QUANTITATIVE EXPERIMENTS WITH ANTIBODIES TO A SPECIFIC PRECIPITATE. I* †

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In addition to the chemical and physical studies (2, 3) which form the basis for the recognition of antibodies as modified globulins, there have been numerous investigations on the antigenic behavior of antibodies and their relation to the normal serum globulins. For example, Braun (4) demonstrated the ability of bacteria agglutinated with horse serum to sensitize guinea pigs to the latter. Landsteiner and Prasek (5) showed that precipitins for horse serum would remove agglutinins from antityphoid horse sera. More recently, antibodies have been produced by immunization with specific precipitates (6, 7). In their use non-specific protein may be removed by thorough washing, leaving relatively pure antibody for injection, especially in those instances in which pneumococcus polysaccharides are used to precipitate the antibody. It will be recalled (8) that these polysaccharides are non-antigenic in the rabbit.

In a comprehensive series of immunological papers (6) Ando and his coworkers have demonstrated that two antigenic fractions of horse serum, designated A and B, are related to antibodies. The antitoxic globulins constitute a part of the A fraction, and the antibacterial antibodies (with the probable exception of the Shiga dysentery anticarbohydrate) belong to the B group. By absorption tests and other methods it was concluded that typhoidal, plague, and Type I pneumococcal antibodies from horse sera are almost, if not completely identical as antigens. Although their investigation was primarily undertaken to see whether euglobulin and pseudoglobulin occur as such in whole horse serum, Marrack and Duff (7) have also tested a number of different horse serum fractions and a purified antibody solution against an anti-specific precipitate rabbit serum.

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The present investigation was undertaken to examine more fully the utility of specific precipitates both as immunizing and as test antigens in an adaptation of the quantitative agglutinin method (9) developed in this laboratory. Quantitative information was desired on the chemical relations not only of antibodies to the different serological types of pneumococcus, but also of antibodies directed against a somatic component, the so called C polysaccharide of the pneumococcal cell. Information was also desired on pepsin-digested antibody. Accordingly, a specific precipitate derived from antipneumococcus Type II horse serum was used as antigen for the injection of rabbits and the resulting sera were tested as described below.

Methods and Materials

Preparation of the Immunizing Antigen Suspension.—100 ml. of anti-Type II pneumococcus horse serum H 513,[1] bleeding Dec. 13, 1938, without preservative, were diluted to 235 ml. with 0.9 per cent saline warmed to 37°C. and a solution of 13.5 mg. of Type II pneumococcus specific carbohydrate[2] (10) was run in with stirring. After an hour the floccules were centrifuged, carefully rubbed up into a thin cream with 25 ml. of saline, and washed five times at 37°C., with centrifuging, until supernatants yielded a constant minimal precipitate on boiling. The specific precipitate was then finely ground with additions of saline, and made up with saline to 175 ml. Merthiolate[3] was added to a final concentration of 1:10,000. The resulting suspension contained 6.96 mg. of protein per ml., or 1.10 mg. of antibody N, corresponding to an average antibody N/S II ratio of 14.3 in the precipitate. Two lots were prepared in this way.

Rabbits were injected intravenously with the suspension, at first in diluted form. Doses of from 3.4 to 10 mg. of protein were given four times weekly for the first eleven injections. The first bleeding was taken 7 days after the last injection. A second course of eight injections, of from 1.7 to 5.0 mg. of protein, was followed by a rest period of a week, after which the rabbits were bled out. The serum was preserved by the addition of 1:10,000 of merthiolate.[3] All experiments were made with a 1:1 dilution of this serum with saline, neutralized to pH 6.9 or 7.0.

Preparation of Test Antigen Specific Precipitate Suspensions.—After precipitation with the appropriate polysaccharides or antigens all suspensions were carefully rubbed up into thin creams and washed with 0.9 per cent saline until supernatants showed no precipitate or only a slight constant turbidity on boiling. Usually about five washings sufficed. The precipitates were uniformly suspended in saline containing 1:10,000 merthiolate. The antibody N/antigen ratio was calculated from the amount of antigen used, the volume of suspension obtained, and the nitrogen analysis, for all antigen was assumed to be precipitated as its amount was chosen in each instance so that an excess of antibody remained (*cf.* 11).

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[1] Obtained through the courtesy of Dr. A. B. Wadsworth and Dr. H. W. Lyall of the New York State Department of Health Laboratories.

[2] Subsequently referred to as S, with the appropriate type numeral.

[3] Manufactured by Eli Lilly and Company, Indianapolis.

Precipitate (*a*): S II-anti-S II specific precipitate with high antibody N/S II ratio: 1.32 mg. of S II were added at 37°C. to a dilution of 18 ml. of horse serum H 513. Ratio antibody N/S II = 10.8.

(*b*) S II-anti-S II specific precipitate of lower ratio: 2.67 mg. of S II and 20 ml. of serum H 513 were used. Ratio = 6.7.

(*c*) S I-anti-S I specific precipitate with higher antibody N/S I ratio: 3.92 mg. of S I were added to 50 ml. of Type I anti-Pn[4] horse serum diluted with 150 ml. of saline, at room temperature, then left at 0°C. overnight. Ratio of antibody N/S I = 8.8.

(*d*) S I-anti-S I specific precipitate of lower ratio: 2.41 mg. of S I and 15 ml. of serum used. Ratio = 4.0. Another suspension with a ratio of 5.1 was also used.

(*e*) Pn C-anti-C specific precipitate of higher antibody N/Pn C ratio: 4 mg. of C substance (12) derived from the Type I pneumococcus were added to 350 ml. of Type VIII anti-Pn horse serum H 909[5] at 0°C. Ratio approximately 6.

(*f*) Pn C-anti-C specific precipitate of lower ratio: To the supernatant serum from (*e*) was added an additional 14 mg. of C substance. Ratio approximately 2.

(*g*) *Haemophilus influenzae*, type B-anti-influenza (horse) specific precipitate: 75 ml. of anti-influenza, type B, horse serum[6] were diluted to 112 ml. with saline and precipitated with crude *H. influenzae* type B polysaccharide (13).

(*h*) Diphtheria toxoid-antitoxin (horse) floccules: 690 ml. of crude toxoid[5] containing approximately 32 Lf per ml. were stirred into 15.5 ml. of horse diphtheria antitoxin containing 1480 units per ml. After 1½ hours at 42–45°C. the solution was left in the ice box overnight, warmed again at 42°C. for an hour, and then centrifuged at 3,000 R.P.M. for several hours. Yield of washed suspension, 32 mg. N.

(*i*) Ea[7]-anti-Ea (horse) specific precipitate: 33 ml. of horse antiserum to crystalline egg albumin[8] (14, 15) were diluted with saline to 110 ml. and 1.28 mg. Ea N added, at 0°C. Ratio antibody N/Ea N = 7.5.

Other Specific Precipitate Suspensions.—Specific precipitates were prepared from anti-Pn II rabbit serum and from anti-Pn I pig serum. Both were tested quantitatively against the anti-specific precipitate rabbit serum but carried down no nitrogen.

Type II Antipneumococcus Horse Antibody Solution.—93.5 ml. of antipneumococcus horse serum H 513 were diluted to 250 ml. with saline and 24.5 mg. of S II added at 37°C. After 1½ hours the precipitate was centrifuged and washed five times. It was then dissociated for 45 minutes at 37°C. with 10 per cent sodium acetate solution, a modification of the procedure used in (16). The precipitate was centrifuged off, and the supernatant dialyzed against 0.9 per cent saline. Precipitin antibody N/total N in solution = 76 per cent.

Type I Antipneumococcus Horse Antibody Solution.[9]—A similar preparation was made

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[4] Pn used for pneumococcus.

[5] Kindly supplied by Dr. R. H. Muckenfuss of the New York City Board of Health Laboratories.

[6] Supplied to Dr. Hattie E. Alexander through the courtesy of the Serum Laboratory of the Massachusetts State Board of Health.

[7] Ea used for crystalline egg albumin.

[8] Obtained through the courtesy of Dr. A. M. Pappenheimer, Jr.

[9] Prepared in this laboratory by Dr. Bertil Josephson of the Caroline Medical University, Stockholm, Sweden.

from a Type I antipneumococcus horse serum by dissociation with 10 per cent K_2HPO_4 solution. Purity = 77 per cent.

Analytical Procedure.—Varying amounts of the suspensions of the test antigen specific precipitates, of known nitrogen content, were measured out with calibrated pipettes into 1.0 ml. of the rabbit antiserum against Pn II horse specific precipitate at 0°C. In

TABLE I

Precipitation of Rabbit Anti-Pn II Horse Specific Precipitate Serum by Pn II Specific Precipitates, at 0°C., 48 Hours, per 1.0 Ml. of Serum

Suspension N added	Total N pptd.	Antibody N pptd. (by difference)	$\frac{\text{Antibody N}}{\text{Antigen N}}$
mg.	*mg.*	*mg.*	
Test antigen: Horse anti-Pn II sp. ppt., ratio antibody N/S II = 10.8			
0.448*	0.760	0.312	0.70
0.672*	1.078	0.406	0.60
0.896*	1.352	0.456†	0.51
1.120*	1.614	0.494‡	0.44
Test antigen: Horse anti-Pn II sp. ppt., ratio = 6.9			
0.636	0.974	0.338	0.53
0.954	1.372	0.418	0.44
1.272	1.756	0.484§	
1.590*	2.070	0.480§	
Same test antigen resuspended after 8 mos. in ice box			
0.192‖	0.314	0.122	0.64
0.288**	0.464	0.176	0.61
0.384††	0.591	0.207	0.54
0.576	0.810	0.234	0.41

* Single determination only.

† Supernatant yielded 0.05 mg. N with a high ratio horse Pn I specific precipitate suspension.

‡ From an aliquot portion of the supernatant, antigen in excess precipitated N equivalent to 0.024 mg. on the entire supernatant, or 0.521 mg. total antibody N.

§ Supernatants yielded no more antibody to a high ratio horse Pn I specific precipitate suspension.

‖ Triple quantities of antigen and serum were actually used for analysis.

** Double quantities actually used for analysis.

†† One and one-half quantities " " "

the region of antibody excess, multiple quantities of both antigen and antiserum were usually used for greater accuracy. The tubes were allowed to stand for 48 hours at 0°C., with occasional mixing, and were washed twice with saline, and the precipitates analyzed. The difference between the total nitrogen brought down and the antigen nitrogen used was taken as antibody nitrogen (Tables I to VI, VIII, IX). This procedure is essentially that of the quantitative agglutinin method (9), with specific precipitates substituted for bacterial suspensions. The determinations with soluble antigens were made similarly

TABLE II

Precipitation of Rabbit Anti-Pn II Horse Specific Precipitate Serum by Pn I Specific Precipitates, at 0°C., 48 Hours, per 1.0 Ml. of Serum Used

Suspension N added	Total N pptd.	Antibody N pptd. (by difference)	Antibody N / Antigen N
mg.	*mg.*	*mg.*	
Test antigen: Horse anti-Type I pneumococcus sp. ppt., ratio antibody N/S I = 8.6			
0.172*	0.342	0.170	0.99
0.344†	0.629	0.285	0.83
0.688	1.120	0.432	0.63
1.032	1.510	0.478	0.46
1.376	1.876	0.500	
2.064‡	2.560	0.496§	
Test antigen: Horse anti-Type I pneumococcus sp. ppt., ratio = 4.0			
0.222†	0.343	0.121	0.55
0.444	0.636	0.192	0.43
0.888	1.254	0.366	0.41
1.776‡	2.224	0.448	0.25

* Quadruple quantities actually used for analysis.
† Double quantities " " " "
‡ One-half quantities " " " "
§ Analysis of supernatant gave no more antibody.

TABLE III

Precipitation of Rabbit Anti-Pn II Horse Specific Precipitate Serum by Pn C-Anti-C Precipitates, at 0°C., 48 Hours, per 1.0 Ml. of Serum Used

Suspension N added	Total N pptd.	Antibody N pptd. (by difference)	Antibody N / Antigen N
mg.	*mg.*	*mg.*	
Test antigen: Pn C-anti-C sp. ppt. from a horse anti-Type VIII pneumococcus serum, ratio antibody N/Pn C = 5.9			
0.091*	0.315	0.224	2.5
0.137†	0.410	0.273	2.0
0.195‡	0.632	0.437§	2.2
0.390	0.894	0.504‖	
Test antigen: Pn C-anti-C sp. ppt. from a horse anti-Type VIII pneumococcus serum, ratio 1.9			
0.281	0.726	0.445	1.6
0.375	0.849	0.474	1.3
0.750	1.258	0.508‖	

* Triple quantities of antigen and serum actually used for analysis.
† Double quantities actually used.
‡ One and one-half times this quantity actually used.
§ Supernatant gave 0.064 mg. N with high ratio Pn II specific precipitate suspension.
‖ Supernatant added no more antibody N to a high ratio horse Pn II specific precipitate suspension.

by the quantitative precipitin method (17). Excess antigen in the supernatants was determined as previously described (18).

Experiments with Anti-Pn II (Horse) Specific Precipitate Rabbit Serum after Absorption with Ea-Anti-Ea (Horse) Specific Precipitate.—

To 24 ml. of 1:1 diluted anti-Ea horse serum[8] (14, 15) there was added 0.84 mg. of Ea N, an amount well within the flocculating range. The precipitate was allowed to stand for 24 hours and was then centrifuged off, washed five times with 0.9 per cent saline, and resuspended in saline. The yield was 11.2 ml. of a suspension containing

TABLE IV

Precipitation of Rabbit Anti-Pn II Horse Specific Precipitate Serum by Haemophilus influenzae, Type B Specific Precipitate, at 0°C., 48 Hours, per 1.0 Ml. of Serum Used

Suspension N added	Total N pptd.	Antibody N pptd. (by difference)	Antibody N / Antigen N
mg.	*mg.*	*mg.*	
Test antigen: Horse anti-*H. influenzae* type B sp. ppt. susp.			
0.086*	0.242	0.156	1.8
0.115†	0.307	0.192	1.7
0.172‡	0.422	0.250	1.5
0.344§	0.756	0.412	1.2
0.516	1.002	0.486	0.94
0.688	1.214	0.526‖	

* Quadruple quantities actually used.
† Triple " " "
‡ Double " " "
§ One and one-half " " "
‖ Supernatants added no more antibody N to a high ratio Pn I specific precipitate suspension.

0.588 mg. N per ml. 10 ml. of the suspension were added to 10 ml. of the anti-specific precipitate rabbit serum and left for 48 hours at 0°C. with occasional stirring. It was calculated (Table V) that this quantity of precipitate N would remove all of the antibodies reactive with this precipitate. After centrifugation the absorbed serum was set up with a high ratio Pn I specific precipitate suspension, except for the first point, for which a similar Pn II suspension was used. The results, given in Table VI, are calculated to an antibody N content of 0.50 mg. per ml. for comparison with 1.0 ml. of the unabsorbed serum.

Experiments with Pepsin-Digested Type I Antipneumococcus Horse Antibody.—

100 ml. of Type I antipneumococcus horse serum were absorbed with Pn C substance derived from Pn II organisms. In order to start with partially purified Type I antibody the supernatant serum was first precipitated by dialysis for 4 days in the cold

TABLE V

Precipitation of Rabbit Anti-Pn II Horse Specific Precipitate Serum by Various Specific Precipitates, at 0°C., 48 Hours, per 1.0 Ml. of Serum Used

Suspension N added	Total N pptd.	Antibody N pptd. (by difference)	$\frac{\text{Antibody N}}{\text{Antigen N}}$
mg.	*mg.*	*mg.*	
Test antigen: Horse anti-crystalline egg albumin sp. ppt. susp., ratio 5.6			
0.076*	0.228	0.152	2.0
0.101†	0.277	0.176	1.7
0.151‡	0.360	0.209	1.4
0.159§	0.363	0.204	1.3
0.201‖	0.408	0.207	1.0
0.239§	0.455	0.216	0.90
0.302‡	0.535	0.233	0.77
0.478§	0.728	0.250	
0.604	0.862	0.258	
Test antigen: Toxoid-antitoxin floccules from horse anti-diphtheria serum			
0.242‡	0.471	0.229	0.95
0.483	0.744	0.261	0.54
0.966	1.246	0.280	0.29
1.449	1.760	0.311**	

* Quadruple quantities of antigen and serum actually used.

† Triple quantities actually used.

‡ Double quantities " "

§ These values are from an earlier experiment with the same antiserum and a specific precipitate with the same ratio and indicate the close reproducibility of the data. Single determinations were run in this series.

‖ One and one-half quantities actually used.

** Supernatants added no more antibody N to toxoid-antitoxin floccules but gave 0.04 mg. antibody N to Ea-anti-Ea (horse) specific precipitate (0.25 mg. N), calculated to 1.0 ml. serum; further complete absorption with high ratio Pn C-anti-C (Pn VIII) specific precipitate yielded an additional 0.16 mg. antibody N. Total removed from 1.0 ml. serum was 0.51 mg. antibody N.

TABLE VI

Addition of Anti-Pn I and Anti-Pn II Horse Specific Precipitate Suspensions to Rabbit Anti-Pn II Horse Specific Precipitate Serum Absorbed with Ea-Anti-Ea (Horse) Specific Precipitate Suspension; 0°C., 48 Hours. Calculated to 0.5 Mg. Antibody N, for Comparison with Unabsorbed Serum

Antigen N added	Total N pptd.	Antibody N pptd. (by difference)	$\frac{\text{Antibody N}}{\text{Antigen N}}$
mg.	*mg.*	*mg.*	
0.233*†	0.368	0.135	0.58
0.584*‡	0.921	0.337	0.58
0.715*‡	1.094	0.379	0.53
1.078‡	1.552	0.474	
1.430‡	1.909	0.479	

* Single determination only because of limited amount of serum.

† High ratio Pn II specific precipitate suspension used.

‡ " " Pn I " " " "

TABLE VII

Precipitation of Rabbit Anti-Specific Precipitate Serum by Purified Horse Antibody Solutions, per 1.0 Ml. Rabbit Serum, 0°C., 48 Hours

Antigen N added	Antigen N pptd.	Total N pptd.	Antibody N pptd.	$\frac{\text{Antibody N}}{\text{Antigen N}}$	Tests on supernatants
mg.	*mg.*	*mg.*	*mg.*		
Test antigen: Salt-dissociated antibody solution from anti-Pn II horse serum					
0.020_5	Total*	0.185	0.164	8.0	Excess RA†
0.041	"	0.335	0.294	7.2	" "
0.082	"	0.506	0.424	5.2	" "
0.124	"	0.626	0.502	4.0	No Ra or Ha†
0.247	0.244‡	0.782	0.538	2.2	Slight excess HA

$$\frac{\text{mg. antibody N}}{\text{mg. antigen N}} \text{ pptd.} = 10.9 - 19.5\sqrt{\text{antigen N}}$$

Test antigen: Salt-dissociated antibody solution from anti-Pn I horse serum					
0.027_6	Total*	0.235	0.207	7.5	Excess RA
0.055	"	0.374	0.319	5.8	" "
0.083§	"	0.480	0.397	4.8	" "
0.110	"	0.560	0.450	4.1	" "
0.138	"	0.614	0.476	3.5	No Ra or Ha
0.165	"	0.658	0.493	3.0	" " " "
0.193	"	0.702	0.509	2.6	Trace HA

$$\frac{\text{mg. antibody N}}{\text{mg. antigen N}} \text{ pptd.} = 9.9\tfrac{1}{2} - 17.3\sqrt{\text{antigen N}}$$

* Assuming all N of the purified horse antibody solution to be precipitable by an excess of the antiserum.

† RA = rabbit antibody. HA = horse antibody used as antigen in the experiment.

‡ From analysis on supernatant.

§ Double quantities of antigen and serum used.

TABLE VIII

Precipitation of Rabbit Anti-Specific Precipitate Serum by Specific Precipitates from Undigested and Digested Antibody, per 1.0 Ml. Serum, 0°C., 48 Hours

Suspension N used	Total N pptd.	Antibody N pptd. (by difference)
mg.	*mg.*	*mg.*
Test antigen: Sp. ppt. from undigested Pn I horse antibody soln.		
0.244	0.596	0.352
0.488	0.991	0.506
Test antigen: Sp. ppt. from digested Pn I horse antibody soln.		
0.368	0.721	0.353
0.736	1.199	0.463
1.104	1.603	0.499

against 0.029 M phosphate buffer at pH 6.8, a method essentially that of Felton (19). The precipitate was collected, washed with cold buffer solution, and redissolved in 50 ml. of 0.9 per cent saline.

Pepsin was partially purified, but not crystallized, according to (20). 25 ml. of the antibody solution were adjusted to pH 4.70 with HCl, cooled to 0°C., and 2.5 ml. of the pepsin solution (6.15 mg. N per ml.) added (*cf.* 21). An immediate precipitate formed. The mixture was allowed to stand at 0°C. with frequent stirring for 5½ hours and was then neutralized to pH 7.0 to destroy the enzyme. 20 ml. of the partially digested antibody solution were precipitated with 1.2 mg. of S I, and the specific precipitate was thoroughly washed before being made up into a test antigen suspension. Ratio antibody N/S I = 6.1. As a control, 3.9 ml. of the undigested antibody solution were precipitated with 0.57 mg. of S I. The specific precipitate was washed and resuspended. Ratio N/S I = 6.2. Both the "digested" and "undigested" specific precipitate suspensions were used as test antigens against the anti-Pn II (horse) specific precipitate rabbit serum (Table VIII).

Qualitative Experiments with Anti-Pn II (Horse) Specific Precipitate Rabbit Serum.—

1. Several drops of anti-Pn II horse serum were added to a relatively large amount of the anti-specific precipitate rabbit serum. This left in solution an excess of rabbit antibody but precipitated all of the Pn II antibody in the horse serum added, since a test portion of the supernatant did not agglutinate a Pn II bacterial suspension even after several hours. The original precipitate was washed and evenly resuspended in saline. When a few drops of 1:10,000 S II solution were added reagglutination took place (*cf.* 22 *a*), the precipitate settling much more rapidly than a control suspension without polysaccharide. The effect could be greatly enhanced by substituting a washed Pn II bacterial suspension for the polysaccharide.

2. 0.5 ml. of anti-Ea horse serum was precipitated with 0.02 mg. of Ea N. The precipitate was washed five times with saline. 0.2 mg. Ea N was then added; complete solution of the precipitate required about 2 days. One drop of this inhibition zone solution added to the anti-specific precipitate rabbit serum gave immediate precipitation. A control tube with the rabbit serum and Ea alone was negative.

3. A guinea pig was prepared[10] with an intraperitoneal injection of 0.20 ml. of undiluted anti-Pn II (horse) specific precipitate rabbit serum. 24 hours later an intravenous injection of 0.15 ml. of Pn I rabbit antiserum was given. There were no reactions up to 25 minutes. A second injection of 0.20 ml. of Pn II rabbit antiserum was then administered again without result for 25 minutes. Finally 0.12 ml. of normal horse serum were given intravenously. Within a minute the animal showed typical anaphylactic signs, with respiratory difficulty and dilated pupils. Paralysis and death occurred in 4 minutes. Control pigs given only an intravenous injection of 0.20 ml. normal horse serum or anti-precipitate rabbit serum showed no signs of injury immediately or after 24 hours.

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[10] The assistance of Dr. Joseph C. Turner in this experiment is gratefully acknowledged.

DISCUSSION

One of the greatest drawbacks in the use of serum proteins in immunological studies is the heterogeneity of the fractions usually obtained by chemical or physical means. This difficulty may be avoided in great part, however, if advantage is taken of the immunological properties of antibodies since these may be separated from the normal serum proteins or from one another by specific precipitation. Studies along these lines, already explored in part by Ando and collaborators (6), and by Marrack and Duff (7), have been carried out in the present instance with an immunizing antigen consisting of the specific precipitate from S II and an anti-Type II pneumococcus horse serum.

The antipneumococcus horse serum H 513 used for preparation of the immunizing suspension contained 2.24 mg. of antibody nitrogen per ml. by direct analysis, but only enough polysaccharide was added to precipitate 86 per cent of the antibody. This resulted in the high antibody N/S II ratio of 14.3 in the precipitate, a value about twice that at the antigen excess end of the equivalence zone for this serum. The antibody in the precipitate could therefore still combine with considerable S II (see pages 126, 127). It seemed desirable that the immunizing antigen contain free groupings capable of reacting with polysaccharide, in order to test whether such reactive groupings could produce antibodies in the rabbit directed specifically toward them.

With one exception specific precipitates derived from Pn II, Pn I, Pn VIII (anti-C), and *H. influenzae* horse antisera, when used in excess as test antigens, removed the same amount of antibody N, within experimental error, from the anti-Pn II horse specific precipitate rabbit serum (Table IX). The amount of the rabbit antibody N removed by a given amount of suspension N varied somewhat with each suspension. Specific precipitate suspensions having the higher ratios of antibody N/S were more effective per mg. of (horse) protein N in removing antibody from the anti-precipitate rabbit serum than those of lower ratio (Tables I, II, III). The total amount of rabbit antibody removed by excess suspension was not affected, however, except that one of the two low ratio Pn I specific precipitates used removed only 64 per cent of the total antibody present (Table IX). It is not yet possible to state whether the decrease in the antibody N removed per mg. of suspension N of lower ratio added is due to actual combination of the larger quantities of S or antigen with groupings antigenically active toward the anti-precipitate rabbit serum, or whether such groupings are sterically shielded and prevented from reacting by the combination of S or antigen with neighboring groupings possessing only antibody function.

A noticeable decrease in the antibody N/antigen N ratio occurred in the reaction of the lower ratio Pn II specific precipitate suspension with anti-precipitate serum when repeated 8 months after preparation of the suspension (Table I). This effect, which might be due either to changes in physical state or to intermolecular rearrangements, may be related to the greater difficulty with which some precipitates dissolve in excess antigen after aging (23).

The Pn C-anti-C specific precipitate suspensions were unusually active in precipitating antibody, as judged by the ratios in the last column of

TABLE IX

Summary of Maximum Amount of Antibody N Precipitated from Rabbit Anti-Pn II Horse Specific Precipitate Serum by Various Antigens, at 0°C., 48 Hours, per 1.0 Ml. Serum

Test antigen (horse)	Maximum antibody N precipitated per ml. serum
	mg.
Anti-Pn II, high ratio sp. ppt	0.52
" Pn II, low ratio sp. ppt	0.48
" Pn I, high ratio sp. ppt	0.50
" Pn C, higher ratio sp. ppt	0.50
" Pn C, lower ratio sp. ppt	0.51
" *H. influenzae*, type B, sp. ppt	0.53
Ea-anti-Ea sp. ppt	0.26
Diphtheria toxoid-antitoxin floccules	0.31
Pn II anticarbohydrate solution	0.54
Pn I anticarbohydrate solution	0.51
Sp. ppt. from partially digested (pepsin) Pn I anticarbohydrate	0.50
Anti-Pn II (rabbit) sp. ppt	0
" Pn I (pig) sp. ppt	0

Table III. The viscosity of the C substance is materially lower than those of the type specific polysaccharides of pneumococcus, indicating either a lower molecular weight or different shape. Either alternative might permit reaction of the C substance with a larger number of horse antibody molecules per unit of weight used.

The reaction of Ea[7] with its antibody in the horse is singular in that it exhibits the zone phenomenon characteristic of toxin-antitoxin reactions in the horse (14, 15), rather than the typical precipitin reaction given by Ea-anti-Ea in the rabbit (11). Like diphtheria antitoxin, antibody to Ea in the horse is largely in the water-soluble pseudoglobulin fraction in contrast to the other, water-insoluble, horse antibodies employed in this work. It might be expected, therefore, that the specific precipitates of these two classes of proteins should exhibit differences in behavior toward

the anti-Pn II horse specific precipitate rabbit serum. Thus the Ea-anti-Ea specific precipitate removed only 51 per cent, and the diphtheria toxoid-antitoxin floccules 61 per cent of the rabbit antibody taken out by the pneumococcus specific precipitates (Tables V, IX). That the first two antigens remove essentially the same antibodies is further demonstrated by the failure of Ea-anti-Ea specific precipitate to combine with appreciable antibody in rabbit anti-Pn II specific precipitate serum which had been absorbed with toxoid-antitoxin floccules (Table V, footnote).

Moreover, the antibody N removed from the antiprecipitate rabbit serum by the Pn C-anti-C specific precipitate alone (Table III) equalled the sum of that precipitated by toxoid-antitoxin floccules (Table V) and the portion subsequently recovered from the supernatant with Pn C-anti-C specific precipitate (Table V, footnote), showing that the antibodies which combine with the water-soluble protein group also form a part of those uniting with the water-insoluble group and differ only in cross reactivity.

The narrow flocculating limits of the antitoxic type of reaction do not permit the preparation of specific precipitate suspensions of much higher ratio than those of the equivalence zone. That the lower amount of antibody nitrogen removed by specific precipitates derived from the water-soluble proteins (Table V) is not merely a low ratio effect is indicated, however, by other evidence. The experiments of Ando and his collaborators (6) show distinct antigenic differences between their water-soluble A group, of which the antitoxic antibodies form a part, and the water-insoluble B group which contains the antibacterial antibodies. Furthermore, Marrack and Duff (7) have found that water-soluble pseudoglobulin from horse serum precipitated less antibody from anti-specific precipitate rabbit serum than did the water-insoluble euglobulin. When their anti-specific precipitate serum was absorbed with pseudoglobulin, the supernatant still gave a positive test with euglobulin, whereas absorption of the serum with euglobulin removed all of the antibodies to pseudoglobulin, although a positive test was still obtained with a salt-dissociated antibody solution.

The quantitative course of the reaction of the anti-specific precipitate serum with various specific precipitates (Tables I to V) or with dissociated antibody solutions (Table VII) may be represented by the linear equation

$$\frac{\text{Antibody N}}{\text{Antigen N}} \text{ in the precipitate} = 3R'' - 2\sqrt{\frac{(R'')^3(\text{Antigen N})}{A}}$$

in which $3R''$ is the intercept on the y axis and the last term on the right is the slope of the line, A = maximum precipitable antibody N, and R'' = antibody N/antigen N ratio at the maximum. This empirical equation has been found to represent quite accurately the behavior of anti-dye

sera (24) and certain anti-egg albumin sera (11). When the ratio antibody N/antigen N in the precipitate was plotted against the square root of the amount of antigen N precipitated, the data fitted closely to straight lines.

Except for the C-anti-C suspensions this relation also held in the present study. The intercepts on the y axis, $3R''$, had values ranging from 0.68 for the low ratio (4.0) Pn I specific precipitate suspension, to 3.5 for the horse anti-Ea system. The corresponding slopes were -0.31 and -5.6 respectively. The constants for the soluble antigens were somewhat larger, as might have been expected owing to the change in physical state of the antigen.

From the data and equations in Table VII it will be noted that the antigenic behavior of the Pn I antibody solution is almost indistinguishable from that of the Pn II antibody solution. Although only a few points in the region of antibody excess are available in the data of Marrack and Duff (7), the agreement between the constants for their antibody solution and ours is of the expected order for two different sera (*cf.* 11).

After absorption with Ea-anti-Ea (horse) specific precipitate and removal in this way of roughly one-half of the antibody the anti-specific precipitate rabbit serum still resembled the unabsorbed serum in its quantitative reactivity toward a high ratio Pn specific precipitate when compared at the same antibody content (Table VI, also Table II). These results, while necessarily subject to a greater experimental error than those on the cross reactions of S III and S VIII with anti-Pn VIII and anti-Pn III horse sera (25), point to the same conclusion that absorption with a cross reacting antigen may leave the remainder of the antibody with unchanged reactivity toward the homologous antigen as judged by the antibody N-antigen curves. In these cases the cross reaction removed essentially an average sample of the homologous antibody.

The coexistence of separate groupings which function as antigen and antibody on the same molecule of horse globulin is indicated by experiments showing that Pn II horse antibody precipitated with an excess of anti-specific precipitate rabbit serum can still combine with S II, and that the horse antibody in a solution of the Ea-anti-Ea (horse) specific precipitate in an excess of Ea still reacts with the anti-specific precipitate rabbit serum (page 133). This accords with and extends earlier data on other systems (26).

Although all of the antigens used in the form of specific precipitates (Tables I to VI) and antibody solutions (Table VII) were horse proteins, each had at the same time the unique property, as an antibody, of reacting specifically with an antigen. It was therefore of interest to examine whether this antibody specificity would affect in any way their function

as test antigens. It has not been possible, however, to demonstrate any variation due to the type specific antibody groupings either by the sensitive quantitative agglutinin method or by passive anaphylaxis tests in the guinea pig. The anti-Pn II horse specific precipitate rabbit serum was also tested with a specific precipitate derived from a Pn II rabbit antiserum (Table IX). There was no addition of antibody N, indicating that the anti-precipitate serum contained no antibody which would cross react with an antigen containing an antibody grouping specific for S II. A test similar to that with Pn II rabbit serum specific precipitate was also carried out with a specific precipitate prepared from a Pn I pig antiserum (Table IX) and yielded negative results. Although no information could be obtained from this antigen concerning cross reactions due to Pn type specificity, there was a possibility that there might be a cross reaction due to the similar sedimentation constants and molecular weights (27) of Pn anticarbohydrate from pig and horse antisera.

The antigenic reactions, then, of this representative water-insoluble group of antibodies engendered in the horse are essentially similar (Tables I to IV), when due allowance is made for the effect of the amount of antigen combined in the test antigen specific precipitate suspension. In addition, each suspension removed the same total amount of antibody as did two types of purified pneumococcus antibody solutions (Table IX). The antigenic activity of the antibodies studied appears therefore to be solely that of a particular fraction of horse serum globulin. This confirms in a more rigorous quantitative manner Ando's conclusion (6) that the various antibacterial antibodies in the horse are closely related antigenically. Moreover, the agreement between the results with specific precipitate suspensions and the corresponding antigens in solution confirms anew the rigorous specificity of the analytical methods used, and their lack of dependence on the physical state of the antigen (*cf.* 22*b*).

Since it has been shown (21) that partial peptic digestion of antipneumococcus horse serum results in an increase in the combining power of the antibody (lower antibody N/S ratio, or more S per unit of antibody) it was of interest to compare the properties of a specific precipitate made from digested antibody with those of a specific precipitate suspension of the same antibody N/S ratio prepared from the untreated antibody. Our results on the combination of digested antibody with polysaccharide were similar to those reported by Grabar (21). It may be noted, however, that the extrapolated initial combining ratio (17) of the undigested antibody with the specific precipitate suspensions was 13 and that of the digested antibody was 7.5. To provide a basis for comparison, an antibody N/S I ratio of 6.1 was chosen in the preparation of the specific precipitate suspensions for

both digested and undigested antibody. As is readily seen (Table VIII) less of the specific precipitate from the undigested antibody was necessary to exhaust the anti-specific precipitate rabbit serum, but the total amount of antibody removed was the same when an excess of either suspension was used. While the antibody function of the horse protein was evidently modified by the digestion, and the ratio of antibody N to antigen N was reduced in the reaction with anti-precipitate rabbit serum, the digested pneumococcus anticarbohydrate still retained completely the horse specificity function, or functional groupings necessary for complete removal of the anti-precipitate rabbit antibody (for related observations with diphtheria antitoxin *cf.* 28, 29). It may be noted, too, that the change in antibody activity of the digested serum is in the direction of an apparent increase, per weight unit, in the number of groupings available for reaction with S, while the reaction of the corresponding specific precipitate with the anti-precipitate rabbit serum indicates an apparent decrease in the number of antigenic groups per weight unit. While differences in the physical state of the precipitates from digested and undigested antibody might be wholly or in part responsible for this effect, it would still be in accord with the assumption that the two sets of groupings, those functioning as antigens, and those acting as antibodies, are independent (*cf.* also 29).

SUMMARY

1. Rabbits were injected with the washed specific precipitate from Type II antipneumococcus horse serum. Antibody in the resulting antiserum was determined by the quantitative agglutinin method using various specific precipitates as antigens.

2. Suspensions of Types I and II antipneumococcus horse specific precipitates, as well as the specific precipitates derived from Type VIII Pn (anti-C portion), and *H. influenzae* horse antisera were found to remove the same amount of antibody from the immune rabbit serum.

3. Purified antibody solutions prepared by dissociation methods from Types I and II antipneumococcus horse sera were found to remove the same quantity of antibody as did the homologous specific precipitates.

4. Specific precipitates from anti-crystalline egg albumin and anti-diphtheria horse sera were found to remove only a fraction of the antibody. The reasons for this are discussed.

5. A specific precipitate prepared from pepsin-digested Type I anti-pneumococcus horse serum removed all of the antibody to the homologous antigen from the rabbit anti-precipitate serum, but followed a different quantitative course.

6. From the quantitative course of these reactions and from experiments

with specific precipitates from anti-Pn rabbit and pig sera it is concluded that the only antigenic specificity demonstrable for the antibodies investigated was that due to their common origin, and that the groupings responsible for their antibody function constitute either a small part of the total protein molecule or else are non-antigenic.

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