VARIATION IN THE GROUP-SPECIFIC CARBOHYDRATE OF GROUP A STREPTOCOCCI

I. Immunochemical Studies on the Carbohydrates of Variant Strains

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In 1945 Wilson presented detailed studies of a strain of group A type 27 streptococcus that had apparently lost its group-specific C carbohydrate in the course of mouse passage (1). Wilson showed that the organisms recovered after passage differed from the parent strain in that extracts prepared either by heating at pH 2 or by extraction with formamide no longer gave the typical group-specific precipitin reaction with group A antisera. However, the type-specific antigens of the parent type 27 strain could be demonstrated in the variant, and it was thus reasonably certain that the variant had been derived from the strain originally injected into the mice despite the fact that the group-specific carbohydrate was no longer present. Additional instances of this same phenomenon have been encountered with other group A strains. For example, during a study of a protein antigen (designated R) characteristic of strains previously classified as type 28, two separate strains lost their group reactivity in the course of mouse passage (2). In these instances it was again possible to establish that the variant strains were derivatives of the original strains, since other identifying antigens remained unchanged. While the present study was in progress, several other strains became available which had undergone a loss of group reactivity during animal passage in this and other laboratories.

The nature of this fundamental alteration in the antigenic composition of group A streptococci was not apparent from the earlier studies. The further investigation of the problem reported in the present paper was stimulated by certain findings with regard to the function of the group-specific carbohydrate in the bacterial cell. The group A carbohydrate has been shown to be localized almost exclusively in the streptococcal cell wall and to constitute at least two-thirds of the dry weight of this structure (3). In view of this fact, it seemed unlikely that the carbohydrate could have disappeared from the variant strains without having been replaced by chemically similar material. The possibility was therefore examined that the loss of group

reactivity reflects an alteration in the chemical structure and serological specificity of the cell wall carbohydrate.

In the present study it is shown that the cell walls of the variant strains contain an amount of carbohydrate comparable to that found in ordinary group A strains. However, the carbohydrate differs immunologically from group A carbohydrate, and it has been possible to prepare rabbit antisera that react with the soluble carbohydrate derived from variant strains. The serological behavior and chemical composition of the group A and variant carbohydrates are compared in detail.

Materials and Methods

Streptococcal Strains.—A total of nine different streptococcal strains was encountered that had lost reactivity with group A antiserum in the course of animal passage. In addition, a tenth strain was investigated that had undergone a somewhat different type of modification during mouse passage. In the case of all but one of the ten strains, the original group A strain from which the variant was derived was available for study. The origin of the several strains is described in detail in the experimental section.

Preparation of Cell Walls.—The cell walls were prepared by the method previously described involving the grinding of acetone-dried streptococci in a ball mill followed by treatment with trypsin, chymotrypsin, and ribonuclease (3). The results have been confirmed using the method of Salton (4) in which aqueous suspensions of organisms are disrupted by shaking with glass beads in a Mickle disintegrator. In both procedures, differential centrifugation is used to eliminate undisrupted cells and fine particulate material.

Preparation of Carbohydrates.—The soluble carbohydrate fractions were obtained from cell wall preparations by treatment with concentrates of *Streptomyces albus* enzymes. Purification was carried out by the method described for the group A cell wall carbohydrate (3).

Analytical Methods.—Quantitative hexosamine analyses were made by a modification of the Elson and Morgan procedure (5) after hydrolysis with 2 N HCl at 100°C. in sealed ampoules. Methylpentose was determined without prior hydrolysis of the sample by the cysteine–sulfuric acid method of Dische and Shettles (6). The monosaccharide components of the hydrolyzed carbohydrates were identified by descending paper chromatography with butanol-pyridine-water (3:2:1.5). The spraying reagents employed were the ammonia–silver nitrate and hexosamine reagents as described by Partridge (7).

Total nitrogen was determined by the direct nesslerization micro-Kjeldahl procedure of Koch and McMeekin (8).

Preparation of Specific Precipitating Rabbit Antisera.—Initial attempts to prepare antisera to the carbohydrate of the variant strains by the routine procedures were unsuccessful. It was eventually found that satisfactory antisera could be obtained consistently if the immunizing suspensions of streptococci were treated with proteolytic enzyme and injected in relatively large amounts. Both trypsin and pepsin were found to be effective in enhancing the antigenicity of the cell wall carbohydrate. There is some indication that in the case of R-containing strains, two of which were employed in this study, trypsin is relatively ineffective for this purpose and pepsin is the enzyme of choice. The procedure employed in preparation of the vaccine and immunization of rabbits is illustrated in the following:—

The cells from 1.5 liters of a culture of the streptococcal strain in Todd-Hewitt broth were recovered by centrifugation, suspended in 20 ml. saline, and killed by heating for ½ hour in a water bath at 56°C. The suspension was centrifuged and the sediment resuspended in 25 ml. 0.01 N HCl in which 25 mg. crystalline pepsin was dissolved. The final pH of the suspension

was adjusted to approximately pH 2. After incubation at 37°C. for 2 hours, the suspension was neutralized with 1 N NaOH. The cells were centrifuged, washed three times with saline (0.85 per cent NaCl), and finally resuspended in 50 ml. saline. When trypsin was used for the digestion, the organisms were suspended in M/15 phosphate buffer pH 8, containing 25 mg. crystalline trypsin.

The immunizing procedure was that currently in use for the preparation of streptococcal antisera in rabbits except that the suspension was 12.5 times more concentrated than that usually employed. The schedule of intravenous injections was: 0.5 ml. of the suspension on 3 successive days followed by 4 days of rest for the first course. The dose was then increased to 1.0 ml. and given on the same schedule for 3 additional weeks. Five days after the last injection, 50 ml. of blood was taken from the ear. If the titer of the serum obtained proved to be adequate, the animal was exsanguinated; if not, an additional course of injections usually led to the production of good antiserum.

Precipitin Analysis.—Qualitative precipitin analyses for comparison of the serological properties of the carbohydrates were carried out by the capillary precipitin technique (9) using undiluted antiserum and serial dilutions of the carbohydrate solutions.

Quantitative precipitin studies were also made in comparing the carbohydrates. In this case the serological reactions were carried out in 12 ml. pointed centrifuge tubes. After incubation for 2 hours at 37°C. and refrigeration overnight, the precipitates were centrifuged in the cold and washed three times with cold physiological saline. The washed precipitates were redissolved in 0.1 N NaOH and the amount of antibody protein present determined by measuring the optical density of the solution (or an appropriate dilution in 0.1 N NaOH) in the Beckman quartz spectrophotometer at 287 mμ. No correction for antigen was necessary, since the carbohydrates employed show no appreciable absorption at this wave length. Parallel nitrogen determinations were carried out in several experiments and showed that the optical density of the redissolved precipitates is directly proportional to the nitrogen content. The mean extinction coefficient (optical density per milligram of N per milliliter) was approximately 10, in agreement with the results of other workers (10).

Chemical Analysis of the Products of Serological Reaction.—In investigating the relationship between the carbohydrates under study, it proved valuable to determine the chemical composition of the specifically precipitated antigens. In the earlier experiments, the redissolved precipitates were analyzed directly for their rhamnose and glucosamine contents. When this procedure was used, it was necessary to apply correction factors for the apparent methylpentose and glucosamine content of the antibody globulin. The correction factors employed were determined by the analysis of rabbit gamma globulin and were similar in order of magnitude to those reported by Beiser and Kabat (11) for human gamma globulin.

In the case of carbohydrates with low glucosamine content, the correction factor proved to be too large to provide accurate results, and the following procedure was devised for recovering the carbohydrate for analysis:—

The washed antigen-antibody precipitates were dissolved in 0.1 N acetic acid and the antibody globulin was precipitated by the addition of an equal volume of 25 per cent trichloracetic acid. The precipitate was removed by centrifugation and the supernatant fluid mixed with 10 volumes of acetone. The recovery of carbohydrate in the acetone precipitate exceeded 75 per cent of the amount used to obtain the specific precipitate, and it could be analyzed for its content of rhamnose and glucosamine without the use of correction factors. The procedure possesses the additional advantage of permitting parallel serological analysis of the recovered carbohydrate.

The chemical and serological properties of carbohydrate remaining in the serum supernatant in those experiments in which antigen excess was present were studied by procedures similar to those described above. The protein in the serum supernate was precipitated with trichloracetic acid, and the soluble carbohydrate recovered by acetone precipitation.

TABLE I

Survey of Stock Laboratory Strains of Group A Streptococci
Search for Variant Strains

No. of strains				No. of Strains				Total
Serological type	Non-passage	Passage	Variants found	Serological type	Non-passage	Passage	Variants found	
1	55	5	1	R*	16	5	2 entered as types 2 and 13	
2	25	5	2					
3	46	5	1	29	13			
4	27	6		30	32	4		
5	21			31	2	1		
6	19	1		32	11	2	1	
8	7	2		33	4	1		
9	2	1		34	3	1		
11	14	1		35	2	2		
12	38	5		36	6	1		
13	18	5	2	37	1			
14	22	5		38	5	3		
15	13	3		39	15	1		
17	49	4		40	3	1		
18	22	2		41	1	1		
19	67	6		42	3	1		
22	13	7		43	4	1		
23	29	1		44	12	3		
24	14	3		46	13	1		
25	10	1		47	5	2		
26	40	5		London	8	1		
27	3	2	1	Corby	1	1		
Total typed					714	108	8	
Untyped					146	9	2	
Total from individual infections					860	117	10	977
Typed and untyped duplicates: repeated cultures from same patient								242

Other strains of special series including repeated cultures from individual patients	No. of non-passage strains	Serological types	
GL series (epidemic)	197	A few types 1 and 3; chiefly types 17, 19, 30	
RS series	258	Some typed, some untyped	
RSC series	123		
RP series	18		
Total	596		596
Total strains tested in survey			1815

* R = strains containing R antigen, previously designated as type 28 on the basis of this antigen. R is not the type-specific M antigen, and many strains possessing this antigen apparently fall into several of the recognized types on the basis of their M antigens.

EXPERIMENTAL

Survey of Stock Laboratory Strains of Group A Streptococci.—A large collection of streptococcal strains was on hand in this laboratory in the form of frozen and dried cultures. This collection was surveyed in a search for

TABLE II

Origin of Mutant Strains of Group A Streptococci

Serological type	Designation of original group A strain	Designation of mutant strain	Origin of mutant strain
1	K43, K43/30*	K43/30/2 rabbit passages	Rabbit skin lesion. Obtained by Dr. G. E. Murphy
2	C132, C132/8	C132/38	Mouse passage. Found among stock cultures
2(R)	C649A	C649A/120	Mouse passage. (Lancefield and Perlmann (2))
3	C203/29	C203 further mouse passage	Mouse passage. Obtained by Dr. Harry Eagle
13(R)	C510	C510/51	Mouse passage. (Lancefield and Perlmann (2))
13	(Usher)‡	Usher/21§ rabbit passage	Rabbit passage. Obtained by Lord Stamp
27	T27	T27A/46	Mouse passage. Obtained by Dr. A. T. Wilson (1)
32	C121	C121/39 (intermediate C polysaccharide)	Mouse passage. Found among stock cultures
Unclassified	B421	B421/14	Mouse passage. Obtained by Dr. R. F. Watson
Unclassified	B422	B422/10	Mouse passage. Obtained by Dr. S. Rothbard

* In strain designations throughout this paper the figure preceding the slanted line is the strain number, and the figure following the slanted line indicates the number of mouse passages, unless otherwise indicated.

‡ No longer available. Presumably it had the group A original C polysaccharide before passage.

§ After 30 additional animal passages this strain again produced the original C polysaccharide instead of the polysaccharide characteristic of the variant.

variant strains. The results of this survey of a total of 1815 strains are summarized in Table I.

Each of the variant strains listed had been subjected to animal passage, and in no instance was a variant of this type encountered among the strains that had not been injected into laboratory animals after isolation from human sources. It would appear that the selection of this type of variant must be extremely rare during residence in the natural human host even during an

epidemic when the strain is undergoing rapid human passage. The strains examined include a large number from epidemics of streptococcal disease as well as serial cultures from the same patient, and it thus seems reasonable to suppose that variants would have been found if they occur with any frequency. In addition to these 1815 group A strains, the laboratory collection of 33 strains designated as "non-groupable" were tested, but none was found that was related to the variants under study.

The data presented in Table I cannot be interpreted as providing information concerning the frequency with which variants are isolated during animal passage, since a systematic study of this point was not attempted. Many strains that were subjected to mouse passage are not represented in the collection of preserved strains.

TABLE III

Comparative Analyses of Group A and Variant Cell Walls

	Total N	Reducing sugars (calc. as glucose)	Hexosamine
	per cent	*per cent*	*per cent*
Group A cell walls	7.2–7.7	58–62	18–22
Variant cell walls	7.1–7.6	55–61	9–12

The ten variant strains shown in Table I are described in more detail in Table II. It will be noted that while most of the variants were isolated after mouse passage, the same selection can occur in the rabbit. The type 1 strain, K43, had been subjected to 30 serial passages in mice without any change in the group-specific carbohydrate, and the variation was first detected after two subsequent rabbit passages. Similarly, the variant of strain Usher appeared in the course of rabbit passage (12).

Properties of the Cell Walls Prepared from Variant Strains.—Cell wall preparations obtained from the variant strains are similar in appearance and general properties to those obtained from group A strains. As in the case of group A cell walls, chemical analysis indicates that the structure is composed primarily of a carbohydrate-protein complex. The cell walls analyzed had been treated with proteolytic enzyme, which removes associated proteins such as the M and T antigens. The comparative analyses of several preparations of group A and variant cell walls are presented in summary in Table III. The total nitrogen content falls in the same range in both cases. The hexosamine analysis of the variant cell walls gives consistently lower values than those obtained with group A cell walls, but the values for total reducing sugars of hydrolyzed samples are essentially the same. It is clear, therefore, that the total carbohydrate content of the variant cell wall is not significantly less than that occurring in the parent group A strains.

Chemical Analysis of the Cell Wall Carbohydrate of Variant Strains—Previous work has shown that the group-specific carbohydrate of group A strains is composed of two monosaccharide constituents, rhamnose and glucosamine, the glucosamine occurring as the *N*-acetyl derivative (3, 13, 14). Analysis of hydrolysates of the carbohydrate of variant strains by paper chromatography revealed the presence of the same two monosaccharide components, and no evidence was found for the presence of other monosaccharides. However, quantitative studies showed that the proportion of the two components differs markedly from that found in the group A carbohydrate. The quantitative data obtained with several preparations are presented in Table IV. The rhamnose content (59 to 62 per cent) is consistently higher and the glucosamine content (10 to 15 per cent) consistently lower than in the group A carbohydrate. The magnitude of this difference in the proportion of the two monosaccharides can be expressed by the rhamnose:glucosamine ratio. The average ratio for group A strains is 1.6, while that of the variant strains ranges from 4.0 to almost 6. From these findings it would appear that the differences in serological behavior of the two carbohydrates is based on the occurrence of different chemical linkages between the same two monosaccharide components.

TABLE IV

Chemical Analysis of Variant Carbohydrates

Source of preparation	Glucosamine	Rhamnose	Ratio Rhamnose: glucosamine
	per cent	*per cent*	
C510/71 (prep. 1)	10.5	61.0	5.8
C510/71 (prep. 2)	14.1	60.0	4.3
T27A (prep. 1)	15.1	59.7	4.0
T27A (prep. 2)	11.8	61.8	5.2
K43 var. (prep. 1)	12.7	62.5	4.9
K43 var. (prep. 2)	10.8	59.0	5.5
C649A/120	15.6	61.9	4.0

The Occurrence of a Strain Intermediate between the Group A and Variant Strains.—In the course of the survey of the occurrence of variant strains in the laboratory collection of streptococci, one strain was encountered which yielded extracts that reacted equally well with group A and variant antisera. This strain, designated C121/39, had been subjected to mouse passage, and the parent strain that had not been passed in mice gave the reactions typical of group A organisms. It was shown that the behavior of C121/39 was probably not due to the occurrence of a mixed culture of group A and variant cells since a large number of single colony isolates retained the prop-

erty of giving extracts which reacted with both antisera. Furthermore, immunochemical studies to be presented below show that the organisms do not merely produce a mixture of the two types of carbohydrate but that a new entity is formed possessing reactivity with antibody to both the group A and variant carbohydrates. The immunization of rabbits with strain C121/39 gives rise to antibodies which precipitate with both carbohydrates.

Chemical analysis of carbohydrate isolated from the intermediate strain again showed the presence of the same two monosaccharide components, rhamnose and glucosamine. Quantitative data are presented in Table V. It

TABLE V

Chemical Analysis of Intermediate Carbohydrate

Source of preparation	Glucosamine	Rhamnose	Ratio Rhamnose: glucosamine
	per cent	*per cent*	
C121/39 (prep. 1)	15.6	53.9	3.4
C121/39 (prep. 2)	16.7	44.7	2.7
C121/39 (prep. 3)	20.4	49.4	2.4

TABLE VI

Comparison of Chemical Composition of the Three Carbohydrates

	Glucosamine	Rhamnose	Ratio Rhamnose: glucosamine
	per cent	*per cent*	
Group A	23–28	42–49	1.5–2.0
Intermediate	15–20	45–54	2.4–3.4
Variant	10–15	59–62	4.0–6.0

will be seen that the values, though somewhat variable from lot to lot, are intermediate between those of the group A and variant carbohydrates. Similarly, the ratio of rhamnose to glucosamine falls in a range midway between that of the group A and the variant. These facts are summarized in Table VI, which presents a comparison of the analytical values for the three kinds of carbohydrate.

Qualitative Serological Studies.—Rabbit antisera containing precipitating antibody to the variant carbohydrate were prepared with eight of the variant strains. Precipitin tests carried out with these sera using the soluble carbohydrate as antigen indicate that the carbohydrates of these strains are serologically identical with one another. Thus, the strains that have lost reactivity with group A serum in the course of animal passage have in each case undergone the same alteration in the cell wall carbohydrate. The chem-

ical and serological change that occurred in the carbohydrate of the intermediate strain appears to be in the same direction but to have resulted in less loss of group A reactivity.

The serological relationships between the three carbohydrates are illustrated by the results of qualitative precipitin tests carried out in capillary tubes. These are presented in Table VII. Purified carbohydrates from each of the three kinds of strain, *i.e.* typical group A (A), variant (V), and intermediate (I), were tested in serial dilution with undiluted group A and variant antisera. The group A and variant carbohydrates each show strong reactivity with their homologous antisera and a minor degree of cross-reactivity with the heterologous serum. On the other hand, the intermediate carbohydrate gives good reactions with both antisera.

TABLE VII

Comparison of the Qualitative Precipitin Tests with the Three Varieties of Carbohydrate

Antigen		Tests in A antiserum							Tests in V antiserum						
Source	Designation	Initial concentration of antigen, mg./ml.													
		1.0	0.1	0.05	0.025	0.012	0.006	0.003	1.0	0.1	0.05	0.025	0.012	0.006	0.003
Typical group A	A	++++	+++	+++	++±	+	±	Tr	Tr	−	−	−	−	−	−
Variant	V	−	±	Tr	Tr	Tr	Tr	−	++++	++±	++	+±	+	±	Tr
Intermediate	I	++++	+++	++	+±	+	±	Tr	+++	++	+	+	Tr	Tr	−

Readings made after 2 hours at 37°C.

The cross-reactivity between the group A and variant carbohydrates was a common finding with the various antisera prepared in the course of this study, but there was considerable variation in the degree of cross-reaction displayed by individual antisera. While the heterologous reactions were usually most marked with those antisera containing the highest concentration of homologous antibody, certain of the potent antisera showed no appreciable cross-reaction whatever. The precipitates with heterologous carbohydrate are characteristically slow to develop in comparison with the rapidly forming homologous precipitates. Thus readings made immediately may show no evidence of the cross-reaction although the homologous precipitates are well developed; and even after 2 hours' incubation at 37°C. the cross-reactions are appreciably less evident than after overnight refrigeration.

Quantitative Precipitin Analysis.—Further information concerning the homologous and heterologous reactions of the group A and variant carbohydrates was obtained by quantitative precipitin analysis. The results obtained with a variant antiserum (prepared with strain C649A/120) are presented in Fig. 1.

1.0 ml. amounts of the antiserum were mixed with 0.1 ml. of solution containing antigen in the amount indicated, and the precipitates obtained were handled as described under Materials and Methods. The precipitates were redissolved in 1.0 ml. 0.1 N NaOH and diluted 1:20 in 0.1 N NaOH for spectrophotometric analysis.

It is shown in Fig. 1 that the amount of antibody precipitated by the homologous variant carbohydrate increases sharply with the increase in antigen concentration to a maximum at about 0.25 mg./ml. antigen, and subsequently falls sharply in the region of antigen excess. On the other hand, the amount of antibody precipitated by the heterologous group A carbohydrate increases gradually with increasing antigen concentration, and a flat

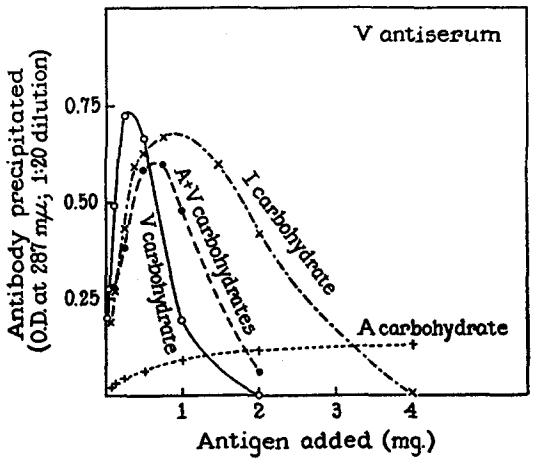

FIG. 1. Quantitative precipitin analysis of variant antiserum with homologous and heterologous carbohydrates.

curve is described which is still at its maximum at antigen concentrations as high as 10 mg./ml. This maximum is less than 20 per cent of the amount of antibody absorbed by the homologous antigen at 0.25 mg./ml.

Fig. 1 also includes data on the absorption of the same variant antiserum with the intermediate carbohydrate. It will be observed that the amount of antibody removed at the point of maximal precipitation approaches that of the homologous carbohydrate, but that a broader curve is described which falls less sharply in the region of antigen excess. The shape of the curve is in part due to the fact that only a portion of the total intermediate carbohydrate added is reactive with the variant antiserum (see Table IX) and the values on the abscissa refer to the actual weight of carbohydrate added. This point is illustrated by the fourth curve, which represents the data obtained by absorption of the same antiserum with a mixture of equal parts of the group A and variant polysaccharides. The results are plotted in terms

of the total amount of carbohydrate in the mixture, and as a consequence the curve appears broader than that for the variant carbohydrate alone. It is of interest, however, that the maximum amount of antibody precipitated by the mixture is distinctly less than that removed by the homologous carbohydrate alone, suggesting that the presence of group A carbohydrate exerts an inhibitory influence.

Similar quantitative precipitin curves obtained with a typical group A antiserum are presented in Fig. 2. The precipitates from 1.0 ml. of serum were redissolved in 1.0 ml. of 0.1 N NaOH and the spectrophotometric readings were made on 1:40 dilutions of this solution. The group A antiserum

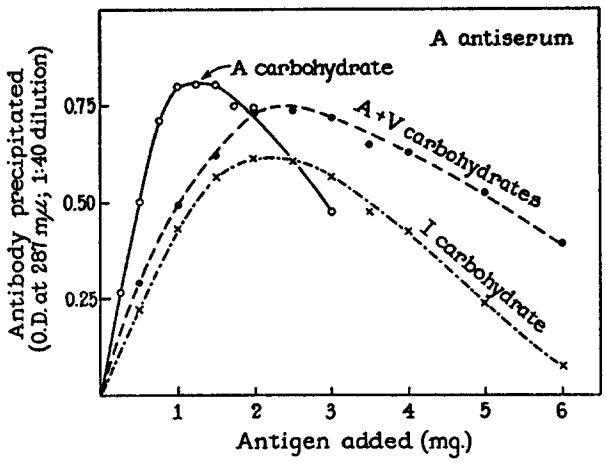

FIG. 2. Quantitative precipitin analysis of group A antiserum with homologous and heterologous carbohydrates.

employed gave only traces of precipitate with the heterologous variant carbohydrate, so that in this case the cross-reactivity was insignificant and was not measured quantitatively. This reflects the usual finding in that most of the group A antisera tested showed only slight cross-reactivity while the majority of variant antisera reacted significantly with group A carbohydrate. To some extent, therefore, the serological cross-reactivity of the two carbohydrates is unequal.

The curve with homologous group A carbohydrate illustrated in Fig. 2 rises to a rather broad maximum and falls less sharply in the region of antigen excess than the curve for variant carbohydrate (Fig. 1). When the intermediate carbohydrate is used as antigen with this serum, about 25 per cent less antibody is removed than by the homologous antigen. The breadth of this curve is again referable to the fact that only a portion of the carbohydrate is reactive with group A antibody; and the results with a mixture of

equal parts of group A and variant carbohydrates give a similar curve, although in this case the maximum amount of antibody absorbed is greater and approaches that absorbed by the A carbohydrate. There is, however, some evidence for inhibition by the heterologous carbohydrate similar to that observed with the variant antiserum (Fig. 1).

Relationship between the A, V, and I Polysaccharides.—The relationship of the intermediate carbohydrate to the group A and variant carbohydrates was considerably clarified by the combined serological and chemical analysis of the specific precipitates formed with group A and variant antisera and of the soluble carbohydrate remaining in the serum supernates. This is illustrated in the following two experiments:—

Experiment 1.—Three aliquots of group A antiserum were absorbed with polysaccharide preparations as follows: (1) homologous group A polysaccharide at the equivalence point; (2) a mixture of equal concentrations of group A and variant polysaccharides, the former again at the equivalence point; and (3) intermediate polysaccharide at the same concentration as that of the mixed A and V polysaccharides. The precipitates were separated and washed, and the polysaccharide recovered in the free form from both precipitates and serum supernates by the procedures described under Materials and Methods. Each sample was analyzed quantitatively for glucosamine and rhamnose content, and was tested for serological reactivity. The results are summarized in Table VIII, in which are presented the rhamnose:glucosamine ratio of the recovered carbohydrates and their reactivity with group A and variant antisera.

The carbohydrate recovered from the precipitate obtained with group A polysaccharide showed a rhamnose:glucosamine ratio characteristic of A polysaccharide and reacted only with group A antiserum. Since the reaction was carried out at the equivalence point, there was no appreciable quantity of carbohydrate in the serum supernate. In the case of the mixed A and V polysaccharides, the material recovered from the precipitate was chemically and serologically typical of group A carbohydrate while that isolated from the serum supernate was serologically typical V carbohydrate. The rhamnose:glucosamine ratio of the latter was somewhat low for V carbohydrate, but in other single experiments of this type chemically typical V carbohydrate was obtained. It would appear that the serum effectively separates the two components of the mixture. In contrast to these findings, carbohydrate recovered from the precipitate with I polysaccharide reacted well with both group A and variant antisera, and thus retained its intermediate properties. This provides convincing evidence that the I carbohydrate does not represent a simple mixture. The analysis of the carbohydrate remaining in the serum supernatant is of interest, since it showed that the material not precipitated had a relative specificity for V antiserum and had the chemical characteristics of V polysaccharide.

Experiment 2.—A similar experiment was carried out with V antiserum absorbing with V carbohydrate at the equivalence point, with a mixture of A and V carbohydrates, and with I carbohydrate. The results are recorded in Table IX.

It is apparent from Table IX that the V antiserum is also capable of resolving the mixture of A and V polysaccharides. As in the previous experiment, however, the material recovered from the precipitate with the I polysaccharide retained its ability to react equally with group A and variant antisera and showed an intermediate value for the rhamnose:glucosamine ratio. Again there was carbohydrate remaining in the serum supernate, and on this occasion it had the chemical composition and serological specificity of group A carbohydrate.

TABLE VIII

Precipitin Absorption of Group A Antiserum

A serum absorbed with:	Fraction analyzed	Ratio Rhamnose: glucosamine	Serological reactivity											
			Serial dilutions tested for precipitation with:											
			Anti A serum						Anti V serum					
A polysaccharide	Precipitate	1.54	++++	++++	++++	+++	++	+	−	−	−	−	−	−
	Supernate	—	?*	−	−	−	−	−	−	−	−	−	−	−
A + V polysaccharides	Precipitate	1.79	++++	++++	++++	+++	+	±	−	−	−	−	−	−
	Supernate	3.32	−	−	−	−	−	−	++++	++++	+++	+++	++	+
I polysaccharide	Precipitate	3.29	++++	++++	+++	+++	++	±	+++	+++	++	++	+	±
	Supernate	4.38	±	±	+	+	−	−	++	++++	+++	+++	+	±

* ?, questionable trace reaction.

TABLE IX

Precipitin Absorption of Variant Antiserum

V serum absorbed with:	Fraction analyzed	Ratio Rhamnose: glucosamine	Serological reactivity											
			Serial dilutions tested for precipitation with:											
			Anti A serum						Anti V serum					
V polysaccharide	Precipitate	5.14	?	−	−	−	−	−	+++	+++	+++	+++	+	+
	Supernate	—	−	−	−	−	−	−	−	−	−	−	−	−
A + V polysaccharides	Precipitate	5.49	±	+	−	−	−	−	++++	++++	++++	+++	++	+
	Supernate	1.80	++++	++++	++++	+++	+	+	−	−	−	−	−	−
I polysaccharide	Precipitate	3.49	++++	++++	++++	+++	+++	++	++++	++++	++++	+++	+++	+
	Supernate	1.67	++++	++++	+++	+	+	±	±	±	−	−	−	−

The findings of these two experiments indicate that the polysaccharide prepared from the intermediate strain is heterogenous both chemically and serologically. Evidence has been presented previously indicating that this is also true of group A carbohydrate prepared by dissolution of the cell wall with *Streptomyces albus* enzymes (3). In the case of the I polysaccharide, it is apparent that the major component is capable of reacting with both group A and variant antisera, but there are obviously minor components

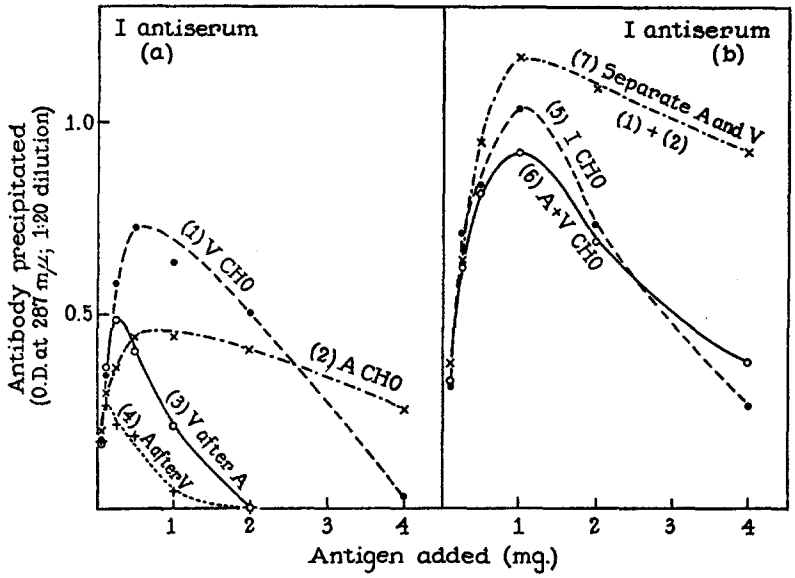

FIG. 3. Quantitative precipitin analysis of antiserum to the intermediate strain with homologous and heterologous carbohydrates. The results using A and V carbohydrates separately on the same samples of antiserum are illustrated as well as the results using the mixed carbohydrates.

present which are relatively specific for each of the antisera and which have rhamnose and glucosamine contents similar to the A and V polysaccharides.

Quantitative Precipitin Studies with Intermediate Antiserum.—Quantitative precipitin analysis of antisera prepared by immunization of rabbits with the intermediate strain (C121/39) indicates that the antibodies formed have a complexity which reflects that of the intermediate carbohydrate. The findings are consistent with the interpretation that much of the antibody elaborated is precipitable by *either* the A or the V carbohydrate, but that antibodies are also present which can be precipitated by only one or the other of the antigens. The homologous I carbohydrate appears to precipitate all of the antibodies simultaneously.

Representative data leading to this conclusion are presented in Fig. 3. The specific precipitates were obtained as previously described and redis-

solved in 0.1 N NaOH at the original volume of the serum. Spectrophotometric readings were made on 1:20 dilutions of the redissolved antigen-antibody precipitates. The precipitin curves obtained when the A and V carbohydrates were used separately to precipitate the intermediate antiserum are given in Fig. 3 *a* (curves 1 and 2). It will be seen that a significantly larger amount of antibody is removed by the variant carbohydrate. Treatment of the serum with either of these carbohydrates leaves antibody in solution that is precipitable by the other. Curve 3 of Fig. 3 *a*, for example, gives the findings on the amount of precipitate removed by V carbohydrate from intermediate serum that had been previously absorbed with the amounts of A carbohydrate indicated on the abscissa. The values on the abscissa also represent the amount of V carbohydrate used. Similarly, curve 4 was obtained with A carbohydrate and serum that had been absorbed with V carbohydrate. The maximum figures occurring on these two curves represent that portion of the total antibodies that is precipitable only by one or the other of the two carbohydrates.

The curve obtained with this antiserum and the homologous I carbohydrate is given in Fig. 3 *b* (curve 5), and it is clear that appreciably more specific antibody is absorbed than by either the A or the V carbohydrate alone. A mixture of equal parts of the A and V carbohydrates (curve 6), however, removes only slightly less of the total precipitable antibody than the homologous antigen, and a similar curve is described. The curve designated as "Separate A and V" (curve 7) is a summation of curves 1 and 2 of Fig. 3 *a*, and the excess of these values over those obtained with the homologous I antigen suggests that a portion of the total antibodies must be precipitated by both the group A and variant antigens.

These findings can be interpreted as evidence that the intermediate antiserum contains antibody with double specificity. The amount of antibody of this kind is reflected by the difference between the total precipitated by treatment of separate aliquots of I antiserum with A and with V carbohydrate (curve 7) and that precipitated by a mixture of A and V carbohydrates (curve 6). In addition, two independent estimates of the quantity of doubly reactive antibody should be obtainable from the data given in Fig. 3 *a*. Thus, the total amount of antibody removed by V carbohydrate (curve 1) less that which was precipitated by V after previous absorption of the serum with A carbohydrate (curve 3) would be expected to reflect the amount precipitable by either antigen. Similarly, in the case of A carbohydrate, the difference between curves 2 and 4 should give the same information. The fact that similar results are obtained by these three methods for estimating antibody with double specificity is illustrated in Fig. 4. The differences between the values obtained at each point on the three pairs of curves are plotted on the same coordinates as the original data. Considering the inherent error in this type of analysis and the possibility of mutual inhibitory effects of

the A and V carbohydrates as illustrated in Figs. 1 and 2, the results are in remarkably good agreement.

It would appear, therefore, that not only does the I carbohydrate include a moiety that is reactive with both A and V antisera, but that immunization with the intermediate strain results in the production of antibodies that are precipitable by both A and V carbohydrate. This finding increases the de-

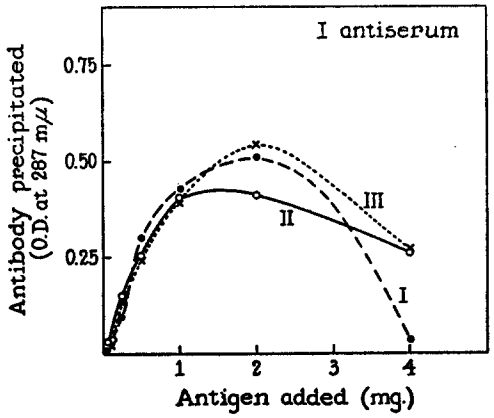

FIG. 4. Representation of antibody in the intermediate antiserum precipitable by either A or V carbohydrate. The figures are derived from the data summarized in Fig. 3 as described in the text:—

I = V − (V after A); *i.e.*, curve 1 minus curve 3.

II = A − (A after V); *i.e.*, curve 2 minus curve 4.

III = (Separate A and V) − (A + V); *i.e.*, curve 7 minus curve 6.

Curve III is plotted on the basis of one-half the total amount of carbohydrate employed in order to make it comparable with curves I and II.

sirability of elucidating the chemical nature of the groupings responsible for A and V specificity.

DISCUSSION

The studies reported in the present paper demonstrate that the loss of reactivity with group antisera which has been observed following repeated animal passage of certain group A streptococci depends on an alteration in the chemical structure and serological specificity of the group-specific C carbohydrate. There is no information concerning the mechanism of this change, but it seems reasonable to suppose that it represents a rare mutational event with subsequent selection in the tissues of the animal. All variant strains that have been encountered had been previously subjected to animal passage. Up to the present time, none have been obtained on direct isolation from human sources and none were found among the large collection of non-passaged strains available in this laboratory. In addition, it should be pointed out that although large numbers of strains of group A streptococcus are

subjected to mouse passage for the purpose of enhancing virulence and M protein production in this and other laboratories, the isolation of these variants appears to be a relatively infrequent event. In order for selection of the variant to occur, it is probable that the organism must possess greater virulence for the animal host than other members of the streptococcal population. If this view is correct, isolation of the variant would depend on the simultaneous occurrence of alteration in the cell wall carbohydrate and enhancement of virulence or ability to grow in animal tissues.

There is no indication that the tendency to produce variants is characteristic of certain strains of group A streptococci. Wilson reported in his paper on the loss of group carbohydrate during mouse passage that a second series of passages of the original group A strain resulted in an increase in virulence without a concomitant loss in reactivity with group A serum (1). As a further example of the irregularity of the emergence of this type of variant during mouse passage, the experience with two strains containing R antigen (C510 and C649A) can be cited (2). Although, as indicated in the introduction and in Table II, both of these strains gave rise to variants in the course of one series of mouse passages, the original group A strains were later subjected to a second series of 100 mouse passages without any alteration in their group reactivity. In both cases virulence for mice was greatly enhanced as a result of serial passage.

Only one instance of reversion of a variant strain to a typical group A strain has been encountered. This occurred in the laboratory of Lord Stamp, who found that a variant strain, Usher, had regained its reactivity with group A antiserum after repeated mouse passage (12). As in the case of all other variant strains, the Usher variant had originally been isolated during animal passage. Thus, both the variation and its reversal have been observed only under these artificial laboratory conditions.

The striking serological difference between the carbohydrate of group A and variant strains, each of which contain the same two constituent monosaccharides, is of interest from the immunochemical point of view. This difference in serological specificity probably has a chemical basis in the nature of the combination between the monosaccharide units, and it seems clear that the immunologically dominant groupings of the two polysaccharides are distinct from one another. However, the cross-reactivity that was observed between the two carbohydrates indicates that at least one of the chemical groupings occurs to some degree in both carbohydrates. The intermediate strain is of interest in this connection, since it behaves as though its cell wall carbohydrate has a more or less equal representation of the two serologically active groups. The evidence presented shows that part of the carbohydrate isolated from the intermediate strain possesses both of these groupings in a reactive form in a single molecule, so that the antigen will combine equally well with antibodies against either group A or the ordinary variant strains.

Furthermore, part of the antibody formed by rabbits immunized with the intermediate strain appears to have double specificity and to be precipitated by either group A or variant carbohydrate. These findings are of importance from the point of view of the debated occurrence of antibody of this type and should be examined further when more information is available concerning the chemistry of the two serologically active units involved.

SUMMARY

The phenomenon of apparent loss of group-specific carbohydrate in the course of mouse passage of group A streptococci has been subjected to further study, and several additional variants showing this property have been described. The loss of group reactivity is shown to be due to an alteration in the chemical structure and serological specificity of the cell wall carbohydrate. This alteration appears to be essentially the same in each of the variants available for study. The carbohydrate of the variant strains (V) contains the same two monosaccharide components as the group A carbohydrate (A), but they are present in different proportions. Precipitating sera reactive with V carbohydrate have been prepared, and the A and V carbohydrates have been compared by qualitative and quantitative precipitin analysis.

A second type of variation has been encountered during mouse passage. This variation is characterized by the occurrence of a cell wall carbohydrate (I) intermediate in chemical and serological properties between the A and V carbohydrates. The I carbohydrate reacts with both A and V antisera and does not appear to be a simple mixture of A and V carbohydrate. Similarly, antisera against the intermediate strain contain antibodies reactive with both A and V carbohydrates, and evidence is presented indicating that in part this represents antibody with double specificity.

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