QUANTITATIVE STUDIES ON ANTIBODY PURIFICATION

II. The Dissociation of Antibody from Pneumococcus Specific Precipitates and Specifically Agglutinated Pneumococci*

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In the first paper of this series (1), it was shown that quantitative study of the effect of salts on the precipitin reaction between pneumococcus polysaccharide and homologous antibody (2) had provided a theoretical basis for the dissociation of pneumococcus specific precipitates with strong salt solutions, since, in these solutions, the polysaccharide combined with less antibody than at physiological concentrations. As a result, it was found possible to pass in a single step from unconcentrated Types I and II antipneumococcus horse sera and Type III antipneumococcus rabbit sera to antibody solutions of which 85 to 93 per cent of the total nitrogen was immune nitrogen.

Less satisfactory results were obtained with Type III antipneumococcus horse sera, but in the present studies it is shown that this difficulty can readily be overcome. The application of the dissociation procedure to precipitates formed by the specific polysaccharide of Type I pneumococcus and Type I antipneumococcus rabbit sera gave irregular results, and attempts at the analysis of these irregularities are recorded. Experiments on the dissociation of Type VIII and C-anti-C precipitates are described and preliminary data are given on the extension of the dissociation procedure to specific precipitates formed by pneumococcus polysaccharides in antisera from pigs and sheep, and in a bovine antiserum. The last, a low grade

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serum, from an animal injected with Types I, II, and III pneumococci, readily yielded antibody to each in a high state of purity, the Type III antibody being analytically pure, or 100 per cent specifically precipitable.

For the formation and dissociation of pneumococcus specific precipitates it is necessary to isolate the specific polysaccharides, although it is probable that relatively crude preparations would suffice. Since there is now ample evidence that specific precipitation and specific bacterial agglutination are manifestations of the same chemical reactions (for a discussion, see (3)), it seemed probable that the salt dissociation procedure could be applied more simply to agglutinated pneumococci. It had been shown that the effect of salts on the agglutination of pneumococci by specific antiserum (3) is similar to their effect on specific precipitation, and the possibility of dissociating antibody from agglutinated pneumococci had already been demonstrated by Chickering (4) and Huntoon (5*a*), and quite recently by Chow and Wu (6). Salt dissociation of antibody from agglutinated *Salmonella* has also been studied by Duncan (5*b*). It is shown below that salt dissociation of specifically agglutinated pneumococci is readily effected, and that in some instances both the yield and degree of purity of the resulting antibody are higher than of the antibody from specific precipitates formed in the same sera. Moreover, the barium hydroxide modification (1) of Felton's alkali dissociation procedure (7) is also applicable to the residual undissociated agglutination complex.

Finally, a discussion is given of the significance of the findings and of several questions raised by recent work of Chow and Wu (6).

EXPERIMENTAL

1. Materials and Methods.—The specific polysaccharides used were prepared by the relatively mild procedures described in (8). Pneumococcus Type I suspensions consisted either of heat-killed organisms, or formalinized and heated suspensions as recommended recently (9) on account of their stability. Before use all suspensions were washed with saline until the washings were practically free from specific polysaccharide[1] in order to avoid the occurrence of precipitin reactions with the agglutination reaction it was desired to study. The horse sera used were

[1] Referred to throughout as S, with the appropriate numeral to designate type.

obtained from the New York City Department of Health through the courtesy of Dr. Wm. H. Park, Dr. K. G. Falk, and Miss A. W. Walter.

Analyses for precipitin nitrogen were made according to (10–12) by addition of a slight excess of homologous S to duplicate 1 to 5 ml. portions of the chilled antibody solution and determination of the nitrogen in the washed precipitate after 48 hours in the cold. Since the total nitrogen in the supernatants was extremely low, one washing with 2 to 3 ml. of chilled saline was considered sufficient. Blank tubes were run similarly with antibody solution alone, and micro Kjeldahl estimations were run separately on the supernatants (plus washings) of the blank tubes and on the usually negligible residues, the sum of the two giving the total nitrogen of the antibody solution. In the analyses of Type I antibody the small amount of S I nitrogen precipitated was deducted from the total nitrogen precipitated in order to give antibody nitrogen. As large aliquot portions as possible of the supernatants from the precipitin determinations were analyzed for agglutinin nitrogen according to (13), by addition to a measured volume of a suspension of homologous type specific pneumococci (Pn) and estimation of the increase in nitrogen over that in the Pn suspension alone after centrifugation and a single washing.[2] In some instances the total antibody nitrogen was determined by the agglutination method. For convenience analyses were calculated to the somewhat uncertain third decimal place.

2. Dissociation of Antibody from Specific Precipitates Formed in Antipneumococcus Sera of Various Species.—15 to 860 ml. of type specific antipneumococcus serum, or, in one instance Felton antibody solution (14), were precipitated at 0°C. unless otherwise indicated in the tables, with an amount of homologous S calculated to bring the system to the beginning of the equivalence zone (15) or to leave a small excess of antibody. After the precipitate had flocked, the mixture was decanted or centrifuged in the refrigerated centrifuge and the precipitate was evenly suspended in chilled saline and washed repeatedly with this until the amount of heat-coagulable protein extracted was at a minimum. Usually 4 to 7 washings were sufficient. Precipitates from Type I antipneumococcus rabbit sera obtained by successive small additions of S I, following a suggestion of Chow and Wu's (6), appeared more difficult to wash than those in which the S I had been added in a single portion. In the latter instance, however, more S was required, and the resulting antibody solution was not necessarily better than when the former procedure was used.

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[2] In applying differential analysis of supernatants for agglutinins (1) to whole sera, Goodner, Horsfall, and Dubos (9) found it necessary to use such small aliquots that the final result was multiplied by ten to give agglutinin in mg. per cc. The errors of three separate analyses are thus multiplied tenfold, and when these are cumulative, the product could easily equal the entire antibody content of low grade sera. It is to be regretted that Table 6 in the paper referred to does not include comparisons with the standard method (13). The necessity for ensuring an excess of Pn is also not stressed.

Many of the precipitates formed and washed at 0°C. were given one or two subsequent extractions with saline at 37°C. until the amount of protein removed was again at a minimum. These extracts were concentrated by dialysis against saline under negative pressure and analyzed for their antibody content (see tables).

After the saline washings the precipitates were extracted at about 37° with 10 to 40 ml. of 15 per cent sodium chloride solution (*cf.* 1) for 1 hour in the presence of a drop of toluene, and after centrifugation were usually extracted again with a smaller volume of the salt solution. In many instances the residual precipitates were suspended in water and treated in the cold with 0.5 to 1 ml. more of saturated barium hydroxide solution than necessary to dissolve the precipitate. After 1 hour in the cold 1 to 4 ml. of 10 per cent barium chloride solution were added and the mixture was neutralized with dilute acetic acid and centrifuged. The supernatant usually contained much antibody (*cf.* 1). Some of the precipitates from rabbit sera were very difficult to dissolve with barium hydroxide unless previously washed several times with water to remove salt and allowed to swell in water. All solutions were dialyzed in the cold against 0.9 per cent saline in the presence of a little toluene and were concentrated at the same time under negative pressure. Dialysis was continued until interferometer readings showed no increased salt concentration in the outer liquid; or, in the case of the barium hydroxide-treated solutions, until the outside saline was free from barium ion.

In using 15 per cent salt solution for the dissociation it was usually noted that a solubility effect was superimposed upon the equilibrium-shifting effect which occurred almost exclusively when 10 per cent sodium chloride solutions were used (1, 2). In the case of the stronger solutions precipitation usually occurred before dialysis had advanced very far. The precipitates were preferably centrifuged off in the cold before continuing the dialysis in order to prevent their reacting with additional antibody as the salt content diminished.

In Table I are given data on antibody recovered from Types III and VIII antipneumococcus horse sera. Since absorption with pneumococcus protein, as in preparation 792 L, seemed of no advantage, other lots of these sera were absorbed only with pneumococcus C substance (16) before precipitation with S. The same low grade Type III serum was used as in (1), so that the higher purity of the antibody seems due to the more thorough washing given the specific precipitate before dissociation. The Type VIII sera were precipitated with S III in order to study differences in the cross reactivity of the dissociated antibody from that of the original serum.

In Table II are given data on the dissociation of C-anti-C precipitates obtained in the preliminary absorptions of the three anti-Pn horse sera used for the experiments recorded in Table I. The C precipitates were washed in the cold as usual. The seventh and final washing of the precipitate from serum 644 still contained considerable protein and antibody. The residues were then extracted several times with saline at 37°C. (solutions C_1 and CC_1) and finally with 15 per cent salt solution at 37°C. (solutions C_2 and CC_2). Recovery of anti-C was very poor, but the 37° saline extracts contained surprisingly large amounts of type specific anti-

body. Attempts to recover anti-C from precipitates in Type I antipneumococcus rabbit sera were even less encouraging, nor could anti-S I be recovered at 37° from the C-anti-C precipitates previously washed at 0°.

TABLE I

Antibody Solutions from the Dissociation of Specific Precipitates from Types III and VIII Antipneumococcus Horse Sera

Serum and preparation number	Antibody N taken	Precipitin + agglutinin N in recovered antibody solution	Recovery of antibody N	Total N	Precipitin N	Agglutinin N	Antibody N	Antibody N / Total N: 37°, 0.9 per cent NaCl extract	Antibody N / Total N: 15 per cent NaCl extract	Antibody N / Total N: Ba(OH)$_2$ dissociated solution
				per ml. of antibody solution						
	mg.	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*
Type III										
792 L	70.5	16.8	24	0.322	0.282	0.015	0.297		92	
N*	39.5	9.1	23	0.251	0.230	0.000	0.230		92	
P†	28.2	6.4	23	0.183	0.167	0.000	0.167		91	
Q	67.7‡	11.2(P)	17	0.322	0.295		0.295(P)			92(P)
{R	41.4	1.1	3	0.074	0.027	0.013	0.040	54		
{T		9.9	24	0.236	0.223	0.009	0.232		98	
{DD	196	1.6	0.8	0.271		0.101	0.101	37		
{EE		24.3	12	0.908	0.860	0.008	0.868		96	
{FF		4.5	2.3	0.310	0.154	0.029	0.183			59
Type VIII; serum precipitated with S III										
644 {D	77.0§	3.1(P)	4	0.194	0.123§		0.123(P)	63(P)		
				0.194	0.169‖		0.169(P)	87(P)		
{E		13.6(P)	18	0.697	0.665§		0.665(P)		95(P)	
				0.697	0.686‖		0.686(P)		98(P)	
909 {D	53.8§	3.4(P)	6	0.210	0.170§		0.170(P)	81(P)		
				0.210	0.195‖		0.195(P)	93(P)		
{E		8.5(P)	16	0.373	0.345§		0.345(P)		92(P)	
				0.373	0.356‖		0.356(P)		95(P)	

(P) after analyses indicates precipitin N only.

* Serum precipitated first with 0.2 mg. S III to remove much of precipitable lipids; precipitate discarded.

† Precipitated at 37°, washed with chilled saline.

‡ Residues, after N and P had been extracted, dissociated by $Ba(OH)_2$ method (1).

§ With S III.

‖ With S VIII.

Experiments on the dissociation of specific precipitates from Type I antipneumococcus rabbit sera are summarized in Table III. Solution 193 C was obtained by 15 per cent salt dissociation of the precipitate from S I and two pooled sera of rather low antibody content, without previous absorption with C substance. The residual precipitate was washed with chilled water, centrifuged, and ground in a mortar with two other pooled low grade sera containing an additional 11.2 mg. of antibody N in 83 ml. After washing, followed by extraction with 15 per cent salt

TABLE II

Antibody Solutions from Dissociation of C-Anti-C Precipitates from Antipneumococcus Horse Sera

Serum and preparation number	Anti-C N taken	Anti-C N in recovered antibody solution	Recovery of anti-C N	Total N	Anti-C N	Anti-S III N	Total antibody N	Antibody N / Total N	
				per ml. of antibody solution				37°, 0.9 per cent NaCl extract	15 per cent NaCl extract
	mg.	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*
Type III									
792 CC_1	56	1.0	1.8	0.152	0.054	0.028*†	0.105	69	
CC_2		0.9	1.6	0.069	0.039	0	0.039(P)		57(P)
						Anti-S VIII N			
Type VIII									
644 C_1		2.3		0.248	0.041	0.191*‡	0.232(P)	94(P)	
C_2		1.1		0.153	0.054	0			35(P)
909 C_1	24	0.5	2.1	0.146	0.029	0.051*	0.080(P)	55(P)	
C_2		0.5	2.1	0.061	0.025	Trace*			41(P)

(P) after analyses indicates precipitin N only.

* After removal of anti-C.

† After removal of anti-C and anti-S III the supernatant gave 0.023 agglutinin N with Pn III.

‡ 0.071 mg. of this also reacts as anti-S III N.

solution (solution D), the residue was dissociated with barium hydroxide and barium chloride as in (1). For the next three solutions 36.5 ml. of serum 14.46, containing 2.12 mg. of anti-S I per ml., were absorbed with C substance, the residual anti-S I content being 1.78 mg. per ml. The serum was then diluted with 2 volumes of chilled saline and precipitated at 0° with eight successive 1 mg. portions of S I. The sixth washing at 0° and a seventh at 37° were combined and dialyzed against saline in the cold under negative pressure (solution F). An eighth washing, also at 37°, contained only traces of protein and was discarded. Both

the strong salt and barium hydroxide extracts of this specific precipitate yielded highly pure antibody (G and H), but repetition of the above procedure on a larger scale with a mixture of lower grade sera (K) gave a poor product. The remaining

TABLE III

Antibody Solutions from Dissociation of Specific Precipitates from Types I and III Antipneumococcus Rabbit Sera

Serum and preparation number		Antibody N taken	Precipitin + agglutinin N in recovered antibody solution	Recovery of antibody N	Total N	Precipitin N	Agglutinin N	Antibody N	Antibody N / Total N		
					per ml. of antibody solution				37°, 0.9 per cent NaCl extract	15 per cent NaCl extract	Ba(OH)$_2$ dissociated solution
		mg.	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*
Type I											
193	C	45.4	3.4	7.5	0.137	0.099	0.010	0.109		80*	
	D	56.6	5.8	10.2	0.251	0.152	0.023	0.175		70*	
	E		4.4	7.8	0.161	0.129	0.009	0.138			86
	F	65.0	2.2	3.4	0.070	0.049	0.004	0.053	76†		
	G		7.1	10.9	0.215	0.204	0.004	0.208		97	
	H		9.8	15.1	0.269	0.251	0.000	0.251			93
	K	177	12.8	7.2	0.976	0.495	0.121	0.616		63	
4.77	L	204	2.9	1.4	0.203	0.151	0.010	0.161	79		
	M		20.0	9.8	1.224	0.833	0.159	0.992		81	
	N		4.9	2.4	0.247	0.213	0.006	0.219			89
	N'		17.0	8.3	1.054	0.897‡	0.048	0.945			90
4.75_2	P	85.2	0.4	0.5	0.039	0.010	0.004	0.014	36		
	Q		1.1	1.3	0.056	0.041	0.004	0.045		80	
	R		5.9	6.9	0.344	0.277	0.019	0.296			86
4.53	B	39.5	2.2	5.6	0.263	0.139	0.017	0.156		59*	
	C		5.7	14.4	0.605	0.418	0.008	0.426			70
	E§	34.3	0.6	1.8	0.088	0.041	0.012	0.053		63	
	F		7.2	21.0	0.538	0.411	0.013	0.424			79
Type III											
199	A	102‖	14.0	13.7	0.551	0.461	0.016	0.477		87*	
	B		2.6	2.5	0.133	0.105	0.000	0.105			79

* Precipitate not extracted first at 37° with saline.

† Sixth washing at 0° combined with a seventh at 37°C.

‡ One analysis discarded.

§ Precipitated at 37°.

‖ Approximate antibody N content.

solutions were prepared from high grade sera containing more than 2.0 mg. of antibody N per ml.[3] but the recovered antibody solutions were rarely more than 80 per cent pure. Possible reasons for this are given in the Discussion. Too little barium hydroxide was probably used for solution N, and the residue was accordingly dissociated once more with the same reagent (N′).

Table IV summarizes a rather comprehensive experiment with a bovine serum, of which nearly 1 liter was available.[4] The animal had been injected intravenously with a mixture of formalin-killed Types I, II, and III pneumococci, and the serum, sterile and without preservative, contained a total of 0.48 mg. per ml. of anti-S I, II, and III. Without previous absorption with C substance 860 ml. of the serum were precipitated at 0° with three successive 2.5 mg. portions of S I. The precipitate was centrifuged off (fraction B) and about 800 ml. of the supernatant were precipitated with three 2 mg. portions of S II (fraction C). The supernatant from this was precipitated similarly with 6 mg. of S III (fraction D). Since appreciable amounts of antibody, especially of anti-S III, remained in the supernatant, this was again precipitated with 2.1 mg. of S III (fraction D_4). The specific precipitates were washed thoroughly in the cold with saline and were then extracted successively with 0.9 per cent sodium chloride solution at 37°C. (B_1, C_1, D_1), 15 per cent sodium chloride solution at 37° (B_2, C_2, D_2), and barium hydroxide followed by barium chloride at 0° (B_3, C_3, D_3). Fractions C_3 and D_3 required larger amounts of barium hydroxide than usual, and much of the precipitate failed to dissolve, so that the greater alkalinity, coupled with longer exposure to alkali, may have been the cause of the unsatisfactory quality of the alkali-treated fractions. Slightly more than one-fifth of the precipitin in the serum was recovered in the various fractions. Fraction D_2 represents the only analytically pure antibody solution recovered thus far in the work, although it will be seen from the tables that others were obtained assaying 95 per cent and higher. Analyses in Table IV for heterologous type precipitins were made on the supernatants from the homologous precipitation; experiments in the reversed order are given in the footnotes to the table.

In Table V are given data on the purification of Type I pneumococcus anticarbohydrate formed in the pig and the sheep. Both sera were very weak, con-

[3] Several rabbit antisera containing more than 4 mg. of anti-S I N per ml. were encountered in the course of the work. One of these contained 5.0 mg. of anti-S I N per ml. and 0.8 mg. of agglutinin N in the supernatant, or over 36 mg. of antibody protein per ml., an amount far higher than any previously reported (*cf.*, for example, 11, 17). This serum contained 14.0 mg. of N and 8.7 mg. of globulin N per ml. (Howe micro method), so that two-thirds of the globulin present, or over 40 per cent of the total protein, actually consisted of antibody.

[4] The material was supplied through the courtesy of Dr. John Reichel, Director of the Mulford Biological Laboratories of Sharp and Dohme, Glenolden, Pennsylvania.

TABLE IV

Antibody Solutions from the Dissociation of Specific Precipitates from a Bovine Type I, II, and III Serum

Serum 1224 D Preparation	Precipitant	Antibody N taken (3 types)	Precipitin + agglutinin N in recovered antibody solution	Recovery of antibody N	Total N	Anti-S I precipitin N	Anti-S II precipitin N	Anti-S III precipitin N	Agglutinin N	Antibody N	Antibody N / Total N: 37°, 0.9 per cent NaCl extract	Antibody N / Total N: 15 per cent NaCl extract	Antibody N / Total N: $Ba(OH)_2$ dissociated solution
					per ml. of recovered antibody solution								
		mg.	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*
B_1	S I	413	4.7	1.1	0.299	**0.223***	0.009	0.001		0.233(P)	78(P)		
B_2			10.5	2.5	0.540	**0.510**	0.005†		0.007	0.522		97	
B_3			9.4	2.3	0.598	**0.575**			0.005	0.580			97
C_1	S II	389	7.0	1.8	0.402	0.000	**0.276**			0.276(P)	69(P)		
C_2			3.8	1.0	0.187	0.003‡	**0.167**	0.000		0.170(P)		91	
C_3			1.3	0.3	0.149	0.000	**0.057**	0.000	0.000	0.057			38
D_1	S III		8.4	2.2	0.449		0.013§	**0.402**		0.415(P)	92(P)		
D_2			23.2	6.0	1.007‖	0.000		**1.007**	0.003	1.010		100	
D_3			1.9	0.5	0.291**		0.002††	**0.102**		0.104(P)			36(P)
D_4‡‡			14.5	3.7	0.913			**0.796§§**		0.796(P)		87(P)	

(P) after analyses indicates precipitin N only.

* S I + S II together gave 0.225, S II first gave 0.036 mg. N per ml.

† S II first, followed by S I, gave resp., 0.013, 0.515 mg. N per ml.

‡ S I before S II gave 0.010 mg. N per ml.

§ S II, then S III gave 0.042, 0.369 mg. N per ml., resp.

‖ S II before S III gave 0.052 mg. N per ml; followed by S I, no N was precipitated.

** On determination only.

†† Anti-S I plus anti-S II.

‡‡ From precipitation of residual serum with additional S III.

§§ S II before S III gave 0.016 mg. N per ml; followed by S I, no N was precipitated.

taining only 0.31 and 0.12 mg. of anti-S I N per ml., respectively, so that a high degree of purity could scarcely be expected for the dissociated antibody. A purer end-product was obtained, using as starting material a Felton solution (14) prepared by pouring the pig serum into 30 volumes of 0.001 M phosphate buffer at pH 5, but the yield by this dual process was very poor.

3. Dissociation of Antibody from Specifically Agglutinated Pneumococci.—In this study Type I pneumococci and Type I antipneumococcus sera were used,

TABLE V

Antibody Solutions from the Dissociation of Specific Precipitates from Type I Antipneumococcus Pig and Sheep Sera

Serum and preparation number	Antibody N taken	Precipitin + agglutinin N in recovered antibody solution	Recovery of antibody N	Total N	Precipitin N	Agglutinin N	Antibody N	Antibody N / Total N		
				per ml. of antibody solution				37°, 0.9 per cent NaCl extract	15 per cent NaCl extract	Ba $(OH)_2$ dissociated solution
	mg.	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*
Pig serum										
198 A	174	1.4	0.8	0.519	0.033	0.046	0.079	15		
B		12.5	7.2	1.920	0.317*	0.514	0.831		43	
C		3.6	2.1	0.353	0.186	0.049	0.235			67
AA	124	2.8	2.3	0.365		0.089	0.089	24		
BB		9.6	7.7	0.723		0.383	0.383		53	
CC		5.9	4.8	0.453	0.323	0.015	0.338			75
D†	124	24.4(P)	20	1.068	0.205					
E‡	23.8	4.4	18.5	0.307	0.187*	0.074	0.261		85	
Sheep serum										
202 A	96	2.4	2.5	0.945	0.054	0.114	0.168	18		
B		2.1	2.2	0.500	0.088	0.148	0.236		47	
C		0.6	0.6	0.078	0.040	0.005	0.045			58

* Insufficient S I used.

† Felton solution. 19 per cent of total N precipitable by S I.

‡ D used as starting material for dissociation.

since sera were available with which comparable experiments had been made on the dissociation of specific precipitates. Extension of the method to the purification of antibody of the other types is being undertaken.

Amounts of antiserum ranging from 10 to 680 ml. were diluted with an equal volume of saline, except in the case of the weak pig and sheep sera, of which the largest volumes were used. Freshly washed, heat-killed or formalin- and heat-treated Pn I suspension was added at 0°C. or at 37°C. in 2 to 25 ml. portions,

TABLE VI

Antibody Solutions Prepared by Dissociation from Pneumococci Agglutinated in Type I Antipneumococcus Sera

Serum and preparation number	Antibody N taken	Precipitin + agglutinin N in recovered antibody solution	Recovery of antibody N	Total N	Precipitin N	Agglutinin N	Antibody N	Antibody N/Total N		
				per ml. of antibody solution				37°, 0.9 per cent NaCl extract	15 per cent NaCl extract	Ba(OH)$_2$ dissociated solution
	mg.	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*
Horse										
701,2 A*	42.7	13.2	31	0.430	0.396†		>0.396(P)		>92(P)	
701,2 B		7.3	17	0.314	0.305		0.305(P)			97(P)
7032 A*		10.1		0.575	0.516	0.000	0.516		90	
7032 B		1.8		0.173	0.151		0.151(P)			87(P)
Rabbit										
4.53 A*	56.8	0.8	1.4	0.084		0.039	0.039	46		
4.53 B		3.9	6.9	0.277	0.185	0.021	0.206		74	
4.53 C		22.1	39	1.318	1.125	0.040	1.165			88
4.56$_2$ A‡	70.4	6.5	9.2	0.305	0.242	0.014	0.256		84	
4.56$_2$ B		29.7	42	1.706	1.206†	0.400	1.606			94
4.56$_2$ C§	70.4	4.0	5.7	0.286	0.125	0.053	0.178		62	
4.56$_2$ D		26.7	38	1.374	1.154†	0.118	1.272			93
4.75$_2$ A*	54.0	0	0	0.026	0.000		0(P)	0(P)		
4.75$_2$ B		2.3	4.3	0.112	0.093		0.093(P)		83(P)	
4.75$_2$ C		24.2	45	1.466	1.422		1.422(P)			97(P)
4.75$_2$ D*	59.6	0.4	0.7	0.067		0.023	0.023	34		
4.75$_2$ E‖		2.2	3.7	0.222		0.150	0.150		68	
4.75$_2$ F		24.5	41	1.296	1.130	0.035	1.165			90
Pig										
198 F‡	62	15.3	25	0.802	0.499	0.078	0.577		72	
198 G		2.1	3.4	0.102	0.063	0.013	0.076			75
Sheep										
202 D§	82	5.1	6.2	1.177	0.128	0.105	0.233		20	
202 E		6.1	7.4	0.407	0.237	0.052	0.289			71

(P) after analyses indicates precipitin N only.

* Agglutination carried out at 37°, washed at 0°C.

† Insufficient S I used.

‡ Agglutination and washing carried out at 37°C.

§ Agglutination and washing carried out at 0°C.

‖ Differed from 4.75$_2$ B only in use of mechanical stirrer during 15 per cent salt extraction at 37° for 3 hours.

depending on the bacterial N content, which varied from 2.8 to 0.4 mg. per ml. Addition of the Pn I was continued, with frequent centrifugation, until agglutination of the added cells occurred only slowly, or until the supernatant gave only a faint precipitin test with S I. The agglutination complex was thoroughly washed with saline at the appropriate temperature. After three to seven washings, the supernatant usually contained only traces of heat-coagulable protein. In some of the 0° experiments the material, after washing at 0°, was given one or two additional washings at 37°C. and analyses were made of the resulting solution after concentration by dialysis against saline under negative pressure.

For the extraction of the washed residue it seemed most advantageous to smooth out all lumps during the addition of 10 to 30 ml. of 15 per cent sodium chloride solution, allow the mixture to stand overnight at 37° in the presence of a little toluene, centrifuge, and then wash once at 37° with one-third to one-half of the initial amount of 15 per cent salt solution. An inferior product resulted in the one instance in which mechanical stirring was used. The antibody solutions were dialyzed and concentrated as in section 2. The extracted residues were submitted to dissociation by barium hydroxide and barium chloride (using 4 ml. of 10 per cent solution of the latter) in the cold as in the case of the specific precipitates. The properties of the resulting solutions are summarized in Table VI.

Preparation 701,2 was a mixture of 47 ml. of absorbed serum 701, 12 ml. of serum 702, and 11 ml. of a Felton solution B 78, the properties of which had been described in (3). Preparation 7032, of unknown antibody content, was absorbed with C substance before dilution and use. Although the rabbit sera were not absorbed with C substance before addition of the Pn I the only dissociated antibody tested, 4.75_2C, contained no anti-C. The data obtained with serum 4.56_2 indicate that purer antibody may be dissociated from Pn I agglutinated in rabbit serum at 37° than from cells agglutinated at 0°, although it is probable that in this case also, a preliminary washing with saline at 37°, as in 4.75_2 A and B, would have improved the antibody subsequently dissociated by means of strong salt. In all of the rabbit sera the yields from the salt dissociation of the agglutination complex were small, while the recovery by the barium hydroxide method was extraordinarily high. Antibody recovery at 0° from the very weak sheep antiserum was disappointing.

DISCUSSION

In the first report (1) on a theoretically indicated procedure (2) for the dissociation of precipitates formed by pneumococcus specific polysaccharides and homologous antisera it was shown that antibody assaying over 85 per cent of immunologically reactive material was readily obtainable in a single step from Types I and II antipneumococcus horse sera and Type III antipneumococcus rabbit sera. It is now found that when the initial specific precipitates are adequately

washed Type III antipneumococcus horse sera also yield antibody (anticarbohydrate) solutions in which 92 to 98 per cent of the nitrogen present is immune nitrogen. In two of these solutions (792 N and P, Table I) the anticarbohydrate appeared to be entirely in the native state, since it was quantitatively removable as precipitin, as in untreated sera (18, 1), leaving no residual agglutinin. The purest solution, 792 T, was obtained by dissociation with strong salt after precipitation and washing of the serum at 0°C. and a preliminary extraction with 0.9 per cent salt solution at 37°C. It is probable, from the analysis of sample 792 P, that solutions of equal purity could be obtained by carrying out both the precipitation and washing at 37°, although in this case a portion of the total antibody would remain unprecipitated (*cf.* 12).

It was also found possible to attain the same degree of purity with the cross reacting anticarbohydrate precipitated by S III from two different Type VIII antipneumococcus horse sera (Table I). In both of the solutions obtained by dissociation with strong salt 97 per cent of the precipitin present (anti-S VIII) was specifically precipitable by S III, which had been used for the initial precipitation. It is thus shown that the Type VIII antisera contain a fraction of antibody which reacts practically as completely with S III, the heterologous cross reacting polysaccharide, as with S VIII, the homologous specific carbohydrate. An attempt will be made to obtain enough of this antibody for study, as it should afford a closer insight into the factors involved in this instance of cross reactivity (19).

While the dissociation of the C-anti-C precipitates from Types III and VIII antipneumococcus horse sera yielded little antibody and that of low purity from the standpoint of anti-C content, the solutions showed several features of interest (Table II). Two of the three 0.9 per cent saline extracts made at 37° after precipitation and washing at 0° contained more type specific antibody than anti-C. Thus the precipitin nitrogen, anti-C N plus type specific N, of one of the solutions, 644 C_1, was 94 per cent of the total nitrogen although the anti-C content was only 17 per cent of the total. Since the saline extract therefore contained almost pure precipitin, the anti-S VIII could scarcely have been adsorbed non-specifically on the C precipitate, but was presumably attached owing to a low degree of immunological

and therefore chemical cross reactivity, possibly in much the same way as the fraction of low grade antibody precipitated from many sera when an excess of antigen is added in one portion, but left behind in the serial precipitation of antibody (*cf.*, for example, 17 *a*). In favor of this view is the somewhat higher proportion of precipitin reactive with S III in this extract than in the original serum, and also the failure of similar extracts of C-anti-C precipitates derived from Type I antipneumococcus rabbit sera to show more than traces of anti-S I. The three 15 per cent salt extracts contained only anti-C and no appreciable amounts of anti-S III or anti-S VIII, so that the type specific antibody appears to be bound by the precipitate only at 0° and to be given off entirely on washing with 0.9 per cent saline at 37°. This is in accord with the finding of Goodner and Horsfall (20 *c*) that cross reactive antibody precipitable at 0°, as we had found (1), does not come down with the homologous specific precipitate at 37°. However, the assumption made by these workers that the precipitated cross reactive antibody is held by adsorption is not sustained by the evidence just presented.

With Type I antipneumococcus rabbit sera it was found (Table III) that occasional sera yielded highly pure antibody by the salt dissociation method as readily as did the Type III rabbit sera previously studied (1). Other sera, whether precipitated at 0° or at 37° and regardless of their antibody content, furnished in poor yield antibody solutions in which not more than one-half to three-quarters of the nitrogen was immune nitrogen. Characteristic, also, of some of these solutions was the relatively high proportion of antibody which could not be precipitated by S I but was recoverable as agglutinin, even in the case of sera which had previously been absorbed with C substance.

A possible reason for the relatively unsatisfactory antibody recovery on dissociation of the Type I rabbit precipitates may be the low antibody:S I ratios in these precipitates (21). On account of their high content of S I the shift in equilibrium between antibody and S I on the addition of strong salt (1, 2) might not be as extensive as in the case of other specific precipitates containing less polysaccharide, resulting in poor yields and a relatively higher proportion of impurities. Application of the barium hydroxide modification (1) of Felton's dissociation method (7) to the salt-extracted agglutination

residues gives particularly good results both as to yield and analytical purity (see Table VI).

Appended to Table III are data on antibody solutions obtained directly from low grade, pooled Type III anti-Pn rabbit sera, showing that both dissociation methods may be applied even to sera of low antibody content.

The data in Table IV not only indicate the utility of the salt dissociation method for the purification of antibodies in a low grade bovine anti-Pn serum, but show that, in spite of the injection of the animal with Types I, II, and III pneumococci, antibody to each type was separately recovered in an exceedingly high state of purity. Since the type specific antibodies were separately precipitable from the serum by the appropriate polysaccharide, and the dissociated antibody of each type showed little cross reactivity with the other types, it is again evident (*cf.* 1) that in a polyvalent serum most, if not all, of the antibodies to each pneumococcus type occur as molecules distinct from those of the other types. The data also provide the first instance of the preparation, from raw serum without previous concentration, of analytically pure antibody (solution D_2).

Even though analytically pure antibody has been prepared, it cannot be considered as a single chemical entity, for earlier work has shown that pneumococcus anticarbohydrate (15, 1) and even antibody to a single crystalline antigen such as egg albumin (17 *a*, 23) consist of a separable mixture of antibodies of differing reactivity.

Initial experiments on low grade pig and sheep sera (Table V) were less encouraging. It was found, however, that preparation of a Felton solution from the pig serum as a preliminary step (198 D) permitted the preparation of antibody of a high degree of purity (198 E).

The salt dissociation of agglutinated Pn I proved so satisfactory and simple of execution that it would seem to be the method of choice for the rapid preparation from antipneumococcus sera of highly pure antibody solutions, especially if the agglutination be carried out at 37°C. (Table VI). Horse antisera (without preliminary concentration) readily yielded solutions in which 90 per cent of the nitrogen was recoverable by precipitation with S I. With the rabbit antisera the salt-dissociated antibody was at least as pure as that dissociated

from specific precipitates, and the yield appeared somewhat better. A direct comparison is afforded by the data on serum 4.75_2 in Tables III and VI. In all of the rabbit antisera between one-third and one-half of the total serum antibody could be recovered from the agglutination complex remaining after salt dissociation by use of the barium hydroxide-barium chloride method. Not only were the yields extremely high, but the analytical purity of the resulting antibody ranged from 88 to 97 per cent. Applied to the pig serum the agglutination procedure gave a 25 per cent yield of salt-dissociated antibody of a higher degree of purity than given by the precipitin method (Tables V and VI). The result with sheep serum at 0°, the only temperature used, was not as good.

Although the dissociation of agglutinated Type I pneumococci gave solutions equalling or exceeding in yield and analytical purity those from the corresponding treatment of specific precipitates, and the procedure offers the additional advantage that isolation of specific polysaccharide is unnecessary, it must be borne in mind that antibody solutions prepared from agglutinated pneumococci, especially those dissociated by the alkaline barium hydroxide method, may contain pneumococcus protein. While in many of the solutions this does not seem to be present in sufficient amounts to affect the analytical results, its occurrence might be of biological importance. It is planned to test these solutions for bacterial protein.

Confirmation of the identity of pneumococcus anticarbohydrate agglutinin and precipitin (*cf.* 18) by an independent method is also afforded by several of the experiments summarized in Table VI. It will be noted that in solutions 701,2 B, 7032 A, 4.75_2 C, and 4.75_2 F, 97 to 100 per cent of the antibody nitrogen present was precipitable by S I. Since this antibody had originally been removed from horse and rabbit sera as agglutinin its quantitative recovery as precipitin again shows that the only difference between anticarbohydrate agglutinin and precipitin is a difference in the distribution and state of the pneumococcus polysaccharide serving as a reagent for both: in the case of agglutinin, the polysaccharide reagent is bound to the pneumococcus cell; in the case of precipitin the polysaccharide exists free in solution. The correlation between precipitin and protective antibody (10, 24) is not as simple, especially with antibody produced

in the horse, as Goodner and Horsfall (20) have recently shown. In this added respect they have confirmed our laboratory's finding that pneumococcus anticarbohydrate, especially that produced in the horse (22, 15, 19), is not a single substance, but a series of substances of differing degrees of reactivity.

The generally high proportion of antibody in the 0.9 per cent saline, 37°C. extracts of specific precipitates or agglutinated pneumococci prepared at 0° is in accord with observations that more antibody is analytically demonstrable in pneumococcus antisera at 0° than at 37° (12, 15, 21, 3). Moreover, the dissociation of this fraction of the antibody in physiological saline at 37° is predictable if specific precipitation and agglutination are regarded as reversible chemical reactions (15, 3).

It has frequently been assumed that in specific precipitation and agglutination the antibody taking part is denatured. It has been shown, however, that these immune reactions may be qualitatively (25) and quantitatively (15, 17 *a*, 3, 26) accounted for without making this assumption, and, indeed, there would seem no experimental foundation for its use. Nevertheless, Chow and Wu (6) have used the reversal of an assumed denaturation as the explanation of the recovery of antibody from S-anti-S precipitates and agglutinated pneumococci by their modification of Felton's alkali dissociation procedure (7). While experimental verification of this may eventually be forthcoming, Chow and Wu have claimed "immunological purity" for their recovered antibody on the basis of a correction for the solubility of specific precipitates formed from the antibody. That such a correction is inadmissible is shown by the virtual insolubility at the temperature of precipitation of S-anti-S precipitates from horse sera (15), the lower solubility of corresponding precipitates from rabbit sera (21) than that used for the correction by Chow and Wu, and the frequent preparation in this laboratory (1, and the present work) and by Goodner and Horsfall (20) of antibody solutions showing an analytical purity of 97 to 100 per cent without any deduction for solubility. One such solution, prepared by alkali dissociation, was examined in the ultracentrifuge (27) and showed evidence of molecular degradation in spite of the high degree of analytical purity. If degradation too slight to affect the precipitating value could be caused

by the brief exposure of the specific precipitate to alkali used in the present studies, the longer alkali treatment employed by Chow and Wu might possibly necessitate a correction for impurities due to greater degradation, but such material could not be called pure antibody.

SUMMARY

1. The salt dissociation and barium hydroxide-barium chloride methods are extended to the preparation of highly purified antibody solutions from specific precipitates derived from Type III and Type VIII antipneumococcus horse sera and a low grade polyvalent bovine serum. Analytically pure precipitin (agglutinin) was obtained from the last, and Types I, II, and III antibodies were separated.

2. Difficulties connected with the application of the methods to Type I antipneumococcus rabbit sera are described, as is also the purification of antibody from low grade pig and sheep sera.

3. The dissociation of antibody by both methods from Type I pneumococci agglutinated in antisera produced in the horse, rabbit, pig, and sheep, is described and its advantages discussed.

4. Certain theoretical aspects of the work are also discussed.

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