A FURTHER STUDY OF THE CROSS REACTION BETWEEN THE SPECIFIC POLYSACCHARIDES OF TYPES III AND VIII PNEUMOCOCCI IN HORSE ANTISERA*

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A constantly increasing number of immunological cross reactions are being studied with the aid of the quantitative precipitin and quantitative agglutinin techniques, and knowledge of this phase of immunological specificity is consequently taking on a more precise character. The present contribution to this subject continues earlier work (1) on the pneumococcus Type III-Type VIII cross reaction.

It was shown in the earlier studies that the reaction between the specific polysaccharide of Type VIII pneumococcus (S VIII) and an antipneumococcus (anti-Pn) Type VIII horse serum, in the antibody excess region and through the equivalence zone, could be represented by the equation

$$\text{mg. antibody N precipitated} = 2RS - \frac{R^2 S^2}{A},$$

in which S = S VIII precipitated, A = antibody nitrogen precipitated at a reference point in the equivalence zone, and R = ratio of A to S at the reference point (*cf.* 1, 2), an equation which may be derived from the law of mass action and which has been shown to be applicable in many immune systems (3). However, the cross reaction between the specific polysaccharide of Type III pneumococcus (S III) and Type VIII antiserum was of a distinctly different character. When S III was plotted against antibody N the resulting curve was not characteristic of the above equation but was composed of a steep initial portion, followed by a less steep, linear section. Since cross reactions are not always similar in the reciprocal sense it was thought of interest to make a quantitative study of the S VIII-anti-S III reaction, and data on this are included in the present report. An attempt was also made to isolate the antibody reactive in linear fashion in the cross reactions, as it was thought that this might show a simpler behavior toward polysaccharide than the antibody as a whole. Finally, experiments were run at 37°C. as well as at 0° in order to test the effect of temperature on the cross reaction.

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EXPERIMENTAL

Materials and Methods.—Type III and Type VIII antipneumococcus horse sera[1] were used after absorption with crude Type I pneumococcus "C" substance. The Type III and Type VIII pneumococcus polysaccharides (designated S III and S VIII) were prepared according to references 1 and 4.

TABLE I

Antibody N Precipitated from Type III Antiserum H 792 by Varying Amounts of S III and S VIII

Amount of S used	Antibody N precipitated by S III from 1.0 ml. serum	Ratio antibody N to S III in precipitate	Tests on supernatants	Antibody N precipitated by S VIII from 5.0 ml. serum	Ratio antibody N to S VIII in precipitate	Tests on supernatants	Antibody N precipitated by S III from 1.5 ml. serum dilution freed from cross reactive antibody	Ratio antibody N precipitated to S III in precipitate	Tests on supernatants
mg.	*mg.*			*mg.*			*mg.*		
0.020	0.396	19.8	Excess A*	0.250	12.5	Excess A	0.394‡	19.7	Excess A
0.030	0.538	17.9	" "	0.336	11.2	" "	0.514	17.1	" "
0.050	0.664	13.3	" "	0.454	9.1	" "	0.620	12.4	" "
0.075	0.702	9.4	No A or S	0.534	7.1	" "	0.624	8.3	No A or S
0.10	0.700	7.0	" " " "	0.556	5.6	" "	0.614	6.1	Trace S
0.15	0.726	4.9§	Excess S	0.690	(4.6)	" "	0.654	4.5§	Excess S
0.20	0.748	3.9§	" "	0.756	3.9§	A and S	0.658	3.7§	" "
0.25				0.764		" " "			
0.30				0.816	2.9§	" " "	0.646	3.3§	" "
0.40				0.844‡	2.5§	Excess S			
0.50				0.884	2.5§	" "			
0.75				0.952	2.7§	" "			
1.00				0.950		" "			
Equation‖: mg. N precipitated = 23.4 S − 137 S²							Equation‖: mg. N precipitated = 23.9 S − 143 S²		

* A = antibody.

‡ Single analysis only.

§ Amount S in supernatant determined (6 *b*, *c*).

‖ Calculated to 1.0 mg. A N as follows: in the experimentally determined linear equation, $\frac{A\,N}{S}$ in precipitate $= 2R - \frac{R^2}{A}$ S, A is put = 1.0, changing only the slope of the line.

The precipitin determinations were carried out in the usual way (5, 6) by addition of increasing amounts of polysaccharide to accurately measured volumes of serum. After 2 days at 0°C. the specific precipitates were centrifuged, washed twice in the cold, and analyzed for nitrogen by the micro-Kjeldahl method. All analyses were run in duplicate.

Experiment 1. Homologous and Cross Reactions of Type III Antiserum H 792 (Bleeding 2/3/37).—Data are given in Table I and Fig. 1 on the addition of increasing

[1] These sera were obtained through the kindness of Dr. Ralph S. Muckenfuss and the late Dr. William H. Park of the New York City Department of Health Research Laboratories.

amounts of S III and S VIII to the antiserum. The equations, calculated to the same antibody content, are seen to be identical before and after heterologous absorption, as in a similar experiment with Type VIII serum (1). The cross reaction, also, was

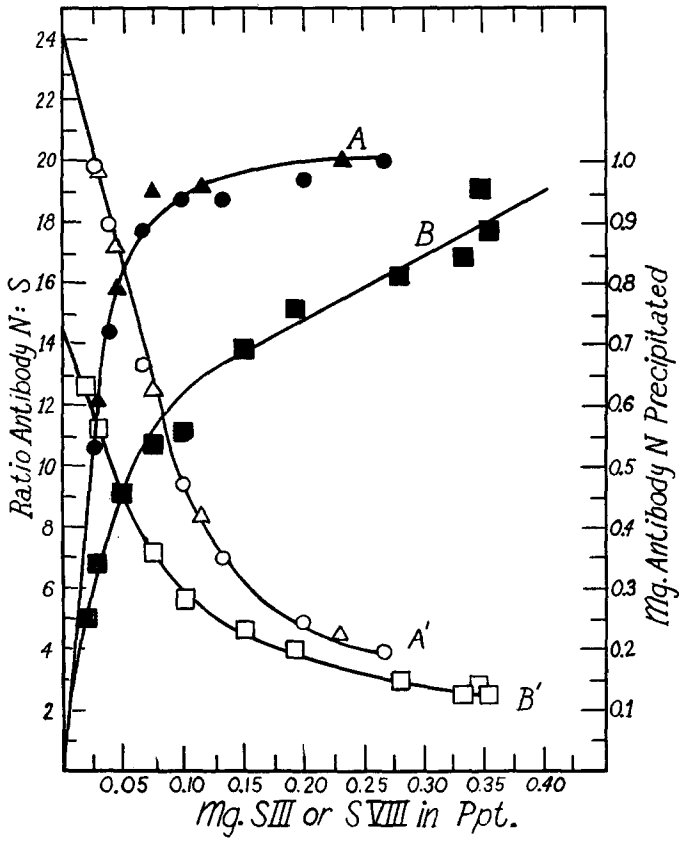


FIG. 1. Reaction of antipneumococcus Type III serum H 792 with S III and S VIII. Curves A and A′ are for the homologous reaction, B and B′ for the cross reaction. A′ and B′ are the respective A N:S ratio plots. Circles give the points obtained with the original serum and triangles those after removal of antibody reactive with S VIII. Not all points in Table I are included in the plot.

similar to that for the antipneumococcus Type VIII-S III reaction (1), consisting of a steep initial segment and a less steep, nearly straight portion, slope 1.1, along which there was considerable scattering of experimental points. Upon addition of larger amounts of S, the combining ratio of antibody N to S reached a lower limit showing that the precipitate attained constant composition. The large factors by which it was necessary to multiply the analytical data for the terminal portion of the curve rendered the slope of this segment uncertain.

TABLE II

Comparison of Total Antibody (3.0 Ml. Serum, 1:1) and Antibody Fractions in Cross Reaction at 0°C. between S III and Anti-Pn VIII Horse Serum 644

S III added	S III in precipitate		Antibody N precipitated	Ratio antibody N to S III in precipitate based on supernatant analyzed with H 644	Supernatants		
	By H 644*	By H 792‡			+ S III	+ Anti-Pn III horse serum	+ H 644
mg.	*mg.*	*mg.*	*mg.*				
0.017_6	Total		0.344	19.5	+++		
0.031_3	"		0.500	16.0			
0.047	"		0.638	13.6	+	±	−
0.071	"		0.706	9.9	+	±	−
0.113	0.110	0.110	0.786	7.1	−		
0.188	0.176	0.174	0.940	5.3	−		
0.245	0.220	0.213	1.04	4.7			
0.261	0.229	0.223	1.10	4.8			
0.353§	0.273	0.258	1.12	4.1			
0.423§	0.273	0.273	1.13	4.1			
0.522	0.30	0.29	1.15	3.8			
Antibody N Precipitated per 6.0 Ml. of H 644 A Solution by Varying Amounts of S III							
0.017_7	Total		0.314	17.7	+++		−‖
0.037_6**	"		0.514	13.7	−		−
0.062_6‡‡	"		0.572	9.1	−		−
0.106§	0.100		0.600	6.0	−		+

Equation: Antibody N precipitated = 21 S − 90 S^2; calculated values for A at the first 3 points are 0.312, 0.521, and 0.569, respectively

Antibody N Precipitated per 6.0 Ml. of H 644 B by Varying Amounts of S III							
0.060_4§§	0.057		0.334	5.9			
0.113**	0.095		0.430	4.5			
0.176‡‡	0.128		0.495	3.9			
0.282‖‖	0.156		0.516	3.3			
0.470‖‖	0.214		0.576	2.7			

* Supernatant analyses in region of excess S III, only, carried out with anti-Pn VIII serum H 644 and quantity found deducted from amount originally taken.

‡ Supernatant analyses in region of excess S III, only, carried out with anti-Pn III serum H 792.

§ 2.0 ml. of serum actually used for analyses, with corresponding amount of S III.

‖ Supernatants in this series were tested with H 644 A.

** 5.0 ml. of serum actually used, with corresponding amount of S III.

‡‡ 4.0 " " " " " " " " " " "

§§ 7.0 " " " " " " " " " " "

‖‖ 3.0 " " " " " " " " " " "

Experiment 2. Fractionation of Antipneumococcus Type VIII Horse Serum H 644, (1/5/37).—The equation describing the homologous reaction of this serum was

$$\text{mg. N precipitated} = 21.5\ S - 108\ S^2,\ A\ N = 1.07\ \text{mg.},$$

practically the same as that of a bleeding from the same horse taken one year earlier (1). The cross reaction data on this serum are given in Table II and Fig. 2. In this system, as in the S VIII-anti-S III reaction, the combining ratio of antibody to polysaccharide approached a constant value. The course of the reaction curve, A, in Fig. 2, was similar to that of the earlier bleeding (1). The slope of the linear portion, 2.4, was practically the same as that, 2.5, found in (1).

To 50 ml. of serum H 644 a quantity of S III was added corresponding to a point at the beginning of the linear portion of the curve in order to separate, if possible, the

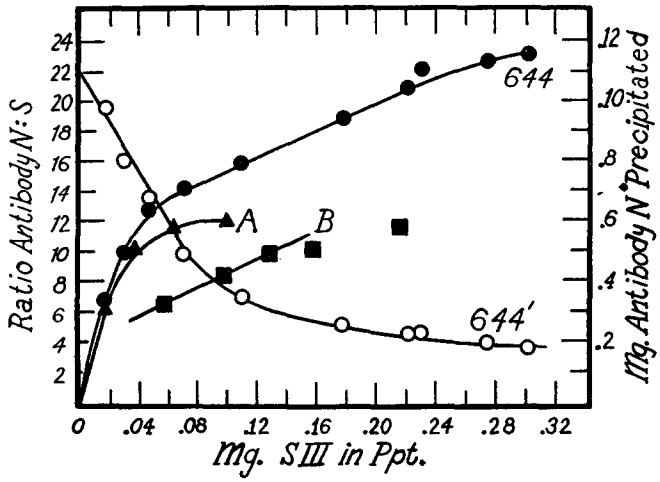


FIG. 2. Reaction of antipneumococcus Type VIII serum H 644 with S III.

antibodies characteristic of the different parts of the reaction plot. The specific precipitate obtained was dissociated by Liu and Wu's modification (7) of Felton's (8) dilute alkali method, and a series of precipitin analyses was carried out with the recovered antibody solution (644 A) and S III. The curve resembled that of the first portion of the cross reaction plot. The supernatant from the original precipitation, designated 644 B, was also analyzed by the quantitative precipitin method. The slope of the 644 B line was 2.2, or almost the same as that of the original serum. The results are given in Table II and plotted in Fig. 2.

Antipneumococcus Type VIII horse sera bled from horse 909 in 1937 and in 1939 were employed for further repetitions of the cross reaction experiments. The data are omitted since they closely resembled those given in Table II and Fig. 2. The slope of the linear portion of the curve for the 1937 bleeding was 2.9 and that for the 1939 bleeding was 2.8. The antibody:S III ratios also approached a lower limiting value as in the case of serum 644.

Experiment 3. Cross Precipitation at 37°C.—Data obtained at 37°C. on sera H 644

and H 909 (1939 bleeding) are given in Table III. The tubes were incubated for 0.5 hour, centrifuged at 37°C., and washed twice with saline in the cold. The supernatants were chilled and allowed to stand overnight in the ice box. Additional precipitate appeared in the supernatants which contained excess antigen. The sum of the antibody N precipitated at 37°C. and that deposited after chilling did not equal the quantity precipitated in analyses run at 0°C. throughout (*cf.* also (6*a*)).

Quantitative analyses in the two cross reacting systems showed a much greater error near the antibody maximum when run at 0° than at 37°C. Even with strict temperature control successive analyses often differed by as much as 0.05 mg. of N. This tendency seemed greater in certain sera than in others, and although the reason for it is not known, it recalls Goodner and Horsfall's observation (9) of coprecipitation

TABLE III

Antibody N Precipitated at 37°C. from 3.0 Ml. (1:1) Type VIII Antiserum H 644 by Varying Amounts of S III

Amount of S III added	Antibody N precipitated	Ratio antibody N to S III	Additional antibody N precipitated by subsequent chilling to 0°C.	Tests on supernatants
mg.	*mg.*		*mg.*	
0.020	0.250	12.5	0.003	Excess A
0.030	0.324	10.8	0.003	" "
0.050	0.424	8.3	0.003	" "
0.075	0.530		0.003	A and S
0.10	0.584		0.003	" " "
0.15	0.668		0.021	" " "
0.20	0.734		0.047	" " "
	2.0 Ml. Type VIII Antiserum 909			
0.127	0.504		0.084	Excess S
0.177*	0.528		0.070	" "

* Three-quarters of this amount and 1.5 ml. antiserum were actually used.

of Types I and II antibodies from polyvalent and mixed antipneumococcus horse sera at 0° while more strictly specific precipitation occurred at 37°C.

DISCUSSION

The quantitative theory of the precipitin reaction (2) involved the assumption that the numerous antibodies in antisera might be treated mathematically as a single substance of average reactivity, multivalent with respect to antigen, which, in turn, was multivalent with respect to antibody. In this way the aggregation and the multiple combining proportions characteristic of immune reactions such as the precipitin and agglutinin reactions could be quantitatively accounted for (3). It was, however, realized that antisera actually contain a complex mixture of antibodies differing possibly both in the number and kinds of groupings reactive with the antigen. Numerous instances were given (3) in which partial absorption of antisera with homologous antigen left behind antibodies different in reactivity from the assumed average in the original serum and characterized by a different equation.

In the first paper on the Type III-Type VIII cross reaction, however, it was shown that cross absorption did not result in an analogous fractionation of the antibodies with respect to their reactivity toward homologous polysaccharide (1). Although about one-third of the antibodies in a Type VIII antipneumococcus horse serum could be precipitated by S III, the precipitin reaction between S VIII and the Type VIII anticarbohydrate followed exactly the same course and gave the same equation, when calculated to the same antibody content, whether carried out in whole serum or in serum deprived of the considerable proportion of antibody cross reactive with S III. This has since been confirmed in parallel studies on other Type VIII antisera. It will be noted from Table I and Fig. 1 that a similar state of affairs obtains in Type III antiserum, the homologous reaction and equation, recalculated to the same amount of antibody, remaining the same after removal of the antibodies precipitated by S VIII. This may be taken to indicate a random distribution, among the antibodies of different reactivities, of groupings or configurations capable of reacting with heterologous specific polysaccharide.

While the homologous reaction pairs, S III-anti-S III and S VIII-anti-S VIII yielded curves of the type previously observed in horse sera (1, 2), the heterologous pairs, S III-anti-S VIII (1) and S VIII-anti-S III followed a different but mutually similar course. After an initial steep, curving portion in the plots (Figs. 1 and 2) resembling the homologous reactions there was a relatively sharp transition into a less steep, linear portion suggestive of the reaction plot found by Scherp for Type I meningococcus specific polysaccharide in polyvalent antimeningococcus horse sera (10), and in exaggerated measure, conforming to Goodner's presentation (11) of even homologous reaction curves as a series of small linear segments. Even along the linear portion of the cross reaction curve the composition of the specific precipitate varied, but tended to become constant at lower antibody N to S ratios as the amount of specific polysaccharide was increased. With 10 times the quantity of S giving maximum cross precipitation partial inhibition ensued in the S III-anti-S VIII reaction while in the S VIII-anti-S III system precipitation was merely delayed, not diminished. A further 5-fold increase in S resulted in complete inhibition of precipitation in the former system and nearly complete inhibition in the latter. In both reactions, then, soluble compounds are possible with lower antibody N:S ratios than the final constant-ratio insoluble compound, just as in homologous precipitin reactions (*cf.* also (12)).

It was noted that removal of the cross reacting antibodies corresponding to the initial curved segment of the plot resulted in the failure of about one-half of the remaining cross reacting antibody to precipitate with S III. A similar effect has been observed in the serial precipitation of many antisera by small amounts of antigen and has been interpreted on the assumption that the nonprecipitable portion of antibody is univalent with respect to antigen and can take part in aggregate formation only in the presence of sufficient multivalent

antibody (3, 13). On this basis, then, the entire linear portion of the cross reaction curve would be due to the addition of univalent (and by itself non-precipitable) antibody to S III-A aggregates formed with the multivalent antibody of the initial segment of the curve. In the attempted separation of the antibody into portions characteristic of the two segments it would seem that sufficient multivalent antibody remained with the linear portion, 644 B, to permit precipitation by S III of roughly one-half of the total cross reacting antibody. This is borne out by the failure of the line B in Fig. 2 to pass through the origin. The interpretation given also accounts for the drift in composition noted in Table II.

If one considers the linear segment as a separate curve, apart from the remainder of the plot, it is found that the quantity of antibody precipitated is directly proportional to the amount of S III added. Since combining proportions of antigen and antibody remain constant over this range, it is possible to calculate an immunological equivalent weight for S III in the cross reaction. The proportionality constant of antibody N to antigen, or the slope, equals 2.5 for serum H 644; multiplied by 6.3 this yields a protein: polysaccharide weight ratio of about 16. If 1,000,000 be taken as the molecular weight of horse antibody to pneumococcus (14), and its valence with respect to S III equal to 1, as discussed above, the equivalent weight of S III is found to be 62,000, which equals about 180 glucuronoglucose units. This would represent a minimum value for the molecular weight of S III. A similar calculation for S VIII, based on Experiment 1, leads to the value 140,000.

Of the numerous instances of cross reactivity studied some have been found to be reciprocal and others unilateral, but criteria are lacking by which predictions can be made as to which behavior a given cross reaction will show. The specificities and cross reactivities of azoantigens containing sugars and sugar acids as determinant groups have been studied by Avery and Goebel (15) and by Goebel (16) and have been related to the chemical structure and spatial relationship of the sugar haptens employed. The present study permits a correlation between the chemical constitution of two naturally occurring polysaccharides, S III and S VIII, and the quantitative behavior of the reciprocally similar cross reactions in both directions between these polysaccharides and horse antisera to Types III and VIII pneumococci.

Since S III (17) and S VIII (18) contain the same aldobionic acid as a structural unit, a measure of cross reactivity is to be expected. Evidence has been given, however, that the serologically reactive unit is a larger portion of the polysaccharide molecule than a single chemical structural unit (19, 1). While S III is a polymer of the aldobionic acid unit, S VIII contains, in addition, approximately two glucose molecules for every aldobionic acid residue (1, 18). Cross precipitation in either the S III-anti-S VIII system or the S VIII-anti-S III system would therefore involve only that fraction of the antibodies carrying reactive groupings of suitable configuration (*cf.* also Hooker and Boyd (20)).

An appreciable portion of the cross reacting fraction would be expected to contain more than one reactive grouping per antibody molecule. It might also be predicted from a knowledge of the structures of S III and S VIII (as yet complete only for S III (21)), that, weight for weight, the polyaldobionic acid S III would be a more efficient cross precipitant for anti-S VIII than S VIII for anti-S III. This would follow, because in S VIII the common aldobionic acid unit, to a multiple of which the cross reactivity is ascribed, comprises only about 60 per cent of the molecule whereas S III is wholly a polyaldobionic acid. That this prediction is borne out is shown by the slopes of the linear portions of the cross reaction curves. In the S III-anti-S VIII system these slopes are more than twice as great as that of the single S VIII-anti-S III system studied, so that along this portion of the curve more S VIII is required to precipitate a given weight of anti-S III than is necessary in the case of S III to precipitate the same weight of anti-S VIII. This not only holds for the linear segments of the curves, but also for the initial steep portions, as may readily be determined either from the intercepts of the line connecting the antibody N:S ratios of the first points in the curves, or by noting on Figs. 1 and 2 the amounts of antibody N brought down in the cross reactions by 0.01, 0.02, and 0.03 mg. of S III and S VIII, respectively.

Since the cross reactivities of S III and S VIII seem reciprocally equal to the proportion of common aldobionic (cellobiuronic) acid contained in each polysaccharide, the cross reactions being of the same type, but with numerical differences of the order expected from as much of the chemical structures as is known, a further, admittedly speculative, deduction might be drawn.

If one writes the structure of S III in conformity with (21), but schematically as

$$(a)\quad \cdot\left\{\mathrm{Gn}\cdot\mathrm{Gl}\right\}\cdot\left\{\mathrm{Gn}\cdot\mathrm{Gl}\right\}\cdot\left\{\mathrm{Gn}\cdot\mathrm{Gl}\right\}\cdot\left\{\mathrm{Gn}\cdot\mathrm{Gl}\right\}\cdots,$$

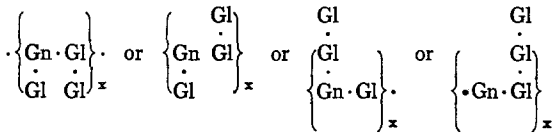
in which Gn = glucuronic acid and Gl = glucose residues, and brackets indicate cellobiuronic acid residues, a number of alternative formulas for S VIII containing the same unit suggest themselves:

$$(b)\quad \cdot\left\{\mathrm{Gn}\cdot\mathrm{Gl}\right\}\cdot\mathrm{Gl}\cdot\mathrm{Gl}\cdot\left\{\mathrm{Gn}\cdot\mathrm{Gl}\right\}\cdot\mathrm{Gl}\cdot\mathrm{Gl}\cdot\left\{\mathrm{Gn}\cdot\mathrm{Gl}\right\}\cdot\mathrm{Gl}\cdot\mathrm{Gl}\cdot\cdots,$$

$$(c)\quad \cdot\left\{\mathrm{Gn}\cdot\mathrm{Gl}\right\}\cdot\underset{\mathrm{Gl}}{\underset{\cdot}{\mathrm{Gl}}}\cdot\left\{\mathrm{Gn}\cdot\mathrm{Gl}\right\}\cdot\underset{\mathrm{Gl}}{\underset{\cdot}{\mathrm{Gl}}}\cdot\left\{\mathrm{Gn}\cdot\mathrm{Gl}\right\}\cdot\underset{\mathrm{Gl}}{\underset{\cdot}{\mathrm{Gl}}}\cdots,$$

$$(d)\quad \cdot\left\{\mathrm{Gn}\cdot\overset{\mathrm{Gl}}{\overset{\cdot}{\underset{\mathrm{Gl}}{\underset{\cdot}{\mathrm{Gl}}}}}\right\}\cdot\left\{\mathrm{Gn}\cdot\overset{\mathrm{Gl}}{\overset{\cdot}{\underset{\mathrm{Gl}}{\underset{\cdot}{\mathrm{Gl}}}}}\right\}\cdot\left\{\mathrm{Gn}\cdot\overset{\mathrm{Gl}}{\overset{\cdot}{\underset{\mathrm{Gl}}{\underset{\cdot}{\mathrm{Gl}}}}}\right\}\cdots.$$

In (*c*) and (*d*) the side-chain glucose residues are arbitrarily placed; if they were shifted, or placed in tandem in (*d*) the structural type would remain much the same. The great similarity of the cross reactions would seem to require as great a similarity in the structure of the two polysaccharides as possible. (*a*) and (*d*) have the same polyaldobionic acid chain, so that (*d*) or one of its alternative forms,



would be a distinctly probable structure, if serological data alone are considered. But unless S III and S VIII have greatly different molecular weights this type of structure seems less probable than one of the linear forms since the viscosities of the two polysaccharides in undegraded form (4, 1) are not very different. If one compares (*b*) and (*c*) with (*a*) and assumes similar linkages and spatial arrangements it becomes evident that in (*b*) every cellobiuronic acid unit would correspond spatially with alternate cellobiuronic acid units in (*a*), while (*c*) would show a periodicity of this kind only between alternate units in (*c*) and every third unit in (*a*). Therefore, taking all available chemical, physical, and quantitative serological evidence into consideration, (*b*) would seem the most probable structure for S VIII although (*d*) and (*c*) cannot, of course, be excluded. A definite answer must await methylation studies such as have led to the elucidation of the structure of S III.

Another rather unexpected by-product of the quantitative study of these cross reactions emerges from a consideration of the initial steep section of the curve in the S III-anti-S VIII reaction. In the equation of the line connecting the first four antibody N:S ratio points on curve 644′, Fig. 2, the slope, $-R^2/A = 171$, and 2R, the intercept on the ordinate, = 22. A, therefore, = 0.7. Since only the slope is affected in putting A = 1.0, the equation of the curve, obtained by multiplying both sides of the linear equation by S, becomes

$$\text{mg. antibody N precipitated} = 22\,S - 120\,S^2, \text{ at } A = 1.0$$

The equation of the homologous S VIII-anti-S VIII reaction calculated to the 1.0 mg. A level is

$$\text{mg. antibody N precipitated} = 21.5\,S - 116\,S^2$$

or practically identical with that of the cross reaction. Unfortunately the data given in (1) cannot be used for an additional test of this relationship since only one point on this part of the curve was determined. However, the dissociated antibody (644 A) from the fractionation of serum 644 (Table II, and Fig. 2, curve A) also has the same equation,

$$\text{mg. antibody N precipitated} = 21\,S - 114\,S^2,$$

as that of the homologous reaction when calculated to 1.0 mg. of cross reacting antibody N. The virtual identity of the equations indicates that an appreci-

able fraction of the antibody reacts in exactly the same way with S III and with S VIII. As far as can be determined by the sensitive quantitative method, this fraction of the antibody fails to distinguish between the two polysaccharides. This serves again to emphasize both the close structural relation between the two polysaccharides as well as the sharp differences in reactivity between different fractions of the antibodies elicited in an animal by immunization with a single antigen (*cf.* also (3)). These results also extend an earlier observation that the antibody dissociated from a specific precipitate of S III and anti-S VIII was entirely precipitable by either polysaccharide (22).

A corresponding antibody fraction with identical reactivity toward S III and S VIII was not found in the antipneumococcus Type III serum studied (Table I and Fig. 1). It will be noted that the equation of the initial segment of the cross reaction curve, calculated to 1.0 mg. of cross reacting antibody N in the same way as in the preceding instance,

$$\text{mg. antibody N precipitated} = 14.5\ S - 52.6\ S^2$$

is distinctly different from that of the homologous reaction,

$$\text{mg. antibody N precipitated} = 23.4\ S - 137\ S^2$$

at the same antibody N level. The failure of the two cross reactions to show strictly reciprocal behavior in this sense, while not predictable on the basis of available information, is at least in accord with the points stressed, since, as noted above, S VIII is only in part a polycellobiuronic acid such as S III.

Thus the S III-anti-S VIII and S VIII-anti-S III cross reactions show, in general, a reciprocal character that might have been expected from the close structural similarity of the specific polysaccharides comprising the hapten portion of the distinctive antigens of the two pneumococcus types. Quantitative analysis of the cross reactions, has, however, brought to light distinct differences in the course of the two reactions and permitted a correlation between these differences and the known chemical structures of the two polysaccharides.

It is also evident that at least three distinct kinds of anticarbohydrate[2] are evoked in horses in response to the stimulus of a type specific pneumococcus antigen such as, for example, that of Type VIII. Two of these forms make up the cross reactive fraction, which usually comprises one-quarter to one-third of the total. As already noted, this portion is completely precipitable by S III or by S VIII. It may be fractionated by means of the cross reacting polysaccharide, S III, into (1) a portion characterized by a sharply ascending reaction curve, and (2) a fraction showing a linear segment and ultimately constant composition of the specific precipitate. This, if it could be obtained entirely separate from the other fraction, would appear to be univalent with

[2] Antibodies to the somatic C-substance need not be considered here, since they were removed from all antisera in advance.

respect to S III, although not with respect to the homologous S VIII. Finally, after separation of the cross precipitable antibody from the antiserum, the principal antibody fraction (3), at least two-thirds of the total, is found to be rigidly type specific in that cross precipitation between Types III and VIII does not occur. Even this antibody is not homogenous, but is composed of fractions of varying reactivity toward the homologous polysaccharide as shown by the identity of its characteristic equation with that of the antiserum as a whole. The concept "antibody" would therefore seem to refer, not to serum globulin modified in a single manner, but to a series of modified globulins separable and identifiable as distinct fractions and limited in number mainly by the cross reactivities and quantitative criteria available for their characterization. These complexities are, however, far from hopeless, as they may be accounted for even quantitatively on the basis of varying numbers and kinds of reactive molecular groupings, for example, as in the present communication and in earlier papers from this laboratory.

SUMMARY

1. The cross reaction of the specific polysaccharide of Type VIII pneumococcus with Type III antipneumococcus horse serum has been studied quantitatively and found similar to the S III-anti-S VIII reaction.

2. Contrasted with the general similarity of the two-segment reaction curves were distinct qualitative and quantitative differences in the course and character of the reciprocal reactions with respect to each segment.

3. These differences could be interpreted in terms of the known chemical differences between the specific polysaccharides of the two types. A minimum molecular weight of 62,000 was calculated for S III and 140,000 for S VIII.

4. It was also found possible to fractionate the Type VIII antibody into portions characteristic of each segment of the cross reaction curve. At least three different kinds of Type III and Type VIII anticarbohydrates were identified.

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