A QUANTITATIVE THEORY OF THE PRECIPITIN REACTION

VII. The Egg Albumin-Antibody Reaction in Antisera from the Rabbit and Horse*

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In Paper III of this series (1) it was shown that the quantitative theory of the precipitin reaction (2) was applicable to the reaction between a native protein, crystalline egg albumin,[1] and its homologous antibodies in the rabbit. Data were also given for the sera from a single rabbit after three successive courses of injections. It was found that in the linear equation characterizing the behavior of the serum as far as the point of maximum precipitation:

$$\frac{\text{mg. antibody N precipitated}}{\text{Ea N precipitated}} = 2R - \frac{R^2}{A}(\text{Ea N}) \quad \ldots\ldots\ldots\ldots\ldots\ldots [1]$$

in which R is the ratio of antibody N to Ea N precipitated at a reference point in the equivalence zone and A is the amount of antibody N precipitated at the reference point, the value of 2R, the intercept on the y axis, increased with progressive immunization. This was considered to indicate that the antibody formed as a result of the later courses of injections was capable of reacting with more and more groupings on the Ea molecule. In accord with this interpretation was the marked broadening of the equivalence zone with each additional course of injections.

The generality of these observations has been indicated for the other antigen-antibody systems studied in this laboratory, and reference to these

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[1] Subsequently referred to as Ea. Antibody is referred to as low grade, incomplete, imperfect, or "univalent" in instances in which it is incapable by itself of precipitating with added antigen; as multivalent in instances in which, carrying presumably two or more immunologically reactive groupings, it combines with antigen to form insoluble aggregates. "Univalent" is not used in the literal sense.

will be made below. It seemed of interest, however, to follow the properties of the antibodies in anti-Ea sera through even more extensive courses of Ea injections, particularly in view of the failure of data obtained by Taylor, Adair, and Adair (3) to show a similar increase in 2R, as noted in Text-fig. 2 of reference 1.

It will also be remembered that on exhaustion of anti-Ea sera with successive small portions of Ea there remained a portion of the antibody which was no longer capable of precipitating Ea, but which could be detected by its capacity of combining with Ea-anti-Ea precipitates (1). This was shown to be in accord with the theory that specific precipitation is the result of the interaction of multivalent antigen with multivalent antibody. The non-precipitating residual or low grade antibody would then be "univalent" or characterized by so few specifically reactive groupings as to be incapable of forming the large aggregates yielded by multivalent antibody, yet capable of adding to such aggregates, when these are present, by means of the single or limited number of specific groupings available.

The observation of Dr. A. M. Pappenheimer, Jr., that precipitins were not formed in the early stages of Ea injections into a horse suggested the possibility that only such low grade, or incomplete antibody might be present. Dr. Pappenheimer furnished a sample of plasma from the horse and appreciable amounts of the incomplete antibody were actually found. Its quantitative estimation and the demonstration of its combination with Ea are given in the present paper.

At the same time the presence of incomplete antibody was detected by Dr. Pappenheimer by its inhibitory effect on the precipitation of Ea by rabbit anti-Ea, and he has described in the paper immediately preceding this both this early reaction and the precipitin reaction which appeared later (4). Additional observations made with horse anti-Ea bleedings kindly placed at our disposal by Dr. Pappenheimer are also recorded in the present communication, including experiments with a bearing on reaction velocities in Ea anti-Ea systems.

EXPERIMENTAL

The experimental procedure was the same as that reported previously (1, 2, and other papers). Rabbit 4.28 received an initial course of 10 intravenous injections, each of 10 mg. of alum-precipitated egg albumin, over a period of 21 days. Serum 4.28 I was drawn 5 days after the last injection. In general, bleedings were taken 5 to 7 days after the final injection of a course, and a 10 to 14 days' rest period was allowed before the next course of injections. The second course for rabbit 4.28 consisted of 10 injections of from 10 to 15 mg. of Ea during 15 days. The third course consisted of 8 injections of 2 to 8 mg. of Ea each during 10 days. The fourth comprised 11 injections of 10 to 20

mg. each in 27 days. The initial course for rabbit 4.30 consisted of 14 injections of 10 mg. of Ea in a period of 21 days. Courses II, III, and IV were the same as for rabbit 4.28. The fifth course was one of 10 injections, of from 5 to 20 mg., during 15 days.

Antibody nitrogen was determined by the quantitative method (1, 2), increasing amounts of standard egg albumin solution being added in duplicate to tubes containing the antisera at 0°. After mixing, the tubes were allowed to stand for 48 hours and were centrifuged and washed twice with cold saline. Nitrogen was determined by a modification of the micro Kjeldahl method. In the region of antibody excess, the amount of Ea nitrogen added was deducted from the total N precipitated to give the antibody nitrogen. In the region of excess antigen, separate analyses were made of the amount of Ea in the supernatants in order to correct for the incomplete precipitation of the antigen.

Data are given in Table I for both sera in the reaction range including the equivalence zone. The analyses are calculated to 1.0 ml. of undiluted or 1:1 antiserum, although the actual amount taken for analysis varied with the strength of the sera. Equation [1] may be written in the form

$$\text{mg. antibody N pptd.} = 2R(\text{Ea N}) - \frac{R^2}{A}(\text{Ea N})^2 \quad [2]$$

and the constants for this equation are given after the data for each course in Table I. In calculating these, use was made of numerous other points in the region of antibody excess which are not reported in the table.

In Table II the equations for the sera have been recalculated to a common basis of 1.0 mg. of precipitable antibody nitrogen per ml. Values are also given for the ratios at the antibody excess and antigen excess ends of the equivalence zone, as nearly as could be determined by tests on the supernatants for antigen and antibody. The last column gives the difference between these ratios at the ends of the zone as a measure of the breadth of the equivalence zone.

Experiments with Bleedings 3, 4, 5, and 8 of Pappenheimer's Anti-Ea Horse 728 (4).—(*a*) Bleeding 3 (*cf.* Tables I and II, preceding paper). After a preliminary test in which it was found that the serum did not precipitate Ea at any of the dilutions tried, the following series of quantitative analyses was set up.

Rabbit anti Ea pool *a*, *ml*	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Horse 728_3 serum, *ml*		1.0						1.0	1.0	1.0
Normal horse,*			1.0				1.0			
Ea N, *mg*				0.11	0.12	0.14	0.14	0.14	0.16	0.18
Saline, *ml*	2	1	1	2	2	2	1	1	1	1
N pptd., *mg*	0.00	0.01	0.01	1.12	1.14	1.04	1.08	1.37	1.40	1.32
Less Ea N and blank				0.11	0.12			0.18†	0.20†	
Antibody N pptd., *mg*				1.01	1.02			1.19	1.20	
Less rabbit antibody N								1.02	1.02	
Low grade horse antibody N								0.17	0.18	
Supernatants + Ea				−	−	−	−	−	−	−
" + anti-Ea pool *a*				−	±	++	++	−	+	+±

* From a horse injected with bovine tubercle bacilli. The serum contained no precipitins for bovine tubercle bacillus protein or carbohydrate fractions.

† The difference between the runs with and without normal horse serum, 0.04 mg. N, (columns 7 and 8) was added to the Ea N as a blank.

TABLE I

Addition of Increasing Amounts of Egg Albumin to Rabbit Antiserum at 0°C.

Ea N added	Ea N pptd.	Total N pptd.	Antibody N by difference	Ratio antibody N:Ea N in ppt.	Tests on supernatant
mg.	*mg.*	*mg.*	*mg.*		
		*Serum 4.28**			
		Course I, undiluted			
0.021_5	Total	0.243	0.221	10.3	Excess A
0.032	"	0.323	0.291	9.0	No A or Ea
0.043	0.039	0.356	0.317	8.1	Excess Ea
	mg. antibody N pptd. = 13.1(Ea N) − 129(Ea N)²				
		Course II, undiluted			
0.048	Total	0.556	0.508	10.6	Excess A
0.063	"	0.644	0.581	9.2	No A or Ea
0.070	"	0.694	0.624	8.9	" " " "
0.079	0.077	0.722	0.645	8.4	Excess Ea
	mg. antibody N pptd. = 15.6(Ea N) − 103(Ea N)²				
		Course III, undiluted			
0.042	Total	0.464	0.422	10.1	Excess A
0.048	"	0.512	0.464	9.7	No A or Ea
0.063	"	0.586	0.523	8.3	" " " "
0.070	0.068	0.620	0.552	8.1	Excess Ea
	mg. antibody N pptd. = 15.3(Ea N) − 117(Ea N)²				
		Course IV, 1:1			
0.032	Total	0.367	0.335	10.5	Excess A
0.035	"	0.369	0.334	9.5	No A or Ea
0.038	"	0.393	0.355	9.3	" " " "
0.040	"	0.407	0.367	9.2	" " " "
0.043	"	0.408	0.365	8.5	Trace Ea
	mg. antibody N pptd. = 13.7(Ea N) − 120(Ea N)²				
		Serum 4.30			
		Course I, undiluted			
0.010_8	Total	0.120	0.109	10.1	Excess A
0.014_3	"	0.150	0.136	9.5	Trace Ea
	mg. antibody N pptd. = 13.0(Ea N) − 273(Ea N)²				
		Course II, undiluted			
0.029	Total	0.323	0.294	10.1	Excess A
0.035	"	0.364	0.329	9.4	Trace Ea
	mg. antibody N pptd. = 13.7(Ea N) − 122(Ea N)²				

* The writers are indebted to Miss Dorothy R. Meeker for many of the analyses on this serum.

TABLE I—*Concluded*

Ea N added	Ea N pptd.	Total N pptd.	Antibody N by difference	Ratio antibody N:Ea N in ppt.	Tests on supernatant
mg.	*mg.*	*mg.*	*mg.*		
		Serum 4.30—Continued			
		Course III, undiluted			
0.036	Total	0.440	0.404	11.2	Excess A
0.043	"	0.473	0.430	10.0	No A or Ea
0.057	"	0.544	0.487	8.5	" " " "
0.065	0.062	0.538	0.476	7.7	Excess Ea
	mg. antibody N pptd. = 17.1(Ea N) − 154(Ea N)²				
		Course IV, 1:1			
0.038	Total	0.479	0.441	11.6	Excess A
0.043	"	0.510	0.467	10.9	No A or Ea
0.065	0.064	0.566	0.502	7.8	Excess Ea
	mg. antibody N pptd. = 17.4(Ea N) − 156(Ea N)²				
		Course V, 1:1			
0.106	Total	1.54	1.43	13.5	Excess A
0.128	"	1.72	1.59	12.4	No A or Ea
0.157	"	1.81	1.65	10.5	" " " "
0.170	"	1.92	1.75	10.3	" " " "
0.210	"	2.01	1.80	8.6	Trace Ea
	mg. antibody N pptd. = 19.5(Ea N) − 54.0(Ea N)²				

TABLE II

Variation of 2R and Equivalence Zone Ratios with Length of Immunization

Serum	Equation, calculated to 1.0 mg. of precipitable antibody N	Equivalence zone ratios		Breadth of equivalence zone in A:Ea ratio units
		Antibody excess end	Antigen excess end	
4.28 I	13.1(Ea N) − 42.8(Ea N)²	<10	>8.1	<1.9
4.28 II	15.6(Ea N) − 60.9(Ea N)²	10.5	8.4	2.1
4.28 III	15.3(Ea N) − 58.5(Ea N)²	10.0	8.2	1.8
4.28 IV	13.7(Ea N) − 46.9(Ea N)²	9.7	8.7	1.0
4.30 I	13.0(Ea N) − 42.2(Ea N)²	<10.1	9.5	<0.6
4.30 II	13.7(Ea N) − 46.9(Ea N)²	10.1	9.4	0.7
4.30 III	17.1(Ea N) − 73.1(Ea N)²	<<12.9 >11.2	8.1	>3.1
4.30 IV	17.4(Ea N) − 75.7(Ea N)²	<12.3 >11.7	7.9	>3.8
4.30 V	19.5(Ea N) − 95.1(Ea N)²	13.6	8.6	5.0

The first figure in the reaction equation is 2R, the intercept on the y axis, or initial combining ratio.

The use of 0.5 or 1.5 ml. of rabbit serum instead of 1.0 ml. resulted in a decreased coprecipitation of the horse antibody. The tests on the supernatants and the amounts of Ea N used with the rabbit serum alone and with the mixture of rabbit and horse anti-Ea sera show that between 0.02 and 0.03 mg. of Ea N combines with the horse anti-Ea and is precipitated in the mixed specific precipitate.

(*b*) Flocculation reactions in serum from bleeding 8, anti-Ea horse 728. The quantitative flocculation experiments described by Pappenheimer in the preceding paper (4) were carried out at 42°C., followed by standing overnight in the cold. In those illustrated in Table III the tubes were allowed to stand at 0° for 48 hours, as in the precipitin reaction. There appears to be little difference in the end result in spite of the difference

TABLE III

Addition of Increasing Amounts of Egg Albumin to Serum from Bleeding 8, Horse 728; 0°C., 48 Hours; Calculated to 1.0 Ml.

Ea N added	Ea N pptd.	Total N pptd.	Antibody N pptd.	Ratio antibody N:Ea N in ppt.	Supernatants	
					Appearance	Tests with Ea and rabbit anti-Ea
mg.	*mg.*	*mg.*	*mg.*			
0.018*		0			Turbid	
0.020*		0.01			"	Excess A, trace Ea?
0.030*	Total	0.29	(0.26)	(8.7)	Slightly turbid	No A or Ea
0.045†	"	0.350	0.305	6.8	Clear	" " " "
0.050†	"	0.359	0.309	6.2	"	" " " "
0.060†	"	0.373	0.313	5.2	"	" " " "
0.067*	"	0.377	0.310	4.6	"	" " " "
0.075†		0.267			Turbid	
0.080‡		0.153			"	

Neutralization of the serum, originally pH 8.58, did not significantly alter the N precipitated.

* 3 times the indicated quantities of serum and Ea were used.

† 2 " " " " " " " " " "

‡ 2.5 " " " " " " " " " "

in temperature at which the two sets of data were obtained. Similar figures were yielded by sera from bleedings 4 and 5.

(*c*) Reversibility of the soluble Ea-horse anti-Ea compound or compounds formed in the region of antibody excess. It appeared of interest to test the reversibility of the effect of the horse anti-Ea serum, noted by Pappenheimer (4) of temporarily inhibiting precipitation in the Ea-anti-Ea rabbit system, the more so as it is shown in section *a* that the low grade antibody in bleeding 728_3 adds to the Ea-rabbit anti-Ea precipitate when suitable proportions are used. For this purpose it was convenient to use serum from one of the later bleedings, containing antibody which was completely precipitable in the equivalence zone,[2] but the amount of Ea was chosen so that a soluble pre-zone compound would be formed. The experimental data are summarized in Table IV.

[2] The supernatants contained no antibody precipitable on addition of both Ea and rabbit anti-Ea.

DISCUSSION

From the data presented in Tables I and II it is evident that successive courses of immunization may be accompanied by a rise in the initial combining ratio of antibody to antigen (from 13 to 19.5 in the case of rabbit 4.30) and a broadening of the equivalence zone (from about 0.6 to 5 ratio units), much as had been shown in a previous instance (1). On the other hand, the properties of the antibodies engendered in another rabbit, 4.28, remained relatively constant under the same treatment and at the same time. It would be difficult to postulate experimentally founded reasons for such differing behavior. It might, however, be assumed, within the framework of modern theories of antibody formation (5, 6) that the antibody-producing mechanism of rabbit 4.30 was more efficient than that of 4.28, not only in the sense of producing a slightly greater quantity of antibody, but more especially in its ability to form antibody capable of reacting with an increasing number of groupings on the antigen molecule as immunization proceeded (*cf.* 1). It would therefore seem important, in the selection of rabbits for the production of therapeutic sera, to keep under prolonged immunization only those animals showing increases in the initial combining ratios and a broadening of the equivalence zone. As shown in (1), these effects continually increase the efficiency of the antibody in the region of antigen excess, from which side therapeutically administered serum is operative.

The quantitative study of anti-egg albumin produced in the horse has thrown light on several aspects of the precipitin reaction. According to Pappenheimer (4), the toxin-antitoxin type of flocculation reaction appears to be the normal form of the protein-antiprotein reaction in the horse. This phase of immune reactivity, as shown both in (4) and in the present paper, is preceded by a phase in which only imperfect or low grade antibody is present and all Ea-anti-Ea compounds are soluble. The relatively sudden transition between the two phases occurring between bleedings 3 and 4 (Table I in reference 4) does not necessarily imply that all of the antibody has been converted into the multivalent form capable of building up insoluble aggregates, since it has been shown repeatedly that a fraction of low grade or incomplete antibody is present in antisera in many antigen-antibody systems (1, 2, 7, 8, 9). In the presence of normal or multivalent antibody the low grade, or univalent antibody adds to the aggregates which separate from solution after the addition of multivalent antigen.

Comparison of Table III in Pappenheimer's paper (4) and Table III in this study shows little difference in the Ea-horse anti-Ea reaction at 42°C., followed by 0°, and at 0° throughout, except in speed. In the failure of

the combining ratios and precipitable antibody to depend on temperature the system resembles protein-antiprotein (1, 7, 8, 9) and carbohydrate-anticarbohydrate (10) systems in the rabbit rather than the precipitin reaction between pneumococcus specific polysaccharide and homologous anticarbohydrate in the horse (2).

Pappenheimer has shown that combination of Ea with the low grade or imperfect antibody in bleeding 3 temporarily inhibits precipitation of Ea by rabbit anti-Ea. We have found similar inhibition by the multivalent antibody in later bleedings provided the amount of Ea chosen is small enough to keep the Ea-horse anti-Ea system in the region of excess antibody, or pre-zone, in which soluble compounds are formed. It appeared that a study of the reversibility of this reaction might throw light on the differences in mechanism in the Ea-HA[3] and Ea-RA systems and might also be of service in elucidating the mechanism of the Danysz effect (11) and in kinetic studies of antigen-antibody reactions. Exploratory experiments were accordingly made and these are summarized in Table IV.

When the horse and rabbit anti-Ea sera were mixed at 0°C. before addition of a pre-zone quantity of Ea, it was evident that formation of soluble Ea-HA compounds competed with the precipitation of insoluble Ea-RA compounds since only 0.12 mg. of nitrogen was precipitated out of a maximum of 0.18 mg. If, however, specific precipitation is due to the combination of multivalent antigen with multivalent antibody it is preceded by the formation of soluble compounds (2, 1). The two competing forward reactions in the case of the mixture of anti-Ea sera would be,

$$m\ \mathrm{Ea} + x\ \mathrm{RA} \rightarrow \mathrm{Ea}_m(\mathrm{RA})_x, \text{ soluble,} \quad [3]$$

and

$$n\ \mathrm{Ea} + y\ \mathrm{HA} \rightarrow \mathrm{Ea}_n(\mathrm{HA})_y, \text{ soluble,} \quad [4]$$

These would appear to proceed at comparable velocities, for in these experiments the concentration of RA was roughly three times that of HA and the subsequent polymerization of $\mathrm{Ea}_m(\mathrm{RA})_x$ into large insoluble aggregates to the extent of 0.12 mg. of N is about the amount expected under this assumption.

The slow transformation of soluble $\mathrm{Ea}_n(\mathrm{HA})_y$ into a precipitate, indicated by a steady increase in nitrogen precipitated from the mixed system, may be interpreted in several ways. It is possible that reaction [4] represents an equilibrium in which the reverse direction furnishes free Ea which may combine with the RA present according to [3], a reaction followed by precipitation and consequent removal of the reactants. This would be expected to proceed until the fraction of the Ea originally combined according

[3] HA is used to designate horse anti-Ea, RA to denote rabbit anti-Ea.

to [4] had reacted according to [3]. Possibly even more nitrogen would have been precipitated after an interval longer than 6 days, for the precipitates may have consisted in part of Ea-RA-HA aggregates.[4] Their texture was looser and they settled with greater difficulty on centrifugation than did Ea-RA precipitates even when these were formed in the presence of normal horse serum.

TABLE IV

*Inhibition of Specific Precipitation in Ea-Rabbit Anti-Ea Systems by Horse Anti-Ea Sera, and Its Reversal at 0°C., Calculated to 0.01 Mg. Ea N and 1.0 Ml. Serum**

Serum† to which 0.01 mg. Ea N per ml. was added first	Interval before second serum was added	Serum† added to initial reaction mixture	Interval before analysis	Total N precipitated
	hrs.		*days*	*mg.*
R, pool *c*			2	0.18
R, H_4 mixture	0		0.1‡	0.12
" "	"		0.7	0.14
" "	"		1.7	0.15
" "	"		2.7	0.16
" "	"		6	0.18
H_5	0.005	R§	2	0.02
"	0.8	"	2	0.01‖
H_4	4	R	3	0.02
"	"	"	7	0.11‖
"	"	"	12	0.16‖
"	"	"	20	0.17‖
R	0.02	H_5	6	0.19
"	1	"	"	0.19
"	"	H_4	"	0.18
"	"	"	1.7	0.17

* 0.03 mg. Ea N and 3.0 ml. of each serum were actually used in most instances.

† R = rabbit pool *c*; H = horse; subnumeral indicates bleeding.

‡ Flocculation occurred in about 3 hours in this mixture. In R plus normal horse serum the precipitate settled in about 20 minutes.

§ Pool *b*, yielding 0.19 mg. N per ml. with 0.01 mg. Ea N.

‖ Supernatant not clear.

The second set of experiments summarized in Table IV illustrates the remarkable speed of formation of the soluble Ea-HA compounds even at 0°, since the amount of reversal brought about by addition of RA after

[4] That Ea-RA-HA aggregates are actually present is now indicated by the following: A portion of precipitate, washed 5 times and containing 0.75 mg. of N, removed 0.21 mg. of N from 1 ml. of a rabbit antiserum to a horse globulin fraction. A control of Ea-RA, with 0.43 mg. of N, precipitated in the presence of an equal volume of normal horse serum, removed only 0.02 mg. of N from the same antiserum.

about 20 seconds was no greater than when RA was added after a week. The slow reverse reaction accompanied by the precipitation of $Ea_m(RA)_x$ appeared to approach completion in about 2 to 3 weeks, and its velocity is therefore of the order of 10^{-4} or 10^{-5} that of the forward reaction.

The third set of experiments in the table again illustrates the speed of the Ea-RA reaction. The indicated high velocity of antigen-antibody combination even at 0° might seem surprising. Earlier experiments in this laboratory have, however, shown a high reaction rate for pneumococcus I-agglutinin combination at 0° (12, page 888). Initial high reaction velocities are in accord with the quantitative theories of the precipitin and agglutinin reactions (2, 12) and would be compatible with the view that the postulated initial bimolecular reactions might take place between ionized groupings on antigen and antibody.

In earlier papers (2, 1, 12, etc.) the formation of insoluble aggregates has been considered a continuation of the initial specific combination by a series of comparable, competing bimolecular reactions. The great rapidity of the formation of soluble compounds and the relatively slow building up of insoluble aggregates does not necessitate a modification of this view. Steric and unknown (*cf.* 17) factors, increasing molecular size, and the decreasing number of available reactive groupings with progress of the reaction, could be expected to retard the later stages of an initially rapid combination of multivalent antigen with multivalent antibody.

The rapid formation of soluble compounds, $Ea_n(HA)_y$, indicates a close analogy with the toxin-antitoxin reaction, as already noted by Pappenheimer (4). A somewhat similar coupling of a rapid reaction to form a soluble TA complex, followed by a slow reaction resulting in flocculation was proposed by Pappenheimer and Robinson (13) in accounting for the slow flocculation time and for the Danysz effect in the toxin-antitoxin reaction. The slow reverse reaction noted in our experiments would account for the slow reversibility of the Danysz effect stressed by Arrhenius and Madsen (14) and by Healey and Pinfield (15).

In Ea-HA mixtures in the pre-zone a precipitate formed readily when sufficient Ea was added to bring the system into the equivalence, or precipitating zone (*cf.* 13, 15 for the toxin-antitoxin system). This may be taken to indicate that in the soluble compounds, $Ea_n(HA)_y$, free reactive groupings are available on the multivalent antibody with which to build up large insoluble aggregates when multivalent Ea is added in the proper proportions. Evidently, in the Ea-HA system the symmetry and close packing necessary for precipitation (*cf.* Marrack (16), also (8, 17)) is possible over a very limited range of molecular composition, for addition of

more Ea results in a soluble post-zone. In the Ea-RA reaction, it will be recalled (1), the initial soluble pre-zone does not occur.

While the effect of adding Ea to the pre-zone may be taken to indicate the presence in this zone of free A groupings, the failure of the addition of rabbit antibody to induce immediate precipitation does not necessarily indicate the absence in these compounds, in the proportions used in our experiments, of Ea groupings reactive with the rabbit antiserum pool used. It is possible that the dissymmetry of the soluble $Ea_n(HA)_y$ compounds is so great that even linkage of two or more molecules by added multivalent RA, through free Ea groupings, does not suffice to bring enough ionized groupings into sufficiently close proximity for mutual discharge and consequent loss of affinity for water (*cf.* 16, 17). If, however, there are no free Ea groupings available for reaction with added RA this would indicate the presence of soluble compounds ranging in molecular composition from $Ea(HA)_4$ to $Ea(HA)_6$, since the maximum immunological "valence" of Ea hitherto observed is 6 (*cf.* 17, 18).

A recent study has been made by Follensby and Hooker (19) of the effect of temperature upon combination, aggregation, and equilibrium in the reactions between diphtheria toxin and antitoxin and between Ea and precipitated anti-Ea. While several of the conclusions reached follow from earlier work and the new experiments, we consider that no evidence is given that "a decrease in temperature from 40–5°C. has little influence upon the speed of combination" or that "with regard to temperature the reaction does not behave like one occurring in a single phase." It is clear from Table IV of our paper that the speed of combination between antigen and antibody even at 0° may be at least ten to 100 times as great as it was possible to measure by the cumbersome method used in the agglutination system (12) or by the technique adopted by Follensby and Hooker, in which measurements were made only after an effective delay of somewhat less than 8 minutes. It is therefore difficult to see how conclusions as to the mechanism of the initial antigen-antibody combination can be based on observations of this nature made long after the initial reaction was essentially complete.

SUMMARY

1. In two rabbits subjected to prolonged injections with crystalline egg albumin the antibodies in one showed progressive changes such as noted in an earlier paper; the antibodies in the other did not.

2. The significance of this behavior in the production of sera for therapeutic use is pointed out.

3. Quantitative studies are reported on the low grade or incomplete antibody present in the early stages of immunization of a horse with egg albumin.

4. Quantitative studies on the flocculating antibody from later bleedings from the horse are given, and the dissociation of the soluble pre-zone compounds by rabbit anti-egg albumin is studied. Rough velocity estimations are reported.

5. The bearing of the findings on the mechanism of precipitin and flocculation reactions and of the Danysz effect is discussed in terms of the union of multivalent antigen with multivalent antibody.

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