixture was allowed to stand in ice water overnight and was centrifuged at 0°. The supernatant solution gave a further precipitate with 10 ml. of isopropyl alcohol (fraction B) and the supernatant from this yielded another small fraction (D) with excess ethyl alcohol. Each fraction was dissolved in a little water and reprecipitated with 10 to 20 volumes of redistilled ethyl alcohol. The yields were: A, 2.42 gm.; B, 0.03 gm.; D, 0.01 gm.

Because fractions B and D possibly represented smaller molecules and showed differences of reactivity (Table II) from fraction A this was further fractionated as follows: 1.85 gm. were dissolved in 100 ml. of 5 per cent sodium acetate solution and mixed in the cold with 25 ml. of isopropyl alcohol. The precipitate (fraction A_1) was centrifuged off in the cold, dissolved in 5 ml. of water, and poured into 50 ml. of redistilled ethyl alcohol, filtered off, washed with redistilled alcohol and acetone, and dried; yield, 0.26 gm. Another fraction, A_2, was obtained from the supernatant from fraction A_1 by addition of 8 ml. of isopropyl alcohol in the cold, and

a fraction, A_3, by further addition of ethyl alcohol to a final volume of 200 ml. Each fraction was isolated from concentrated aqueous solution as above. Yields: A_2, 1.27 gm.; A_3, 0.03 gm. Data obtained with the six fractions are given in Table II. Differences between A_1 and A_2 were small; A_3 appeared to be of lower molecular weight, since it was less reactive.

TABLE III

Quantitative Analyses of Specific Precipitation of Various Antisera by Glycogens from Different Sources

Source of glycogen	Antiserum, type			
	IX	XII	XX	XXII, No. 243
	Amount N precipitated per ml.			
	μg.	μg.	μg.	μg.
Maize *a*, 0.3 mg.		82	35	54
1 "		184*	59	101
2 "	116‡	144	50‡	106
3 to 4 mg.	14(?)	165§		
Rabbit liver, 0.5 mg.		71		58
1.2 "	28			
2.0 "	<121‖	107		
6 "	26	129		
v.G.1, human liver, 0.5 mg.		74		
2.0 "		125¶		
Cat liver, 1 "	88			
2 "	140			
3 "		179		
4 "	186			130

* One determination only. The supernatant from this precipitated 660 μg. N with polyglucose A; the supernatant from this, 939 μg. N with S XII; total of the three precipitations, 1783 μg. N.

‡ 1.5 to 2 mg. sample *b*. With C-absorbed Type IX serum 107 μg. N was precipitated, almost the same amount as for the unabsorbed serum.

§ Maize glycogen *b* used.

‖ One determination only. A freshly prepared solution of the glycogen was used. The other values in the Type IX serum were obtained with a solution 1.5 months old.

¶ One determination only.

Supernatants of Types XX and XXII antisera from which maximal antibody had been precipitated by synthetic polyglucose fraction A (1) gave no precipitate with glycogen fraction A.

Effect of Saliva on the Reactivity of Glycogen toward Antisera.—4 ml. of human saliva was diluted with 4 ml. of saline and centrifuged. 3.5 ml. of the supernatant was added to 3.5 ml. of a saline solution of oyster glycogen, fraction A, containing 10 mg. per ml. After 2 hours at 37° 1.0 ml. portions were added in duplicate to 1.0 ml. each of Type IX and Type XII antisera,

with blank tubes containing antisera and saliva without glycogen. Estimation of reducing sugars in the digested glycogen according to Schales and Schales (12) showed 3 mg. per ml. 7 and 0 μg. of N were precipitated from the antisera by the digested glycogen, respectively, as against 204 and 250 μg. of N precipitated by 5 mg. of undigested fraction A. An analogous test of saliva-treated maize glycogen with Type XII antiserum gave a similar result. The presence of saliva did not prevent precipitation of antibody by the homologous type-specific pneumococcal polysaccharide.

Quantitative Data with Various Glycogens.—These are given in Table III.

Experiments with Reprecipitated Glycogens and with Glycogens after Acetylation and Deacetylation.—In order to find out whether the immunological specificity of glycogen could be removed by purification portions of dog liver and maize glycogens were reprecipitated and then acetylated by dispersion in formamide and treatment with acetic anhydride and pyridine at room

TABLE IV

Reactions of Reprecipitated Glycogens and of Corresponding Acetylated-Deacetylated Derivatives in Antipneumococcal Horse Sera

Antisera	Type IX 1655 μg. antibody N per ml.					Type XXII, No. 566 878 μg. antibody N per ml.		
Antibody N precipitated by glycogen, *mg.*...	1	2.5	5	10	14	1	2	3
	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.
Dog liver, reprecipitated	83	129	157	152*				
Dog liver, acetylated, deacetylated	75	118	148	132*	130*			
Maize, reprecipitated						63	74	75
Maize, acetylated, deacetylated						45	71	73
Maximum per cent of type-specific antibody N precipitated			9					9

1.0 ml. antiserum used in all tubes except those marked*, which contained 0.50 ml. Results calculated to 1.0 ml.

temperature (13). The acetyl derivatives were deacetylated with $Ba(OMe)_2$ (14) and the glycogens recovered by precipitation with alcohol. Comparison of the original and recovered materials is made in Table IV.

DISCUSSION

Intimations that glycogens from various species of higher animals, from clams, and from *Ascaris*, might have antigenic properties may be found in the literature (15). Ikeda showed that the sera of rabbits injected with large quantities of rather crude horse liver or dog liver glycogen fixed complement with either glycogen and also gave weak precipitation. Fixation of complement was much less with rabbit liver glycogen but appreciable precipitation occurred. The possibility that these reactions were due to cross-reactivities of protein impurities is not ruled out by the negative experiments recorded with liver proteins probably denatured by extraction with alkali and precipitation by trichloroacetic acid. Possibly, too, the reactions were similar to the relatively weak ones observed in the current studies with supposedly normal sera (page

344, Table I). Nor is there any statement that the reactions were abolished by enzymatic destruction of the glycogen. This objection also applies to Campbell's studies on clam and *Ascaris* glycogens, while there is also the possibility that other immunologically specific polysaccharides were contained in the preparations. The antigenicity of glycogen therefore remains in doubt.

The present studies, however, clearly establish the reactivity of amylopectin and of glycogens of diverse origins, even from the horse, toward certain preformed antibodies, notably those in horse antisera to the pneumococcal Types II, IX, XII, XX, and XXII. Because of the precipitation of antibodies in the designated sera by certain synthetic polyglucoses (1) and the dextrans (16), all of which possess glucose units linked together by α-1,6 and α-1,4 bonds, glycogen and amylopectin were tested in the same sera after Stacey's finding that the glucose in the specific polysaccharide of the Type II pneumococcus consists of glucose linked at the 1,4 and 6 positions (3). The presence of α-1,4,6 branch points in glycogen and amylopectin made this reactivity appear almost inevitable on the basis of chemical relationship and the quantitative theory of specific immune precipitation (17) and agglutination (18) which postulates multiple reactive groupings on both antigen and antibody. Earlier instances of the relation between the chemical constitution of immunologically reactive polysaccharides and their cross-reactivity in different antisera are the precipitation of Types II and III antipneumococcal sera by gum arabic and its derivative from which one-half of the arabinose had been removed (19), reactions of other plant gums in antipneumococcal sera (20), the cross-reaction between Types III and VIII pneumococcus which has been studied quantitatively in detail (21), the reaction of the polysaccharide of *Rhizobium radicicolum* in Type III antipneumococcal serum (21 *a*), the reaction between the blood group A substance and Type XIV antipneumococcal sera (22), and the relation of the removal of fucose from this substance to the cross-reaction (23).

The question immediately arises whether the precipitates observed in the various sera are due to glycogen itself or to other polysaccharides present as impurities. Answers were sought by fractionation of oyster glycogen, available in relatively large quantity, by destruction of glycogens with saliva, and by conversion of glycogens into their acetyl derivatives and subsequent deacetylation. Fractionation from sodium acetate solution by means of isopropyl alcohol (4) yielded only small fractions which differed appreciably from the principal one (Table II) in serological properties. All fractions precipitated the antisera, although the curves of precipitation were often relatively different in the different antisera. Possibly fraction A_1 was larger in molecular size, and fractions B, D, and A_3 smaller than the main fraction, A_2.

Since the reactions measured were all cross-reactions, it is not surprising that relatively large amounts of glycogen were required to precipitate the maximal quantity of antibody and that much less antibody was precipitated at room temperature (Fig. 1) or 37° than at 0° (*cf.*, for example, reference 21).

As for the enzymatic destruction of glycogen, treatment of oyster and maize glycogens, the only ones tested, with saliva rapidly and effectively destroyed their capacity to precipitate antisera. Control tests with antisera containing saliva showed that the enzymes did not interfere with the precipitation of the antibody by the homologous type-specific polysaccharide, so that it was in reality the glycogen and its precipitating power that had been affected.

Reprecipitation of maize and dog liver glycogens and conversion of the purified substances into their acetyl derivatives followed by deacetylation, led to products which precipitated almost as much antibody as did the original, purified glycogens (Table IV). The small differences observed were doubtless due to some degree of depolymerization during chemical manipulations which have been shown to degrade a specific polysaccharide such as that of Type III pneumococcus (24).

If it be granted, then, that the observed precipitation of antisera is due to the glycogen itself, it might still be considered that the reactions of the various glycogens were non-specific and that globulins other than antibody were precipitated. Evidence against this interpretation is: (*a*) the negative or weak reactions in two normal horse sera and the Type III, XVIII, XXVII, and XXVIII antipneumococcus horse sera (Table I); (*b*) the data in the footnotes of Tables II and III showing reductions in the amounts of nitrogen precipitated by polyglucose fractions after prior precipitation of the sera with a glycogen, it having been shown in reference 1 that only antibody is precipitated by the polyglucoses; and (*c*) the figures in a footnote to Table III showing that the sum of the quantities of nitrogen precipitated by glycogen, polyglucose, and type-specific polysaccharide, in succession, equals the amount of antibody nitrogen precipitated from unabsorbed serum by an excess of the type-specific polysaccharide alone. The data under (*b*) show also that glycogen precipitates a portion of the same antibody which reacts with synthetic polyglucose fractions A and B.

The true chemical basis of the cross-reactivity of the glycogens in the antisera to pneumococci of Types VII, IX, XII, XX, and XXII must remain in doubt until the presently unknown constituent sugars of the specific capsular polysaccharides of these types are identified and the nature of the linkages between the sugars has been revealed. A beginning is being made on this formidable task. It is quite possible that multiple recurrence of α-1,6 linkages in the glycogen may suffice to ensure cross-reactivity in one or more sera, while α-1,4 linkages, or both α-1,4 and α-1,6 linkages may be required to precipitate the antibody in another, and α-1,4,6 branch points might be essential in others. This would seem to be the case in the cross-reaction of glycogen with Type II antiserum, since the specific polysaccharide of Type II pneumococcus appears to contain all of its glucose linked in the 1,4 and 6 positions (3). While it might on first thought be considered strange that less than 2 per cent of the antibody in the Type II serum was precipitated by glycogen it must be remem-

bered that glucose makes up only about 30 per cent of the Type II specific polysaccharide, while 50 per cent is rhamnose (25) and about 20 per cent presumably glucuronic acid (3). It might more appropriately be considered remarkable that the α-1,4,6 branch points of the glycogens, spaced as they are, 8 to 15 sugar units apart, suffice to fit closely enough in multiples with the Type II antibody to bring about any degree of precipitation.

Presumably the specific polysaccharides of Types IX, XII, XX, and XXII will be found to be more closely related to glycogen, since higher percentages of antibody are precipitated from these sera by glycogen than from the Type II serum. The occurrence of glucose in the polysaccharides of these pneumococcal types is probable but by no means certain. In the first place, glucosamine may be immunologically equivalent to glucose; secondly, Morgan has found instances of inhibition of precipitation of a blood group substance and homologous antibody by sugars and sugar derivatives not obviously related to the antigen (26). Until, therefore, more structural evidence is available any assignment of explicit relationships would appear premature.

SUMMARY

Glycogens from various animal and vegetable sources precipitate antipneumococcal horse sera of Types II, VII, IX, XII, XX, and XXII.

Fractionated glycogen and glycogen recovered after reprecipitation, acetylation, and deacetylation precipitate the antisera, but glycogen degraded by saliva does not.

A fraction of the antibody is precipitated in the antisera by glycogen.

Possible chemical relationships accounting for these instances of cross-precipitation are discussed in terms of the structures of glycogen and the type-specific polysaccharides of pneumococcus and the quantitative theory of specific precipitation.

Amylopectin also gives cross-reactions of smaller magnitude. Quantitative data on these are withheld until irregularities have been eliminated.

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