ANTIPROTEINS IN HORSE SERA

III. Antibodies to Rabbit Serum Albumin and Their Reaction with Antigen*‡

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As far as is known, the injection of any antigen into the rabbit by any route may give rise to antibodies which are of the so called precipitin type. Addition of a small amount of antigen to the antiserum results in a precipitate which increases in amount as more antigen is added until the maximum is reached with a slight excess of antigen (2). No "prezone" or inhibition of precipitation in the region of antibody excess is observed. While antibodies to specific carbohydrates may be elicited in the horse or rabbit by intravenous injections of type-specific pneumococci, it is common experience that tetanus or diphtheria antitoxins are produced in the horse in sufficient amounts only in response to subcutaneous injection. These antitoxins flocculate with the antigen, but the precipitation differs from the precipitin type in that it is confined to a relatively narrow zone, inhibition being observed with excess of either antibody or antigen (*cf.* 3, 4). Antibodies with similar properties have been produced in the horse by the subcutaneous injection of a number of protein antigens: egg albumin (5, 6), hemocyanin (7), and hsiguasin, a crystalline globulin of watermelon seed (8). The few recorded experiments on the intravenous injection of protein antigens into the horse have not been very successful (see (5) for literature).

It was, however, shown in the preceding paper (9) that pneumococcus antinucleoprotein in the horse, elaborated after intravenous injections, reacted according to the precipitin type. One or more of at least four factors might be responsible for the qualitative type of antibody produced: the animal species injected, the route of injection, the duration of the immunization, and the nature of the antigen (whether carbohydrate or one or another type of protein). The work about to be described traces the influence of these factors.

Rabbit serum proteins were selected for study as antigens in the horse partly on account of their ready preparation in the relatively large amounts required and partly because information had already been obtained regarding the reverse

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system, horse serum proteins in the rabbit (10). Rabbit serum was fractionated into albumin and globulin portions, and although neither was homogeneous, it was felt that any resulting multiple antibody response would not be an undue complication since more highly purified test antigens were to be used for the analytical studies of the sera. In some instances, at the termination of a course of injections, it became desirable to change antigens in order to follow the response of an individual animal to the administration of different antigens by the same route. The present report deals with the results obtained with rabbit serum albumin as antigen, while the following paper records the findings with rabbit serum globulin.

EXPERIMENTAL

Methods and Materials

Suspensions for Injection.—Pooled normal rabbit sera were diluted with 2 volumes of water and 1.22 volumes of saturated ammonium sulfate solution were added drop by drop, with stirring to 55 per cent saturation. The precipitate was allowed to stand overnight, was washed once with 55 per cent saturated ammonium sulfate solution, and was reprecipitated in the same way, taken up in water, and dialyzed in the cold until sulfate-free. This solution contained the globulin antigen used.

The supernatants from the two precipitations were mixed and the rabbit albumin was thrown down by addition of acetic acid to maximum turbidity. The precipitate was centrifuged, redissolved in water, and dialyzed against saline in the cold. This solution contained the albumin antigen used.

For injection the albumin or globulin stock solutions, at a concentration of 3.3 mg. of protein per ml., were precipitated by addition of 1 ml. of 1 per cent alum solution per 100 mg. of protein (11), followed by dilute sodium hydroxide to maximum turbidity. One per cent by volume of 1 per cent merthiolate[1] was added as a preservative. For the immunization of horses 1126 and 1127 equal volumes of the albumin and globulin solutions were mixed before precipitation with alum (mixed antigens).

Injection Procedure.—The four horses used for these experiments were selected from the regular stock of the Research and Antitoxin Laboratory of the New York City Department of Health, Otisville, New York. All injections and observations on the animals were carried out by members of that Division. The intravenous injections appeared to be better tolerated than the subcutaneous ones, although for all but one animal the dose of protein had to be reduced for a short period during which the horses experienced some rise in temperature. Injection schedules with albumin or albumin-globulin mixtures are given in Table I.

Test Antigen.—Rabbit serum albumin was prepared by electrophoretic separation,[2] in a large Tiselius cell, of 90 ml. of rabbit serum which had been dialyzed in the cold for 3 days against buffer at pH 7.7. The electrophoretic homogeneity of the albumin was checked by a separate analytical run.

Electrophoretic Patterns.—Electrophoretic patterns were determined on the sera both before and after immunization. The patterns of the postimmunization sera which contained antibody resembled those of typical antitoxic horse sera (12, 13).

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[1] Manufactured by Eli Lilly and Co., Indianapolis, Indiana.

[2] In the Electrophoretic Laboratory of the College of Physicians and Surgeons, Columbia University, under the direction of Dr. Dan H. Moore.

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Examination of the Sera for Anti-Albumin

Horse 999.—Precipitin tests, with 0.0002 to 0.04 mg. N of the rabbit albumin test antigen per ml. of serum, on bleedings taken from horse 999 ten days after the last intravenous injection and after a rest period of over 3 months gave no positive result. After the period of rest from the intravenous injections (Table I) horse 999 was given three preliminary intracutaneous injections (< 5 mg. of protein in all) and then eight subcutaneous doses of rabbit serum albumin totalling 1.15 gm. A test bleeding 9 days after the last injection showed a zone of flocculation with albumin. Injections were continued over a period of 5 months and a bleeding was taken 10 days after the last dose.

TABLE I

Injection Schedule of Horses Receiving Alum-Precipitated Rabbit Serum Albumin or Albumin-Globulin Suspensions

Horse No.	Antigen	Route of injection	From	To	No. of injections	Average amount per injection
						mg.
999	Albumin	Intravenous	May 9, 1940	July 2, 1940	13	200
	"	Subcutaneous	Dec. 4, 1940*	June 24, 1941	41	200‡
1046	Albumin	Intravenous	Oct. 28, 1941	May 13, 1942	43	200§
1126	Albumin + globulin	Intravenous	Oct. 28, 1941	June 12, 1942	48	100‡‖
1127	Albumin + globulin	Subcutaneous	Oct. 28, 1941	June 12, 1942	48	100‡‖

* The first three injections (0.1 to 3.6 mg.) were given intracutaneously.

‡ Dosage reduced for four injections because of febrile reaction.

§ Dosage increased continuously by 10 mg. per injection from 5 mg. to 385 mg.

‖ Albumin portion only; an equal amount of globulin was also present.

Quantitative estimations of total N and antibody N precipitated (Table II) were carried out as in previous studies (14). In a typical instance 5.0 ml. amounts of the serum were measured out at 0°C., and appropriate amounts of electrophoretically separated rabbit serum albumin were added in accordance with preliminary tests on smaller volumes. The volume was adjusted to 9.5 ml. with saline and the contents of the tubes were carefully mixed and allowed to stand either 3 or 7 days in the ice box, with mixing at intervals. The tubes were centrifuged in the cold,[3] the precipitates were washed three times with 4 ml. portions of saline at 0°, and were then analyzed for nitrogen by a modification of the micro-Kjeldahl technique. In one instance the supernatants from the tubes which had stood 7 days were allowed to remain in the ice box for another week, centrifuged, and the precipitates washed. The nitrogen found was added to the corresponding values for the 7 day experiment. Antibody N was estimated by subtracting the added antigen N from the total nitrogen found, under the assumption that all added antigen N was precipitated (*cf.* 2).

[3] In a refrigerated centrifuge supplied by the International Equipment Co., Boston, Massachusetts.

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In the series which was allowed to stand for 3 days, the largest amounts of total N precipitated from 5 ml. of the July (later) and January (earlier) bleedings were 1.06 and 0.430 mg., respectively; ratio, 2.47. In the experiment with the January bleeding, by multiplication of each value of the antigen N added and the total N precipitated by 2.47 the behavior of this serum may be compared directly with that of the later, stronger bleeding (Fig. 1).

TABLE II

Precipitation of Horse Antibody to Rabbit Serum Albumin by Electrophoretically Separated Rabbit Serum Albumin, per 5.0 Ml. Serum, Horse 999, 0°C.

Antigen N added	Total N* precipitated after			Antibody N precipitated‡
	3 days	7 days	2 wks.	
mg.	*mg.*	*mg.*	*mg.*	*mg.*
		Bleeding Jan. 23, 1941		
0.040	0			
0.050	0.116			0.066
0.060	0.303			0.243
0.080	0.394			0.314
0.100	0.430			0.330
0.120	0.422			0.302
0.150	0.156			
0.200	0			
		Bleeding July 2, 1941		
0.096	0.256	0.524	0.586	0.490§
0.144	0.954	0.938	0.940	0.796
0.169‖	0.990	0.913	0.923	0.754
0.192	1.02	0.984	0.984	0.792
0.240	0.986	1.05	1.06	0.82
0.288	0.994	1.05	1.06	0.77
0.336	0.486	0.552	0.586	
0.384	0.018			

* The 3- and 7-day columns represent independent series run in duplicate; column 4 contains data obtained by adding to the values in the preceding column one-half of the small amount of nitrogen which separated from the combined supernatants of each pair of tubes in the 7-day series after an additional week.

‡ All added antigen N assumed to be in the precipitate.

§ Values in column 4 minus corresponding values in column 1.

‖ 4.25 ml. serum and 0.144 mg. albumin N actually used.

In order to study the effect of volume on the precipitability of the antibody a concentrate was prepared from the July bleeding. The fraction precipitated from 500 ml. of serum by one-third saturation with ammonium sulfate was removed, washed, and discarded since it contained little antibody. The supernatants and washings were brought to ½ saturation with ammonium sulfate. The resulting precipitate was taken up in water and dialyzed against water in the cold until free from sulfate. After removal of the water-insoluble portion, the supernatant was concentrated by dialysis under negative pressure to a final volume of 115 ml.

Portions of this globulin concentrate, 999 B, were set up at 0° against electrophoretically separated rabbit serum albumin test antigen. In one series the total volume was kept at

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3 ml., in the other at 10 ml. After 6 days in the ice box both series were centrifuged, given three washings with 3 ml. of cold saline, and analyzed as before. The data are given in Table III.

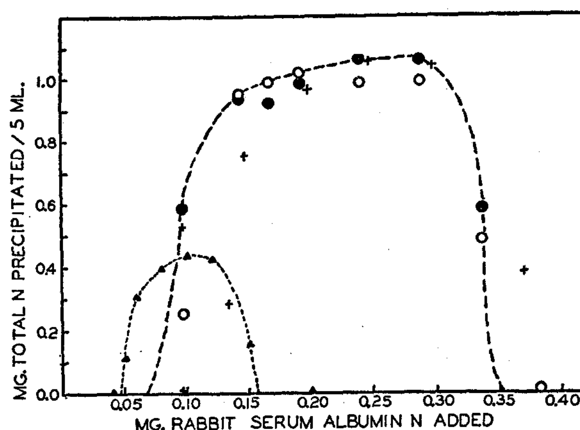

FIG. 1. Precipitation of horse antibody to rabbit serum albumin by electrophoretically separated rabbit serum albumin. Triangles = Jan. 23, 1941, bleeding; crosses = experimental values × 2.47 for comparison with later (July 2, 1941) bleeding. Open circles = July 2, 1941, bleeding, 3 day experiment; shaded circles = same, 14 day experiment.

TABLE III

Effect of Volume on the Precipitation of Horse Anti-Rabbit Serum Albumin

Per 1.0 ml. globulin solution, 999B, bleeding of July 2, 1941, 0°, 6 days. Test antigen: electrophoretically separated rabbit serum albumin

Antigen N added	Total N precipitated at volume of 3 ml.	Total N precipitated at volume of 10 ml.	Antigen N added	Total N precipitated at volume of 3 ml.	Total N precipitated at volume of 10 ml.
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
0.048	0.020	0.012	0.160	0.694*	0.688
0.060	0.140	0.134	0.171	0.698	0.648
0.072	0.502	0.410	0.192	0.636	0.526
0.096	0.612	0.566	0.216		0.334
0.128	0.662	0.652	0.233	0.158	0.052
0.144	0.678		0.274	0.054	0.01

* Ratio antibody N: antigen N at maximum precipitation: 3.3.

Sera from Horses 1126 and 1127.—Two other horses were injected (Table I) in order to test the generality of the findings. Horse 1126 received intravenously a mixture of the rabbit albumin and globulin, while No. 1127 received the same antigens subcutaneously. Only the results obtained on testing with rabbit albumin will be noted here, those dealing with globulin test antigens being reserved for the paper following. After twenty-two injections a test bleeding on horse 1126 taken Dec. 16, 1941, showed a zone of weak precipitation with electrophoretically separated rabbit albumin extending over at least an eightfold range of

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antigen concentration. A single absorption of this serum was carried out with an egg albumin anti-egg albumin (rabbit) specific precipitate which, containing rabbit γ-globulin in an insoluble form, served as a test antigen for antibody to globulin. The added specific precipitate was visibly agglutinated and the reaction with the rabbit serum albumin was noticeably diminished in the supernatant. It is therefore likely that precipitation in this bleeding was due to a small amount of globulin present in the albumin test antigen. The serum from horse 1127 did not give any reaction at this time. Test bleedings taken Feb. 4, 1942, indicated, however, that the subcutaneously injected horse 1127 was producing anti-albumin of the antitoxin type, like horse 999. This is illustrated by quantitative data given below.

Effect of Temperature on the Precipitation of Anti-Albumin.—An antibody concentrate was made from 300 ml. of a bleeding of horse 1127 taken at the conclusion of the immunization schedule. The portion precipitated by ½ saturation with ammonium sulfate was separated by dialysis against distilled water into a water-insoluble fraction (1127G) and a water-soluble portion (1127H). The protein which precipitated between ⅓ and ½ saturation was similarly divided into a water-insoluble part (1127I) and a water-soluble fraction (1127J). Tests indicated that most of the anti-albumin was in fraction J.

TABLE IV

Effect of Temperature on the Reaction of Horse Anti-Rabbit Serum Albumin. Precipitation of 2.0 Ml. of Globulin Solution 1127J by Electrophoretically Separated Rabbit Serum Albumin

Antigen N added	Total N precipitated	
	0°C., 7 days	37°C., 3 hrs.
mg.	*mg.*	*mg.*
0.048	0*	0.002
0.073	0.312	0.166
0.098	0.506	0.490
0.110		0.542
0.146	0.616	0.632
0.171	0.574	0.602
0.219	0.006	0.126

* One determination only.

2.0 ml. portions of 1127J were analyzed with various amounts of antigen in a total volume of 4 ml. One series of analyses was set up at 0° and allowed to stand in the ice box for 1 week, with mixing at intervals, followed by centrifugation and washing in the cold. A second series was run at 37° and left in the water bath for 3 hours before centrifugation and washing at 37°. The data for both series are given in Table IV.

Reversibility of Rabbit Serum Albumin Anti-Albumin Combination.—The reversibility of the antigen-antibody compounds formed on either side of the precipitating region (*cf.* Table IV) was tested by addition, at both 0° and 37°, to duplicate portions of 1127J, of amounts of antigen which would not give visible precipitation. In one case insufficient antigen for precipitation was added, in the other an excess of antigen calculated to inhibit precipitation completely. After 3 to 48 hours, depending upon the temperature, additional antigen or serum was added in amount calculated to bring the system to the point of maximum precipitation (corresponding to the addition of 0.146 mg. of antigen N for each 2.0 ml. of solution J). The tubes were then allowed to stand the same length of time as the controls treated initially with the optimum amount of antigen. The data, summarized in Table V, indicate the reversibility of the system.

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Determination of the Chemical Nature of the Anti-Albumin with an Anti-Antibody Serum.—In a previous communication (10) a study was reported of quantitative aspects of the observation that antibodies to crystalline egg albumin and diphtheria antitoxin in the horse could be distinguished serologically from typical antibacterial antibodies such as pneumococcus anticarbohydrate. When these antibodies, in the form of specific precipitates, were used as antigens much as bacterial suspensions in the quantitative agglutination procedure (15) it was found that the first pair removed only 50 to 60 per cent of the antibody in a rabbit antiserum to Type II pneumococcus anticarbohydrate from the horse.

TABLE V

Reversibility of Albumin Anti-Albumin Interaction

Temperature	Volume of antibody solution 1127J used	Rabbit serum albumin N added	Time allowed	Appearance	Second reagent	Further time allowed	Antibody N precipitated
°C.	ml.	mg.	hrs.			hrs.	mg.
0	2.0	0.024	48	Clear	0.122 mg. antigen N	168	0.600*
0	1.0	0.146	48	Clear	1.0 ml. antibody solution	168	0.610
0	2.0	0.146		Precipitate		168	0.616‡
37	2.0	0.024	0.5	Clear	0.122 mg. antigen N	3	0.616
37	1.0	0.146	0.5	Clear	1.0 ml. antibody solution	3	0.634
37	2.0	0.146		Precipitate		3	0.632‡

* One determination lost.

‡ Control determination from Table IV.

Since the antibody to rabbit serum albumin resembled diphtheria antitoxin and anticrystalline egg albumin in its water-solubility and its flocculation with the homologous antigen it appeared desirable to compare the antigenic properties of the three antibodies. A rabbit antiserum to specific precipitate from the capsular polysaccharide of Type II pneumococcus and Type II antipneumococcus horse serum (10) could not be directly absorbed with the albumin anti-albumin floccules, however, since the unfractionated serum contained rabbit albumin which might interfere with the reaction by a solvent effect on the floccules. An antibody globulin solution was therefore prepared from this rabbit serum by twice repeated precipitation with an equal volume of ammonium sulfate, with thorough washing of the precipitate to free it from as much albumin as possible. After dialysis to remove ammonium sulfate, the antibody globulin solution was analyzed with a floccule suspension prepared from serum 999 (Jan. 23, 1941) by precipitation with rabbit albumin, and for comparison, with a Type I pneumococcus specific precipitate from horse serum, with results given in Table VI.

The anti-albumin floccules removed 0.19 mg. of antibody N, or 45 per cent of the total present. As a check, the supernatant was further absorbed with pneumococcus specific precipitates. In view of the number of absorptions, the total, 0.37 mg. N, is in satisfactory agreement with the direct determination of homologous antibody N, 0.42 mg.

Intravenous Injection of Albumin.—It will be noted from Table I that three horses, 999, 1046, and 1126 received rabbit serum albumin intravenously during at least part of the immu-

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nization period. Serum taken from horse 999 after a short course (thirteen injections) gave no precipitate over a range of dilutions of the test antigen. Serum from horse 1046 (bleeding May 14, 1942) after an extensive series of intravenous injections, and from horse 1126 which had received smaller intravenous amounts of albumin, together with globulin, showed some flocculation with rabbit albumin, but compared with the serum of horse 1127 which had received a parallel subcutaneous course (Table I) the zones were much broader and the precipitates, even at the maximum, were much smaller. Globulin concentrates of these final bleedings of horses 1046 and 1126 were also prepared, but the portions precipitated at $\frac{1}{3}$ saturation (1046A and 1126C) and $\frac{1}{3}$ to $\frac{1}{2}$ saturation with ammonium sulfate (1046B and 1126D) were not further fractionated as had been done for serum 1127. For antibody tests on the fractions, portions of the flocculating concentrate 1127J were set up with several amounts of albumin corresponding to a point near the maximum of precipitation (Fig. 1), a point on the descending part of the curve, and a point just beyond it. When the precipitation was carried out in the

TABLE VI

Removal of Antibody from an Anti-Antibody (Pn II Horse Specific Precipitate) Rabbit Globulin Solution by Specific Precipitates from Horse Sera

Suspension used	Antibody N precipitated			
	First absorption	Second *	Third *	Total
	mg.	*mg.*	*mg.*	*mg.*
H 999 floccules	0.184	0.004	0	0.19
Pn I specific precipitate (on supernatant of last absorption above)	0.132	0.036	0.012	0.18
				0.37
Pn I specific precipitate	0.342	0.077	0.005‡	0.42

Pn used for pneumococcus.

* Single determinations on combined supernatants.

‡ Pn II specific precipitate suspension used for this absorption.

presence of concentrates 1046A and B, and 1126C and D, only 1046A failed to show some deviation of the zone of flocculation as compared with that of the control containing 1127J alone. All of the concentrates had been absorbed twice with rabbit anti-egg albumin specific precipitates to free them of at least part of their anti-γ-globulin antibody. The presence of some antibody in the sera of horses 1046 and 1126 was therefore indicated. However, quantitative precipitin tests of 2 ml. portions of solutions 1046B and 1126D with six different amounts of albumin antigen yielded precipitates whose total N ranged from 0.012 to 0.112 mg. Since this was only 0.6 to 0.2 of the N added in the form of antigen, it is evident that the reaction was not due to the rabbit serum albumin itself, but most likely to some impurity, possibly traces of α- or β-globulin, all or most of the antibody to γ-globulin having been previously absorbed.

DISCUSSION

The data show that horses 999 and 1127, which had been injected subcutaneously with rabbit serum albumin, yielded antibody of the flocculating type, with inhibition zones in the regions of antibody and antigen excess. In this respect the anti-rabbit serum albumin resembled other antiproteins elaborated by the horse, except the antinucleoprotein described in the preceding paper (9).

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The electrophoretic patterns of the sera of horses 999 and 1127 clearly showed the appearance, after the subcutaneous injections, of a new component migrating between the β-and the γ-globulins. Similar changes have characterized antitoxic sera in horses (12, 13), as well as some antipneumococcus sera (16).

The first course of injections for horse 999 was given intravenously and no precipitation was observed in serum taken after the course. The succeeding subcutaneous course of injections evoked a prompt response, and it is readily seen (Table II) that the newly formed antibody was of the flocculating type. As is evident from the data on the later bleeding reported in Table II and Fig. 1, the amount of nitrogen precipitated in the cold increased appreciably when the tubes were allowed to stand for longer periods than customarily used, especially when the proportions of antigen and antibody deviated notably from those giving maximum precipitation. It is quite possible that the shape of the curve for the earlier bleeding might have been somewhat altered if the precipitations had been allowed to stand longer.

If the assumption is made that all of the antigen N added is precipitated in the equivalence region, antibody N values may be calculated by subtraction from the total N. In the first bleedings (Table II) the antibody N remained constant (0.30 to 0.33 mg.) when the amount of antigen N added was varied by 50 per cent. Similarly, for the later bleeding, the antibody N varied between 0.82 and 0.77 mg. when the amount of antigen was increased from 0.144 to 0.288 mg. N.

This behavior appears to be characteristic of the flocculation reaction, having been observed as well in the diphtheria antitoxin (4), scarlatinal antitoxin (17), and anti-egg albumin systems in horse sera (5, 6). In all of these there is a region in which the total N precipitated increases linearly with added antigen. The amount of antibody precipitated is the same throughout this region, however, since the increase in total N is due to the precipitation of antigen, all of which is precipitated if homogeneous. In this region the reaction resembles the usual precipitin reaction (*i.e.*, a system without an inhibitory prezone) in that the ratio of antibody N to antigen N precipitated decreases as more antigen is added. There is, however, the distinction that in the precipitin type of reaction the antibody N precipitated usually increases slightly as well.

Pappenheimer and coworkers (4, 17) have used the constancy of the amount of antibody precipitated in the floccules to determine the amount of nitrogen corresponding to a flocculating unit of toxin or to determine, by difference, the per cent precipitability of a flocculating antigen. Similar calculations may be made from the present data. It will be noted from Table III (3 ml. series) that 0.096 mg. of albumin N precipitated 0.612 mg. of total N, while 0.171 mg. of antigen N removed 0.698 mg. N. The increase in total N precipitated, 0.086 mg., is the same, within experimental error, as the increase in the amount of added antigen N, 0.075 mg. The corresponding difference in the total N pre-

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cipitated in the 10 ml. series is 0.082, again evidence that all of the albumin N added was effectively antigenic. Similarly, in Table II an increase of 0.144 mg. antigen N resulted in an increase of 0.12 mg. total N precipitated.

The data of Table IV, obtained with antibody concentrate 1127J, are somewhat at variance with this, since 0.146 minus 0.098 = 0.048 mg. antigen N resulted in an increase of 0.11 and 0.14 mg. of total N at 0° and 37°, respectively. This disproportionate increase in the amount of N precipitated is as yet unexplained.

An early bleeding of horse 999 (January 1, 1941) (Table II) and a later one (July 2, 1941), were compared quantitatively by multiplying the antigen N added and the total N precipitated in the earlier bleeding by 2.47, the ratio found for the total N precipitated (as well as the relative antibody contents) in the two bleedings. It is evident that the points calculated for the earlier bleeding (crosses, Fig. 1) lie quite close to the experimental curve for the later bleeding, at least in the linear region, and that the two maxima occur with the same amount of antigen. Although the heights of the two curves can be made to coincide arbitrarily it does not necessarily follow that the maxima will occur with the same amounts of antigen unless the combining ratios are the same. These may be estimated directly by calculation of the antibody N to antigen N ratios, at for example, the midpoints of the flocculation zones (Table II). A value of 3.3 is obtained for the earlier bleeding compared with 4.1 to 3.4 for the later bleeding, depending upon the mid-point chosen. It is evident, therefore, that the combining properties of the antibody did not change greatly on continued immunization of the animal, as in some other systems (6, 11) even though the absolute quantity of antibody increased 2.5-fold.

Within the time limit selected, 6 days, precipitation at the maximum was not influenced (0.694 to 0.688 mg. total N) by variation of the reaction-volume from 3 to 10 ml. (Table III). This did not apply to points on either side of the maximum, as these usually showed less precipitation at the greater dilution.

In the experiments at 0° and 37°, carried out with antibody concentrate 1127J, the points of maximum precipitation coincided in both series, but more N was precipitated in the region of antibody excess at 0° than at 37° (Table IV). The reverse was true in the region of antigen excess. Whether these effects are due to varying rates of attainment of equilibrium at the two temperatures, or to real changes in the combining proportions, it is evident that the amount precipitated at the maximum is the same at either temperature, and that in this respect the system resembles diphtheria toxin-antitoxin (18) and the egg albumin anti-egg albumin reaction (5, 6) in the horse, as well as protein-antiprotein (11) systems in the rabbit. It will be recalled that carbohydrate-anticarbohydrate reactions in horse sera may show quite pronounced temperature effects (19) and, as will be discussed in the following paper (20), this is true for some antiprotein reactions in this species as well.

The data given in Table V show that the soluble antigen-antibody complexes

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formed in the two zones of inhibition (for diphtheria toxin-antitoxin, *cf.* (21)) react reversibly with more of the component not in excess, since addition of this component in proper amount suffices to bring the system to the point of maximum precipitation.

Although quantitative estimations were not made on all globulin fractions of the serum of horse 1127, it was evident from qualitative flocculation tests that most, if not all, of the antibody to the rabbit serum albumin was present in the water-soluble portions, particularly of the fraction precipitating between $\frac{1}{3}$ and $\frac{1}{2}$ saturation with ammonium sulfate. This fraction of the serum of horses immunized with diphtheria toxin or toxoid also contains most of the antitoxin. Antibacterial horse sera, on the other hand, contain a much larger proportion of water-insoluble antibodies, and antibody to the type-specific carbohydrate of pneumococcus may be almost quantitatively recovered by dilution with slightly acid water (22).

Since the chemical properties of the antibody to rabbit serum albumin, as well as its behavior toward its homologous antigen (Tables II to IV) suggested that it closely resembled diphtheria antitoxin in the horse, it was of interest to see whether the correlation held for its serological activity toward an anti-antibody serum. The latter, produced by injecting rabbits with a specific precipitate derived from Type II antipneumococcus horse serum, had previously been shown to differentiate antitoxic from antibacterial antibodies produced in the horse (10). As is demonstrated in Table VI, the antibody to rabbit serum albumin, in the form of floccules with its antigen, removed only about one-half of the antibody present, as did diphtheria toxin-antitoxin floccules. This serological criterion, therefore, also emphasizes the similarity between the subcutaneously elicited anti-rabbit serum albumin and the similarly induced diththeria antitoxin and anti-egg albumin in the horse. In agreement with the practical experience of others in the production of antitoxin, intravenous injection of horses with the same antigen did not result in detectable production of antibody with these characteristics. It is, however, possible that antibody is produced after intravenous injection in a form not detected by these tests, and it is hoped to undertake further examination of the sera with this in mind.

While, with the exception of hemolysin (23), antibody produced in the rabbit appears to be of only one qualitative type, it is clear that even in this species the response to a given antigen may be markedly influenced by the route of injection. The intracutaneous or subcutaneous injection of pneumococci (24) and of streptococci (25) results mainly in the production of species-specific antinucleoprotein. Type-specific antibodies may be produced abundantly, however, on intravenous injection of these microorganisms.

Thus far, in the horse, the production of the zonal flocculating type of antibody appears to have occurred only after subcutaneous injection. This, however, is not the only type of anti-protein response by the subcutaneous route, as will be set forth in detail in the following paper (20).

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SUMMARY

1. Two horses were injected subcutaneously with alum-precipitated rabbit serum albumin.

2. The resulting antibody resembled diphtheria antitoxin and anti-egg albumin in the horse in giving a sharp zone of flocculation with antigen, in being water-soluble, in reactivity toward an anti-antibody rabbit serum, and in its electrophoretic properties.

3. The effect of continued immunization, and of variation in volume and temperature on the reactivity of the antibody are discussed.

4. Intravenous injection of the same antigen into horses did not give rise to detectable amounts of antibody of the same type.

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