QUANTITATIVE EXPERIMENTS WITH ANTIBODIES TO A SPECIFIC PRECIPITATE

III. Antigenic Properties of Horse Serum Fractions Isolated by Electrophoresis and by Ultracentrifugation*

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The relation of antibodies to each other and to the other serum proteins is a subject of considerable theoretical interest and practical importance. Since antibodies are proteins advantage may be taken of a common property of that class of substances—the ability to act as antigens—in carrying out this comparison. In the first study of the present series (1) rabbits were injected with a washed specific precipitate composed of the Type II pneumococcus specific polysaccharide and the corresponding anticarbohydrate from a Type II anti-pneumococcus horse serum. As the polysaccharide is non-antigenic in the rabbit (2) this procedure is immunologically equivalent to injecting pure antibody protein. The resulting anti-antibody rabbit serum was absorbed quantitatively with a variety of specific precipitates derived from horse serum. In a second paper (3) the data were extended to include the behavior of both horse and rabbit specific precipitates in immune chicken sera.

On the basis of their behavior as antigens the horse antibodies were found to fall into two groups, which corresponded with the immunological classification into antibacterial and antitoxic (prezone) antibodies already noted by Ando (4). The almost identical antigenic properties of the various antibacterial antibodies from horse sera suggested that at least the major portions of the protein molecules must be identical. However from this evidence alone it was not possible to decide whether antibacterial antibodies are normally occurring globulins, slightly modified to enable them to react specifically with the appropriate antigen, or whether they constitute a new antigenic fraction not present in normal serum. Inasmuch as certain fractions containing antibodies have been isolated from immune sera by ultracentrifugation (5, 6) and by

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electrophoresis (7) it seemed possible that an immunological study of these and corresponding fractions from normal horse serum might provide further evidence on this point.

Methods and Materials

Ultracentrifuge.—Most of the preparative and all of the optical runs were made in an air-driven ultracentrifuge (8) kindly placed at our disposal by Dr. Aura E. Severinghaus of the Department of Anatomy of the College of Physicians and Surgeons. Sedimentation constants were determined at room temperatures merely for the purpose of identifying the proteins.

Electrophoretic Apparatus.—Mobilities were determined in the Tiselius apparatus (9), preferably with the Longsworth scanning method, while the Philpot-Svensson cylindrical lens was found convenient in guiding separation procedures. All solutions were dialyzed for at least 2 days in cellophane bags against several changes of buffer at ice box temperatures. The completeness of the dialysis was tested in several instances by calculation of the constancy of the refractive index increments of several dilutions of the protein solution with buffer. The relative refractive index measurements were made with an interferometer.

Anti-Antibody Serum.—This was the rabbit serum pool used in the earlier studies and resulted from injection of rabbits with a washed specific precipitate derived by reaction of Type II pneumococcus specific carbohydrate with Type II antipneumococcus horse serum H513.[1] Ultracentrifugal and electrophoretic analyses of the serum were carried out, both before and after absorption with polysaccharide.

In electrophoresis no definite peak corresponding to a component migrating between the β- and the γ-components was found in the unabsorbed serum as would have been expected from electrophoretic measurements on certain other anti-Pn horse sera (7). Measurements were made of the area under the curves, the average ratios of the α-, β-, γ-areas to the albumin area being 0.36, 0.44, and 1.39 for the unabsorbed serum. After absorption with the Type II specific polysaccharide the corresponding ratios were 0.34, 0.37, and 0.72. The decrease in the first two ratios is probably within the experimental error, although the association of a small amount of antibody with the β-fraction is not excluded. It is apparent, however, that in this particular case most, if not all, of the antibody had the mobility of a γ-globulin (*cf.* 10) (Table II).

Ultracentrifugal data on a globulin solution from this same bleeding have since been reported elsewhere (11). They indicate[2] that the antibody is quite polydisperse; although most of the antibody had a sedimentation constant of $s = 18 \times 10^{-13}$ cm. per second per dyne, there were appreciable amounts with $s = 11$ and 30 (Table I).

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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] We wish to thank Dr. A. M. Pappenheimer, Jr., for his kindness in sending us the complete data before publication. They were made by the Lamm scale method in the Svedberg ultracentrifuge at the University of Wisconsin.

Fractions Isolated from Normal and Immune Horse Sera by Ultracentrifugation.— Serum from normal unimmunized horses contains a small amount of a high molecular weight component of s = 18 × 10^{-13} (12). This fraction increases markedly with immunization in certain instances and part of this new globulin has been identified as antibody (5). The rapidly sedimenting protein from two samples of normal horse serum was therefore isolated for comparison with the purified antibody solutions previously investigated (1).

Preparation 1: 112 ml. of normal horse serum[3] were diluted 1:1 with saline and and enough merthiolate[4] to give a final concentration of 1:10,000, and run in two portions in an angle ultracentrifuge[5] at 36,000 R.P.M. for 7½ hours. The rotor head was chilled before the run. The small amount of jelly at the bottom of the tubes was quickly washed by decantation and redispersed in cold saline. The combined solutions were spun down again in the ultracentrifuge. Yield about 7 mg. of protein. Approximate sedimentation constants were determined in the optical ultracentrifuge (Table I) after which the material was redispersed and used in the quantitative precipitation determinations recorded in Table III.

TABLE I

Approximate Sedimentation Constants of Antigen Fractions from Horse Sera

Preparation	Sedimentation constant × 10^{13} (cm./sec./dyne)	
	Main component	Other components, in order of concentration
Heavy globulin from normal serum, preparation 1	18	11, 5
" globulin from normal serum, preparation 2a	15	6
" " " " " preparation 2b	18	7
Anti-Pn II serum H513, antibody containing components[2]	18	11, 30
γ-Globulin isolated by electrophoresis of normal serum	8.3	—
γ-Globulin isolated by electrophoresis of immune serum	23	8

Preparation 2: 650 ml. of another lot of normal horse serum preserved with 1:10,000 merthiolate were diluted with an equal volume of water, and 1350 ml. of saturated ammonium sulfate run in drop by drop with stirring. The globulin was allowed to stand overnight, centrifuged, and the precipitate dissolved in water and dialyzed against 0.9 per cent saline. The solution was then run in two portions in the ultracentrifuge for 4½ hours at 48,000 R.P.M. The solutions and the centrifuge head were kept in the ice box before the run, and the temperature at the end of the run

[3] We are indebted to Dr. A. J. Weil and Dr. W. G. Malcolm of the Lederle Laboratories for the normal horse sera. The horses were in good health at the time of bleeding, and had not been used for any immunization experiments.

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

[5] We are indebted to Dr. Jacob Furth and Dr. Elvin A. Kabat of the Department of Pathology of the Cornell University Medical College for the use of their air-driven ultracentrifuge.

did not exceed 32°C. The jelly deposited during the first centrifugation was resuspended in saline and recentrifuged with the second portion. Both sediments were again resuspended in saline, merthiolate was added to 1:10,000, and the solutions were allowed to stand for a month to permit complete redispersion. An optical run was then made (preparation 2a, Table I). It showed two components, of sedimentation constants s = 18 and s = 7, the former comprising about 50 per cent of the protein. A portion of this material was kept for quantitative precipitin determinations. The remainder of the solution was again centrifuged in the quantity head at 36,000 R.P.M. for 2½ hours. The resuspended jelly was then allowed to stand for a month before optical analysis (preparation 2b, Table I). The same two components were present but the heavier material had increased about fourfold. An electrophoretic analysis was also performed on this solution (Table II).

Fractions Obtained by Electrophoresis.—Two samples of normal horse serum were subjected to prolonged electrophoresis in buffers containing 0.15 M NaCl and 0.02 M phosphate at pH 7.6. In one case it was possible to remove a part of the γ-globulin, in another a small amount of β-globulin (*cf.* 13). The γ-globulin solution was redialyzed and run again to provide more accurate mobility data, and to verify the electrophoretic homogeneity (Table II). It proved to be quite homogeneous on ultracentrifugation as well (Table I). A portion of the γ-globulin was also removed from a bivalent (Types I and II) antipneumococcus horse serum H6225 (Table II). Insufficient material was available to permit a determination of the amount of antibody present, but from the sedimentation photographs it was estimated that about 75 per cent of the protein was rapidly sedimenting. γ-Globulin was also isolated from a weak goat Type II antipneumococcus serum, but contained very little antibody. A solution of the γ-globulin from normal pig serum was also available.[6] It did not react with the antiprecipitate rabbit serum when tested at concentrations up to 0.28 mg. N per ml. of serum. A diluted unfractionated antipneumococcus horse serum was also tested against the rabbit serum.

Analytical Procedure.—Accurately measured portions of the rabbit antiserum against the horse Type II antipneumococcus specific precipitate were set up at 0°C. with varying amounts of the antigens described above. The analytical methods were those described previously (1, 14). The tubes containing the precipitates were allowed to stand in the ice box, with occasional mixing, for 48 hours, followed by centrifugation in the cold.[7] The precipitates were then washed twice with 3 ml. portions of cold saline. Nitrogen in the precipitates was determined by a modification of the micro-Kjeldahl method. Tests made on the supernatants indicated whether all of the added antigen had precipitated. In those instances in which it had, the antibody nitrogen was taken as the difference between the total nitrogen found and the added antigen nitrogen. In some cases of incomplete precipitation of the added antigen it was possible to determine the amount of antigen left in the supernatants by setting the latter up with another portion of serum (15). Antibody

[6] Obtained through the courtesy of Dr. Elvin A. Kabat, now of the Neurological Institute, Columbia University Medical Center.

[7] In a refrigerated centrifuge supplied by the International Equipment Company, Boston.

nitrogen was then determined by deduction of the corrected antigen value from the total N (Tables III–VII).

Qualitative Inhibition Reactions with Sheep Serum.—A portion of the rabbit anti-precipitate serum was tested with increasing amounts of unfractionated sheep serum globulin solution. A marked precipitate could be obtained at certain dilutions of the antigen; a large excess completely inhibited precipitation. Three portions of the rabbit antiserum were then set up with the same excess of sheep globulin. One was kept as a control. To another was added an equivalent amount of a solution of normal horse globulin, preparation 2b. The last portion was treated with an amount of goat γ-globulin solution which gave a good precipitate in the absence of the sheep globulin. The tubes were allowed to stand overnight, after which it was found that

TABLE II

Electrophoretic Mobilities of Antigen Fractions in Buffers Containing 0.15 M NaCl and 0.02 M Phosphate

Preparation	Descending mobilities × 10^5 cm./volt/sec.			pH	Remarks
	α	β	γ		
Ultracentrifuged globulin from normal horse serum, preparation 2b	−2.4	−1.4	−0.7	6.41	
Anti-Pn II horse serum H513, unabsorbed	−3.4	−3.0	−0.7	7.55	
Same, after absorption with S II	−4.0	−3.2	−0.6	7.55	γ-Fraction markedly decreased
γ-Globulin, from normal horse serum			−1.3	7.60	Single component
γ-Globulin from immune serum			−0.7	7.49	Single component
γ-Globulin from goat serum			−1.2	7.61	Single component

precipitation had occurred only in the tube containing the added normal horse globulin. It was evident therefore that an excess of sheep globulin can completely inhibit the cross-reaction of the rabbit anti-specific precipitate serum with goat γ-globulin. There was apparently no inhibition of the homologous reaction.

FINDINGS AND DISCUSSION

In recent years much information has become available on the physical properties of horse serum proteins. Ultracentrifugal analysis has shown the presence of several globulin components in normal horse serum. The largest in amount is contained in the component with a sedimentation constant, s, of 7, with traces of heavier protein of s = 11; 18, or 32 (12). In antipneumococcus horse serum the concentrations of the heavier fractions, particularly that of s = 18, are markedly increased. Most of this new material is antibody (5, 6).

Another type of analysis can be made with the aid of electrophoretic methods. Tiselius has shown (16*a*) that there are at least three globulin components in normal horse serum. Of these, the α-, β-, and γ-globulins are characterized by mobilities of -3.8, -3.1, and -1.0×10^{-5} respectively, in buffers of ionic strength 0.15 at pH 7.7. Tiselius and Kabat have reported (7) the antibody in antipneumococcus horse sera to have a mobility of -1.6 to -1.8 under the same conditions, although some sera showed an additional antibody of lower mobility, -0.3 to -0.9. Moore, van der Scheer, and Wyckoff on the other hand found (10) that all of the antibody in their antipneumococcus sera had a mobility corresponding to that of the γ-globulin. Confirmation of the presence of antibody migrating between the β- and γ-components in other sera has been made however (17).

Relatively few studies have been made on the sedimentation constants of material isolated by electrophoresis, or the mobilities of fractions prepared by ultracentrifugation. Tiselius has reported (16*a*) that horse serum albumin obtained by electrophoresis had the same sedimentation constant as crystalline serum albumin, and that normal horse γ-globulin is also practically homogeneous, with $s = 7.0 \times 10^{-13}$. The α- and β-globulins examined were more complex; in addition to the main components of $s = 6.7$, there were others with $s = 3.1$ and 18 (*cf.* also 16*b*). Electrophoresis of purified antipneumococcus horse antibody solutions which showed polydisperse material in the ultracentrifuge revealed the presence of increased amounts of material of mobility $\mu = -0.3$ to -0.9, as well as the component $\mu = -1.7$ previously identified as antibody (7). The γ-globulin from normal horse serum has recently been shown to be inhomogenous with respect to solubility (18).

In the present study information was desired on the immunological properties of fractions in normal horse serum which corresponded in physical properties with components present in immune sera. It was first established[2] that the major part of the antibody in the horse serum H513, from which the immunizing specific precipitate was derived, was rapidly sedimenting (Table I). The rabbit antiserum to horse anti-Pn II (anti-antibody serum) therefore contained antibody directed against a protein of high molecular weight. Electrophoretic study of H513 showed that the antibody was mainly in the γ-globulin fraction.

Since it has been established that normal horse serum contains a small amount of a high molecular weight component of the same sedimentation constant as antibody (12, 5) it was attempted to isolate enough for comparison with the antigenic properties of antibody fractions. Sera from two normal, unimmunized horses were used. By differential ultracentrifugation it was possible to free most of the rapidly sedimenting material from the lighter components (preparations 1 and 2a, b). The analytical control of preparation 2 was the more complete, and the product was purer, although neither lot was homogeneous. (Tables I and II.)

Both preparations were set up against the rabbit anti-antibody serum. Preparation 1 removed only about one-half of the total antibody present (Table III). Preparation 2a precipitated more of the antibody, and after further ultracentrifugation precipitated practically all of the antibody N from the

TABLE III

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

Antigen N added	Antigen N pptd.	Total N pptd.	Antibody N pptd.	Antibody N / Antigen N ratio in ppt.*	Tests on supernatants
mg.	*mg.*	*mg.*	*mg.*		
Test antigen: ultracentrifuged globulin from normal horse serum, preparation 1					
0.041‡	Total	0.170	0.129	3.1	Excess antibody, no antigen
0.068	"	0.236	0.168	2.5	" "
0.095‡	"	0.284	0.189	2.0	" "
0.136	0.129§	0.360	0.231	1.8	Slight excess antigen
0.181	0.151§	0.356	0.205	1.4	Excess antigen
Mg. antibody N pptd. = 4.9 (antigen N) − 9.0 (antigen N)$^{3/2}$					
Test antigen: ultracentrifuged globulin, preparation 2b					
0.026‖	Total	0.192	0.166	6.4	Excess antibody, no antigen
0.034**	"	0.249	0.215	6.3	" "
0.051‡‡	"	0.326	0.275	5.4	" "
0.102	"	0.510	0.408	4.0	" "
0.153	(0.150)	0.610	(0.460)	(3.1)	Traces antigen and antibody
0.204		0.638§§			Excess antigen, no antibody
Mg. antibody N pptd. = 9.0 (antigen N) − 15.5 (antigen N)$^{3/2}$					

* Assuming all of the antigen N to be precipitated.

‡ Single determination only.

§ From analysis of the supernatant.

‖ Quadruple quantities of antigen and serum used for analysis to insure greater accuracy.

** Triple quantities used.

‡‡ Double quantities used.

§§ Supernatant gave no precipitate with salt-dissociated Pn I antibody solution.

rabbit serum (preparation 2b, Table III). This indicated that the heavy protein which now constituted the bulk of the preparation was the active antigen, the lighter components in preparation 2a having possibly caused partial inhibition. A comparison of the antigenic properties of these materials with other antigens including the salt-dissociated antibody solutions studied previously (1) is given in Fig. 1.

It is evident that preparations isolated by similar procedures from two different samples of presumably normal horse serum have dissimilar immunological properties. In the one instance a product with antigenic properties approaching those of the original pneumococcus anticarbohydrate was obtained. This finding raises the question whether a horse with no previous

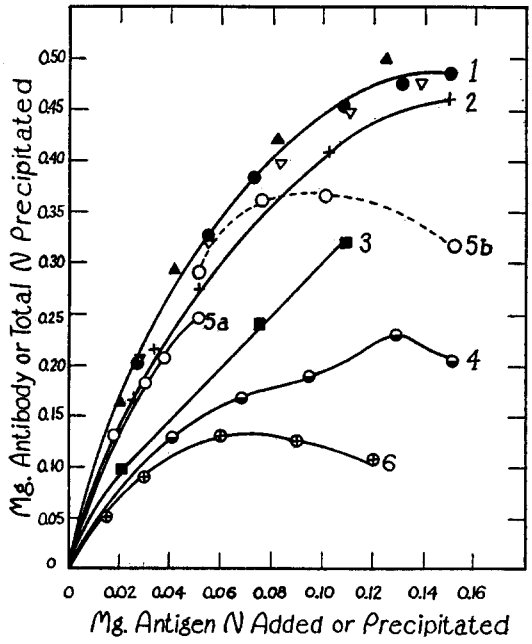

FIG. 1. Precipitation of an anti-antibody rabbit serum by various antigens: Curve 1, ● = Pn I, II horse γ-globulin; ▲ = Pn II salt-dissociated antibody solution (1); ▽ = Pn I salt-dissociated antibody solution (1); Curve 2 = normal horse heavy globulin, preparation 2b; Curve 3 (total N precipitated) = normal horse β-globulin; Curve 4 = normal horse heavy globulin, preparation 1; Curve 5a = normal horse γ-globulin; Curve 5b (total N precipitated) = normal horse γ-globulin; Curve 6 (total N precipitated) = goat γ-globulin.

history of immunization might not at some time have been subjected to an unrecognized infection sufficient to induce antibody formation. The problem could only be solved by extensive experiments.

Less uncertain are the differences in antigenic behavior between a normal γ-globulin and γ-globulin containing antibody (Table IV). In this instance two serum proteins which have the same electrophoretic mobility and are homogeneous by that physical criterion can be distinguished from each other by a second physical method, ultracentrifugation, and by the immunological

method. (Tables I, II, IV, and Fig. 1.) An interpretation of this behavior in terms of the quantitative reaction constants is given below.

A sample of the low molecular weight β-globulin from normal horse serum also cross-reacted with the anti-specific precipitate rabbit serum (Table V).

TABLE IV

Precipitation of Antibody from Rabbit Anti-Pn II Horse Specific Precipitate Serum by Fractions Isolated by Electrophoresis from Normal and Immune Horse Sera per 1.0 Ml. Serum, 0°C., 48 Hours

Antigen N added	Antigen N pptd.	Total N pptd.	Antibody N pptd.	Antibody N / Antigen N ratio in ppt.*	Tests on supernatants
mg.	*mg.*	*mg.*	*mg.*		
Test antigen: γ-globulin solution from normal horse serum					
0.018‡	Total	0.149	0.131	7.3	Excess antibody, no antigen
0.030‡	"	0.213	0.183	6.1	" "
0.038§	"	0.243	0.205	5.4	" "
0.051‖	"	0.296	0.245	4.8	" "
0.076	Incomplete	0.354			Both antibody and antigen
0.101‖	"	0.356			" "
0.152	"	0.306			" "
Mg. antibody N pptd. = 10.8 (antigen N) − 26.6 (antigen N)$^{3/2}$					
Test antigen: γ-globulin solution from an antipneumococcus horse serum					
0.027§	Total	0.229	0.202	7.5	Excess antibody, no antigen
0.055‖	Total	0.381	0.326	5.9	" "
0.073‖	"	0.456	0.383	5.2	" "
0.110	0.108**	0.562	0.454	4.2	Excess antibody, trace antigen
0.137	0.131**	0.606	0.475	3.6	Trace antibody and antigen
Mg. antibody N pptd. = 10.8 (antigen N) − 19.8 (antigen N)$^{3/2}$					

* Assuming that all of the added antigen is precipitated.

‡ 2.5 times this quantity of antigen and serum used for analysis.

§ Double quantities used.

‖ One and one-half quantities used.

** From analysis of supernatant.

A complete curve could not be constructed as the sample was antigenically inhomogeneous, but the data differentiate it clearly from the antibody containing fractions. An attempt was also made to study the immunological behavior of antibody migrating faster than the γ-component, but in two sera which contained appreciable amounts of this component a portion of the antibody occurred in the γ-globulin fraction as well (*cf.* 6). A specific precipitate

made from one of these sera had practically the same quantitative properties as the γ-antibody specific precipitates (1). Another form of antipneumococcus horse antibody having a molecular weight of 150,000 has recently been reported (11). No samples were available.

Marrack and Duff (19) concluded that a whole globulin solution from normal horse serum removed only a portion of the antibody from anti-specific precipitate serum. However, when we used whole antipneumococcus horse serum as antigen the total N precipitated corresponded to that expected from the antibody content of the serum (*cf.* 4).

It will be noted from Fig. 1 that antipneumococcus Types I and II horse antibodies isolated by salt dissociation and used as antigens follow closely the curve for the γ-globulin from an antipneumococcus horse serum. The data may be expressed by an equation which has been found to represent satis-

TABLE V

Precipitation of Antibody from Rabbit Anti-Pn II Horse Specific Precipitate Serum with Normal Horse β-Globulin per 1.0 Ml. Serum, 0°C., 48 Hours

Antigen N added	Antigen N pptd.	Total N pptd.	Antibody N pptd.	Tests on supernatants
mg.	*mg.*	*mg.*	*mg.*	
0.020	Total	0.10	0.08	Excess A, no antigen
0.072	Incomplete	0.24	(0.17)	Excess antigen, no A
0.109	"	0.32	(0.22)	Excess antigen, no A

Single determinations only.

factorily the behavior of a number of other protein-antiprotein systems (20, 21, 1):

$$\text{mg. antibody N precipitated} = k_1 (\text{antigen}) - k_2 (\text{antigen})^{3/2} \quad \ldots\ldots\ldots\ldots (1)$$

This equation must be regarded as empirical, for it is not yet possible to give the constants definite chemical and immunological significance as is the case with the second power relation derived for a number of other systems (14, 21). Several conclusions may be drawn, however, when reactions show identical constants. The variation in the constants for the reactions of the γ-globulin solution (Table IV) and the salt-dissociated antibody solutions (1) are within the limits of experimental error. This would indicate that no significant change in antigenic properties had occurred during the chemical treatments with salt. The somewhat lower constants for the reaction of the heavy normal globulin, preparation 2b, (Table III) are probably due to other antigens of lesser reactivity still remaining (see p. 141).

If the equations for the above are compared with that for the reactivity of the normal horse γ-globulin with the same anti-antibody serum it will be noted (Table IV) that the decrease in precipitating power of the normal globulin to

about one-half of the others is reflected in the different reaction constants obtained. If, however, the equations for the normal and immune γ-globulins are recalculated[8] to the same maximum of 1.0 mg. of antibody N removed, the constants of the equations become identical (Table VII), the equation being expressed as

$$\text{mg. antibody N precipitated} = 10.8\ (\text{Gb N}) - 13.6\ (\text{Gb N})^{3/2} \quad \ldots\ldots\ldots\ldots (2)$$

where Gb N is the amount of normal or immune γ-globulin-nitrogen. The reactivity of the normal horse γ-globulin *per mg. of antibody precipitated* is therefore the same as that of the immune γ-globulin antigen, and it is evident that the portion of the rabbit antibody which is precipitated by both globulins is incapable of distinguishing between them. The remaining portion of the antibody precipitates only the immune globulins or the heavy normal horse protein. These findings may be compared with others from this laboratory, in which it was noted that a cross-reacting antibody solution reacted equally well with two different pneumococcus specific polysaccharides (22) or that serum supernatants from the absorption with heterologous polysaccharide (23) or specific precipitate (1) still yielded the same reaction curve with the homologous antigen as did the original serum, when calculated to the same antibody content.

The following hypothesis may be advanced to account for the cross reaction of normal horse γ-globulin with antisera to the γ-globulin from immune sera. The chemical composition of antibodies and normal globulins from the same species appears to be quite similar (24). The striking differences between the two γ-globulins are the sedimentation constants and therefore the molecular weights, approximately 150,000 and 910,000. If the large size of the immune γ-globulin were due to a polymerization of six specifically altered γ-globulin units, it might account for the cross-reaction of a single normal γ-globulin unit with antiserum toward the polymerized molecule. Such specific groups on the antibody γ-globulin molecule as are responsible for its function as an antibody need not be taken into account here since they apparently are not antigenic (1, 3).

If this view were correct, the cross-reaction of the normal horse γ-globulin with an antiserum to the larger immune γ-globulin would be analogous to the precipitation of horse antisera to undegraded pneumococcus carbohydrate by various degraded pneumococcus polysaccharides (25). In Table VII are given

[8] The amount of antigen required to precipitate the calculated maximum amount of antibody, A, may be found by differentiating equation (1) with respect to the antigen, and equating to zero (*cf.* 21). Specifically, equation (2) becomes $10.8 - 3/2 \times 13.6\ (\text{Gb N})^{\frac{1}{2}} = 0$, and is solved for Gb N. Substitution of this new antigen value in equation (1) gives the corresponding maximum A. Conversion to a basis of 1.0 mg. A may be made by multiplying the slope, k_2, by the $\sqrt{\text{A}}$ calculated above.

data on the reaction of a number of Type III pneumococcus polysaccharides which had been partially hydrolyzed by acids. This treatment is known to break down the polysaccharide chain into shorter units (25) and can be followed by changes in the viscosity or in the amount of reducing groups. It will be noted that in spite of the smaller quantities of antibody precipitated by a given amount of the acid-treated polysaccharides, the reaction constants calculated to a common maximum amount of antibody precipitated (1.0 mg. N) are remarkably similar to each other and to those for the untreated material. In contrast is the behavior of the Type I pneumococcus polysaccharide treated with alkali. Under these conditions changes in the polysaccharide are known to be more complex than simple depolymerization (2*b*, 26). The data avail-

TABLE VI

Cross Reaction of γ-Globulin from Goat Serum with Antibody from Rabbit Anti-Pn II Horse Specific Precipitate Serum per 1.0 Ml. Serum, 0°C., 48 Hours

Antigen N added	Antigen N pptd.	Total N pptd.	Antibody N pptd.	$\frac{\text{Antibody N}}{\text{Antigen N in ppt.}^*}$	Tests on supernatants
mg.	*mg.*	*mg.*	*mg.*		
Test antigen: γ-globulin of goat serum					
0.015‡	Total	0.052	0.037	2.5	Excess A, no antigen
0.030‡	Incomplete	0.092			Slight amount antigen and antibody
0.060§	"	0.132			Excess antigen, trace antibody
0.090	"	0.126			Excess antigen
0.120§	"	0.108			" "

* Assuming all of the added N to be precipitated.

‡ Double quantities of antigen and serum actually used.

§ Single determination only.

able, while less numerous, are quite concordant and show clearly the marked differences in the reaction curves produced by the action of alkali on this antigen.

A slight cross-reaction with the anti-antibody rabbit serum was observed for the isolated γ-globulin from goat serum (Table VI). No sedimentation data were available for this preparation. Comparison could be made, however, of the relative precipitating powers of two antigenic fractions from two different species toward antisera to one of them. From Tables III and VI it may be estimated that the goat γ-globulin removed about 15 per cent of the antibody toward the homologous (horse) antibody γ-globulin. A preliminary analysis of the cross-reactivity of electrophoretically isolated goat albumin against a rabbit antiserum to crystalline horse serum albumin showed that the antibody N precipitated in the equivalence zone was about 10 per cent of

that removed by the horse albumin.[9] The order of magnitude in the cross-reactions between goat antigens and rabbit antisera to the corresponding fractions from horse sera is thus the same.

TABLE VII

Precipitin Reaction Characteristics, with Antibody N Precipitated at One Antigen Level, and Empirical Reaction Constants Calculated to a Maximum of 1.0 Mg. of Precipitable Antibody

Preparation	Antibody N pptd. per ml. by given amount of antigen (experimental)	Reaction constants, equation (1) per 1.0 mg. maximum antibody	
		Intercept, k_1	Slope, k_2
	mg.		
Rabbit anti-specific precipitate serum with horse globulin fractions			
	0.05 mg. protein antigen N		
Pn II antibody solution (1)	0.320	10.9	13.9
Pn I antibody solution (1)	0.305	9.9	11.9
Antibody γ-globulin	0.310	10.8	13.6
Normal γ-globulin	0.240	10.8	13.6
Normal heavy globulin, preparation 1	0.145	4.9	4.1
" " " preparation 2b	0.270	9.0	10.3
Horse anti-Pn III antibody solution and various S III preparations*			
	0.15 mg. S III		
Untreated S III	2.12	39.5	95
S III, HCl A	1.90	40.0	98
S III, H_2SO_4 B	1.68	41.4	102
S III, HCl C	1.62	40.7	100
S III, HCl D	1.36	39.5	96
S III, H_2SO_4 C	1.30	43.5‡ 37.0	111‡ 87
Horse anti-Pn I serum and S I preparations§			
	0.15 mg. S I		
S I, untreated	0.340	12.8	17.6
S I, treated with alkali at 37°	0.220	10.7	13.5

* Calculated from (25) and unpublished experiments in this laboratory by Dr. Forrest E. Kendall. The fractions are described in (25).

‡ Because of the scattering of the points about a straight line, the highest and lowest values calculated from the data are given.

§ From unpublished data by Mrs. H. F. Havas.

The technique most commonly used in determining the degree of zoological relationships is to inject rabbits with the whole serum of one species and to test

[9] The serum used was calibrated by Mr. Manfred Mayer of this laboratory.

the resulting antiserum with whole serum from another species. Interpretation of the results is complicated by a number of factors. Whole serum is a mixture of antigens which may differ considerably in their relative concentrations and in their antigenic powers. An undue proportion of the antibodies produced might therefore be directed toward a minor serum constituent. If this complicated mixture of antigens and their antibodies is examined by the usual dilution methods multiple zones of precipitation often result, with partial or complete inhibition of some of the reacting systems. The use of dilution methods and whole serum would, for example, completely obscure the relation between the antigenic properties of the normal horse γ-globulin and the immune horse γ-globulins traced above.

A more direct method would be to study the antigenic properties of one or more homogeneous fractions isolated by chemical methods or by electrophoresis or ultracentrifugation from the sera to be compared. The interaction between these antigens and their antibodies could then be satisfactorily determined by quantitative absolute methods, such as have already proved useful in comparing the species specificities of mammalian thyroglobulins (27). Further studies along these lines would show whether the percentage of cross-reaction of various serum fractions is constant for related species (as in the instance of the horse and goat albumins and γ-globulins here reported) or variable, with perhaps limiting cases in which only the albumins (or globulins) possessed groupings in common. Whatever the findings, it is possible that this information would be of more fundamental significance for general physiology as well as zoological classification than are the conclusions drawn from the average behavior of complicated mixtures such as whole sera.

It is concluded from the data presented that antipneumococcus antibodies from horse sera resemble in antigenic behavior certain normally occurring horse serum globulins, while they differ in some respects from others having the same sedimentation constant or electrophoretic mobility. Thus the antibodies were practically identical in antigenic properties with a rapidly sedimenting globulin from one sample of supposedly normal horse serum but differed in these properties from a sample with the same sedimentation constant from another horse. Differences in antigenic behavior between the normal and immune γ-globulin of the same electrophoretic mobility were accompanied by differences in sedimentation constants and molecular weights, in accord with quantitative relations among the immune properties suggesting that the differences are but reflections of the variation in chain length.

SUMMARY

1. Rabbit antisera to a Type II pneumococcus specific precipitate from horse serum were tested with fractions prepared by ultracentrifugation and electrophoresis of normal and immune horse serum.

2. In one instance a rapidly sedimenting protein from normal horse serum

had nearly the same quantitative antigenic properties toward the anti-antibody rabbit serum as did the purified pneumococcus antibody solutions previously reported. In another instance a comparable fraction removed only a part of the rabbit antibody.

3. Electrophoretic γ-globulin from an immune horse serum had quantitatively the same antigenic properties as did antibody solutions prepared by salt-dissociation of specific precipitates.

4. Electrophoretic γ-globulin from normal horse serum differed in its antigenic behavior from γ-globulin containing antibody. The data are compared with the antigenic properties of acid and alkali treated pneumococcus specific polysaccharides toward antipneumococcus horse sera. An interpretation in terms of polymers is suggested.

5. The cross-reaction of goat serum γ-globulin against the anti-antibody serum is reported and the extent of the reaction compared with those of goat and horse serum albumins against a rabbit antiserum to the latter.

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