ANTI-EGG ALBUMIN ANTIBODY IN THE HORSE

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The flocculation reaction between diphtheria toxin and antitoxin has usually been regarded as an exceptional or atypical example of the precipitin reaction, chiefly because the specific precipitate is soluble in a relatively small excess of antibody. Practically all the work on protein-antiprotein systems previously reported, excepting toxin-antitoxin reactions, has been carried out with antibody obtained from the rabbit. It seemed possible to us that the exceptional character of the toxin-antitoxin reaction might be due merely to a species difference in the antibody used and that the reaction may well be a typical example of horse antiprotein systems. We have therefore immunized a horse against crystalline egg albumin. Crystalline egg albumin is easily prepared and is generally regarded as a well defined protein. It was hoped that the egg albumin-anti-egg albumin system in the horse might prove a more convenient and less costly analogy to the Ramon toxin-antitoxin flocculation reaction for quantitative studies, since pure diphtheria toxin is prepared only after considerable labor and expense and large amounts are not available. The present communication reports the successful immunization of a horse with three times recrystallized egg albumin. The reaction between egg albumin and its antibody in the horse has indeed been found to give rise to a process characteristic of diphtheria toxin-antitoxin flocculation. Moreover, it has been found that diphtheria antitoxin produced in the rabbit gives rise to a typical precipitin reaction with toxin, similar to the usual protein-antiprotein reaction in the rabbit. A quantitative study of the toxin-antitoxin precipitation reaction in the rabbit has not been made.

We are only aware of a few previous attempts to immunize the horse against isolated proteins. Some years ago this laboratory attempted to immunize a horse against egg albumin for Dr. R. M. Ferry. Hooker and Boyd (1) attempted to immunize a horse against arsanilic acid coupled with protein and Goebel (2) with certain glycosides coupled with protein. The last developed a feeble precipitin against the homologous glycoside-protein after injecting a total of 0.5 gm. intravenously. The other attempts were un-

successful in that no antibody was detected.[1] Heidelberger and Kendall (3) used R-salt-azo-biphenyl-azo-egg albumin and injected 4.5 gm. over a period of one month. They obtained weak precipitins in only a single bleeding.

EXPERIMENTAL

Preparation of Crystalline Egg Albumin.—Crystalline egg albumin was prepared by the classical method of Hopkins and Pincus as modified by Heidelberger (4). It was recrystallized three times. The filtrate from the first crystallization was brought to 0.75 saturation with ammonium sulfate and the precipitate collected and dialyzed against phosphate buffer at pH 7 containing 1 per cent sodium chloride. The resulting solution, containing a number of proteins, was termed the conalbumin fraction.

TABLE I

Antibody Content of Serum 728 during Immunization

Bleeding No.	Date	Serum flocculating with 0.01 mg. Ea nitrogen	Flocculation time, Kf (at 42° C.)	Anti-Ea* nitrogen per cc.
	1938	*cc.*	*min.*	*mg.*
0	June 27	—	—	—
3	Aug. 18	—		*ca.* 0.2,† (0.18)
4	Nov. 25	0.35	15	(0.23)
5	Dec. 7	0.32	18	0.23, (0.21)
6	" 23	0.27	22	—
	1939			
7	Jan. 5	0.28	27	0.25
8	" 26	0.20	20	(0.31)
9	Feb. 9	0.17	27	0.35
10	" 23	0.18	25	—
11	Mar. 9	0.17	28	—
12	" 31	0.16	32	0.37

* Figures in parentheses were determined by Heidelberger, Treffers, and Mayer (10).

† No flocculation. Antibody nitrogen estimated from amount of inhibition of precipitation of egg albumin by anti-egg albumin rabbit serum.

Production of Precipitins in Rabbits.—Rabbits were immunized against egg albumin and pure diphtheria toxoid (5) by intraperitoneal and intravenous injections of 5 to 10 mg. of protein daily for four injections followed by a 10 day rest period. Test bleedings were taken after two to three courses of injection.

Immunization of Horse 728 with Egg Albumin.—The horse was started June 27, 1938, with 7 mg. three times recrystallized egg albumin adsorbed on precipitated calcium phosphate. It received subcutaneous doses increasing to 14 mg. two or three times a week. On July 20, 100 mg. of egg albumin in lanolin-olive oil were injected intraper-

[1] Dr. Hooker and Dr. Boyd inform us that they are now immunizing a horse with hemocyanin and have been successful in obtaining flocculating antibody which reacts similarly to that reported in the present paper.

itoneally. 2 weeks later a test bleeding showed no flocculating antibody. Two further subcutaneous injections of 50 mg. each followed. On Aug. 18 a test bleeding still showed no flocculating or precipitating antibody. Immunization was discontinued at this point and in October the horse received three injections of scarlet fever toxin. At this time it occurred to us that non-precipitating antibody might be present and the Aug. 18 bleeding was reexamined. It was demonstrated that appreciable amounts of non-precipitating antibody were present by inhibition of the precipitin reaction between egg albumin and a strongly precipitating anti-egg albumin rabbit serum. The horse was therefore returned to the egg albumin schedule on Nov. 14 to 17 with two subcutaneous injections of 35 mg. on calcium phosphate. One week later, a test bleeding showed the presence of flocculating antibody. From this time on the horse received two injections of 100 mg. each per week followed by a one week rest period until it was bled finally on Mar. 31, 1939. The final bleeding showed 0.37 mg. anti-egg albumin nitrogen per cc. of serum. During the entire course of injections the horse received a total of 1.9 gm. of egg albumin.[2] Table I shows the increase in antibody during the course of immunization.

Demonstration of Non-Precipitating Antibody.—Initial bleedings and particularly that of Aug. 18, 1938, were tested with egg albumin for flocculating antibody. To 0.5 cc. of serum, 1 cc. of 1 per cent egg albumin and 1 cc. of twofold serial dilutions of this solution down to 1:10,192 (1:1,000,000 egg albumin) were added. No flocculation occurred at any dilution even after standing overnight at 42°C.

0.5 cc. of the bleeding of Aug. 18, 1938, was placed in each of a series of tubes and 0.5 cc. of dilutions of a 0.014 per cent solution of egg albumin was added and the contents mixed. After standing in the water bath at 42°C. for one hour, the tubes were brought to room temperature and 0.5 cc. of a strongly precipitating rabbit serum added. At the same time a control experiment was carried out, using normal horse serum instead of serum 728. Inhibition of precipitation occurred in the series incubated with No. 728 but not with normal serum. This inhibition was not permanent and after standing 24 hours at room temperature precipitation occurred in all cases where it occurred immediately with normal horse serum. A typical protocol with the bleeding of Aug. 18, 1938, and some pseudoglobulin prepared from it by collecting the water-soluble fraction of the one-third to one-half saturated ammonium sulfate precipitate is shown in Table II.

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[2] Horse 728 weighed 1670 pounds. Assuming that the animal contained 25 liters of serum at the time of the final bleeding the total circulating anti-egg albumin antibody must have been in the neighborhood of 50 gm. It is of interest to compare this figure with antitoxin production by two horses which were simultaneously immunized at the Antitoxin Laboratories of the National Drug Company in Swiftwater, Pennsylvania. These two horses each received a total of 60 mg. (20,000 Lf units) of diphtheria toxoid over a period of one month. At the end of that time horse 485 contained 2150 units of antitoxin per cc. of serum, equivalent to 25 mg. antitoxin per cc., which by the above calculation would correspond to more than 600 gm. total circulating antitoxin or more than 10,000 times the weight of antigen injected. This striking case would seem to refute the possibility that any significant portion of antigen is incorporated in the antibody. Horse 481 which received identical treatment to 485 produced less than 15 gm. total circulating antitoxin. We are indebted to Dr. Warren B. Rawlings of the National Drug Company for sending us the protocols of the immunization of these two horses.

Flocculation Reaction.—Flocculation reactions were carried out in the usual manner. 1 cc. of egg albumin solution was added to increasing amounts of antiserum and incubated at 42°C. The first tube to flocculate was recorded. It was usually convenient

TABLE II

Demonstration of Non-Precipitating Antibody

0.5 cc. of each serum fraction incubated with 0.5 cc. of egg albumin solution for one hour at 37°C. Then 0.5 cc. of precipitating rabbit anti-egg albumin serum added to each tube. Egg albumin solution 0.014 per cent. Readings after 10 minutes at 25°C.

Serum fraction	Egg albumin solution					
	Undiluted	1:2	1:4	1:8	1:16	1:32
Normal horse serum	++++	+++	++	+	tr	−
No. 728, bleeding Aug. 18, 1938	+±	tr	−	−	−	−
No. 728, pseudoglobulin	++	±	−	−	−	−
No. 728, serum albumin	+++	++	+	±	−	−

TABLE III

Quantitative Flocculation of Serum 728 with Egg Albumin

1 cc. of a pseudoglobulin preparation representing about a threefold concentration of bleeding 9 was used in each tube. It contained 14.5 mg. nitrogen per cc.

Egg albumin nitrogen added	Nitrogen in precipitate	Anti-egg albumin nitrogen	Ratio $\frac{\text{A nitrogen}}{\text{Ea nitrogen}}$
mg.	*mg.*	*mg.*	
0.035	0	—	—
0.07	0	—	(15.2)
0.10	1.03*	—	—
0.12	1.17	1.05	8.8
0.14	1.20	1.06	7.6
0.15†			7.1
0.17	1.25	1.08	6.3
0.20	1.18*	—	—
0.275	0	—	—
0.40	0	—	—

* Probably incomplete precipitation.

† Optimum flocculation occurred with 0.15 mg. egg albumin nitrogen. Average ratio anti-egg albumin to egg albumin nitrogen from five determinations at the flocculation point on different bleedings was 6.8.

to work with solutions containing 0.005 to 0.01 mg. egg albumin nitrogen per cc. Quantitative flocculation tests on larger amounts of serum were carried out as described for the toxin-antitoxin reaction by Pappenheimer and Robinson (6). The protocol of one such experiment on a pseudoglobulin fraction is given in Table III.

Conalbumin Fraction of Egg White.—Following a suggestion by Dr. Hans Zinsser, the anti-egg albumin serum was set up against dilutions of whole egg white. To our surprise, the serum showed double zone flocculation, the first zone corresponding to the expected egg albumin antibody, the second to an antibody against an unknown substance present in the conalbumin fraction of egg white. Quantitative flocculations on egg albumin absorbed serum indicated that approximately the same amount of antibody was present against each of these egg white proteins. When the globulin is removed from egg white by half saturation with ammonium sulfate and the egg albumin crystallized out by bringing to pH 5.2, 90 per cent of the unknown active substance remains in the supernatant from which it may be precipitated by raising the ammonium sulfate concentration to 0.75 saturation. It was no longer possible to detect the active substance of the conalbumin fraction by flocculation after one recrystallization of the egg albumin. In spite of the small trace present, however, immunization of horse 728 with three times recrystallized egg albumin produced considerable flocculating antibody against the unknown antigen which must therefore be a very powerful antigen in comparison with egg albumin itself. Further studies on the nature of this substance in the conalbumin fraction are in progress.

DISCUSSION

A horse has been successfully immunized against three times recrystallized hen egg albumin. The flocculation reaction between egg albumin and anti-egg albumin horse serum behaves in almost every respect analogously to the Ramon diphtheria flocculation reaction. (*a*) The antibody is concentrated in the water-soluble pseudoglobulin fraction of the serum or plasma which is precipitated between 33 and 50 per cent saturation with ammonium sulfate as is antitoxin. (*b*) The zone of flocculation is narrow and no precipitate forms when the ratio of antibody to antigen exceeds twice that at the flocculation point. The composition of the specific floccules varies over approximately a twofold range in the equivalence zone. (*c*) By fractional addition of egg albumin to the antiserum, the Danysz phenomenon may be demonstrated by flocculation. The magnitude of the Danysz phenomenon is approximately the same as that found for the toxin-antitoxin reaction (6). (*d*) Finally, the ratio of antibody nitrogen to antigen nitrogen at the flocculation point is almost twice that of antitoxin to toxin nitrogen. It follows that the molecular composition of the specific floccules at this and other reference points is nearly the same as that of toxin-antitoxin floccules at the corresponding reference points, since the molecular weight of egg albumin is approximately half that of diphtheria toxin (7). In making this last calculation it is assumed that horse anti-egg albumin pseudoglobulin has the same molecular weight as antitoxic pseudoglobulin. In conclusion, it appears to us that the flocculation reaction, as exemplified by the toxin-antitoxin and egg albumin-anti-

egg albumin reactions, should be considered tentatively as the normal type of protein-antiprotein reaction in the horse, and not, as heretofore (Marrack, 8), as an exceptional example of the precipitin reaction.

The behavior of the antiprotein antibody in the horse is quite different from that of the usual antibacterial antibodies. Thus horse pneumococcus antipolysaccharide is concentrated in the water-insoluble or euglobulin fraction of immune horse serum and forms specific precipitates with homologous polysaccharide in all proportions except very large antigen excess. A possible explanation for the difference in behavior of the two types of antibodies has already been suggested elsewhere (7).

Of particular interest is the formation of a completely soluble antigen-antibody system during the early stages of immunization with egg albumin. It has already been shown from ultracentrifugal studies that diphtheria antitoxin contains a high proportion of antibody with at least two "valences" or reacting sites (7). Thus the formation of a lattice (8) or specific aggregates, is possible with proper proportions of antigen and antibody. The most reasonable explanation of the soluble system encountered in the present work would be the presence of "monovalent" or incompletely reactive antibody during the initial stages of immunization, much as postulated for the Type III polysaccharide-antipolysaccharide and egg albumin-anti-egg albumin systems in the rabbit by Heidelberger and Kendall (9). Such incomplete antibody with only one reacting site would, of course, be unable to build up a lattice. As soon as a reasonable proportion of "bivalent" antibody has been formed precipitation becomes possible. Unfortunately, very little of this incomplete antibody has been available for study since its presence was detected only after immunization had been temporarily suspended and upon renewing the injections, flocculation occurred.

Samples of horse anti-egg albumin serum have been sent to Dr. M. Heidelberger, New York, and to Dr. S. B. Hooker, Boston. In both laboratories the ratio of antibody to antigen nitrogen found at the flocculation point agrees closely with our own result. Some of the results from Dr. Heidelberger's laboratory are reported in the following paper (10).

SUMMARY

The reaction between three times recrystallized egg albumin and its antibody in the horse has been studied. The reaction exhibits a behavior typical of the Ramon diphtheria flocculation reaction.

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