

## A QUANTITATIVE THEORY OF THE PRECIPITIN REACTION

### III. THE REACTION BETWEEN CRYSTALLINE EGG ALBUMIN AND ITS HOMOLOGOUS ANTIBODY\*

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(Received for publication, August 7, 1935)

In the first paper of this series (1)<sup>1</sup> it was shown that the precipitin reaction might be considered the resultant of a series of competing bimolecular reactions, the quantitative outcome of which depended on the relative proportions in which the components were mixed. It was thus found possible to express the entire course of the precipitin reaction between a specific polysaccharide and its homologous antibody by simple equations derived from the mass law. In the second paper of the series (2) it was shown that these considerations were equally applicable to an antigen-antibody system in which the antigen was R-salt-azo-biphenyl-azo-crystalline egg albumin. This antigen, a deep red dye, could be determined with accuracy in precipitates over the entire range of the reaction, thus permitting the separate quantitative estimation of the amounts of antigen and antibody nitrogen precipitated.

In the present communication the information obtained with the aid of the specific polysaccharide and the protein dye is applied to a system involving a colorless antigen, crystalline egg albumin, and its homologous antibody. The antigen used has the advantages of homogeneity, known molecular weight, and of having been studied quantitatively in respect to its behavior in the precipitin reaction by a number

\* The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital.

<sup>1</sup> A statement was omitted in this paper that the sera used were absorbed with pneumococcus protein and "C" substance before purification of the antibody.

of workers including Taylor (3), Culbertson (4, 5), Taylor, Adair, and Adair (6), and Hooker and Boyd (7). It is shown below that the theory presented in Papers I and II of this series is applicable to the egg albumin-antibody system and that the data of the previous workers with this reaction may be used over the portion of the range in which they are sufficiently explicit. A method is given by which both egg albumin and antibody nitrogen may be determined quantitatively in precipitates and supernatants over a limited range in the region of antigen excess. Except in the region approaching complete inhibition it is thus found possible to predict the behavior of an anti-egg albumin serum over the entire remaining range of the reaction after a small number of quantitative analyses for nitrogen have been made. Moreover, the empirical relation which was shown in Reference 2 to permit the calculation of the maximum amount of specifically precipitable antibody has been found to be applicable, though somewhat less exactly, to the egg albumin-antibody system.

Opportunity is also taken to discuss the concept of the equivalence zone, the constancy of the equivalence point, the relation of the findings to methods for the determination of optimal proportions for flocculation, and certain practical considerations regarding the combining proportions of antigen and antibody.

Finally, a quantitative study has been made of the behavior of antibody formed by a single rabbit in three successive courses of injections. The differences found are believed to have bearing on the mechanism of antibody formation and the process of immunization.

#### EXPERIMENTAL

The writers are pleased to note that in all of the four laboratories studying the egg albumin-antibody reaction the amount of specifically precipitable nitrogen is now accepted as a standard. The technique used in this laboratory for analyzing specific precipitates for nitrogen has been given in previous papers (1, 2, 8, 9). In applying the method to the egg albumin (Ea)-antibody (A) system it was found that precipitates, particularly in the equivalence zone, tended to remain somewhat loosely packed, so that it was necessary in such cases to centrifuge the supernatant a second time to recover additional small amounts of specific precipitate. In such cases tubes must be carefully watched if allowed to drain.

The crystalline egg albumin used was prepared according to Reference 10 and was recrystallized three times and dialyzed in the cold until free from ammonium salts. The nitrogen content of Ea was taken as 15.5 per cent.

TABLE I

*Influence of Temperature, Time of Standing, and Volume on Total Specifically Precipitable Nitrogen*

Laboratory No. of serum + amount Ea N	Total N precipitated from 1.0 ml. serum by amount of Ea N indicated in 1.0 ml. saline.—Total volume 2.0 ml. unless otherwise stated					
	37°, 1 hr.	37°, 2 hrs.	37°, 3 hrs.	20°, 0°*	0°, 24 hrs.	0°, 48 hrs.
	mg.	mg.	mg.	mg.	mg.	mg.
1.31 +0.027 mg. Ea N	0.29	0.29	0.29			0.31
2.24 +1.0 mg. Ea N				1.78, 1.74 1.75, 1.72†		
3.87 I, 1:1 + 0.050 mg. Ea N	0.62	0.61				0.63
+ 0.098 mg. Ea N (excess)	0.82	0.81			0.82	0.82
+ 0.124 mg. Ea N				37°, 0° 0.72 0.70 (6 ml.)		
+ 0.305 " " "		0.19		20°, 0° 0.12		0.11‡
3.87 II, 1:1 + 0.050 mg. Ea N						0.84, 0.85
+ 0.098 " " "						0.82§ 1.18 1.15 (9 ml.)
3.87 III, 1:1 +0.079 mg. Ea N						1.31   1.28 (8 ml.)
3.88 + 0.066 mg. Ea N		0.70				0.75

\* 2 hrs. at 20°, overnight in the ice box.

† 4 ml. volume.

‡ Value given by duplicate tubes standing 4 days and 7 days in the ice box, with occasional stirring.

§ Two additional washings with a total of approximately 4 ml. chilled saline.

|| Nitrogen determined according to Reference 11.

In Table I are given data showing the effect of temperature and volume changes on the amount of nitrogen precipitated. Since occasional anti-Ea sera gave 0.01 or 0.02 mg. more specifically precipitable N per ml. when allowed to stand at 0° for 48 instead of 24 hours, all determinations at 0° were allowed to stand 2 days except those in the inhibition zone, for which 4 days were allowed (*cf.* 2).

Egg albumin suspensions were prepared for injection as follows: Dialyzed solutions of crystalline Ea were diluted with saline to less than 1 per cent Ea and were treated with 1 ml. of sterile 1 per cent alum solution per 100 mg. Ea present. The clear solution was neutralized with *N* sodium hydroxide solution until the resulting precipitate no longer appeared to increase, the maximum being reached when the suspension was barely acid to litmus paper. The suspension, which contained very little dissolved Ea, was brought down to the desired concentration (5 mg. Ea per ml. in the case of Rabbit 3.87) with saline and enough 1 per cent merthiolate solution to make a final concentration of 1:10,000. Rabbits were injected with this suspension four times a week for 4 successive weeks (Course I) and generally yielded excellent antisera. Rabbit 3.87 (weight 4.9 kilos) received fourteen injections of 10 mg. each under this schedule, and the serum designated 3.87 I was obtained 5 days after the last injection. Serum 3.87 II was secured 5 days after completion of a further course of eleven injections starting with 2.5 mg. Ea and working up to 15 mg. at the end, and 3.87 III after six more injections following a rest period of over a month.<sup>2</sup> The serum then contained 85 mg. of total protein per ml. (total N  $\times$  6.25), of which 44.7 mg. per ml. consisted of globulin (Howe method). It will be noted that by this time 21.5 per cent of the total protein, or 40.7 per cent of the globulin, consisted of precipitin (maximum specifically precipitable N  $\times$  6.25). Quantitative data on the sera from the three courses are given in Table II and compared in graphic form at a concentration of 1 mg. antibody N per ml. in Text-fig. 2, while Text-fig. 1 gives the total N and antibody N curves of 3.87 I as well as the data on this serum calculated according to equations [3] and [6]. In Table II and other Tables, N estimations (always in duplicate unless otherwise stated) are reported to the third decimal place although its value is uncertain except in the case of Ea N added.

The analysis of supernatants for the very small amounts of Ea in the limited range of Ea excess before the beginning of the inhibition zone is most simply carried out by adding as large an aliquot as possible to the same amount of serum as was used in the determinations of the equation and reading off the corresponding amount of Ea from the total nitrogen curve of the serum (as, for example, Curve IV, Text-fig. 1). The validity of the method is supported by the next to last experiment recorded in Table II, in which it is shown that the presence of super-

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<sup>2</sup> Toward the end of this period, when the animal's serum contained  $<$  0.2 mg. of precipitin N per ml., a single injection of 10 mg. of crystalline horse serum albumin was given. Quantitative analyses at short intervals failed to reveal any "anamnestic" rise in anti-egg albumin.

**TABLE II**  
*Addition of Increasing Amounts of Egg Albumin to 1.0 Ml.*  
*Serum 3.87, 1:1, at 0°*

Ea N added	Ea N pptd.	Total N pptd.	Antibody N by difference	Ratio anti-body N:Ea N in ppt.	Anti-body N pptd., calcd. from eq. [3]	Anti-body N pptd., calcd. from eq. [6]	Tests on supernatant
<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>		<i>mg.</i>	<i>mg.</i>	
<b>Course I</b>							
Mg. antibody N pptd. = 15.8 Ea N - 83 (Ea N) <sup>2</sup>							
Mg. antibody N pptd. = 19.4 Ea N - 36 (Ea N) <sup>3/2</sup>							
0.0091	Total*	0.156†	0.147	16.2	0.137	0.146	Excess A
0.0155	"	0.236	0.220	14.2	0.225	0.231	" "
0.025	"	0.374	0.349	14.0	0.343	0.342	" "
0.040	"	0.526†	0.486	12.2	0.499	0.488	" "
0.050	"	0.632	0.582	11.6	0.582	0.567	" "
0.065	"	0.740	0.675	10.4	0.677	0.664	Excess A, trace Ea
0.074	"	0.794	0.720	9.7	0.714	0.710	No A or Ea
0.082	"	0.830	0.748	9.1	0.738	0.746	No A, < 0.001 Ea N
0.090	0.087	0.826	0.739	8.5	0.746	0.763	Excess Ea, analyses in Table III
0.098	0.089	0.820	0.731	8.2			" "
0.124	0.087	0.730	0.643	7.4			" "
0.135	(0.072)‡	0.610†	(0.538)	(7.5)			" "
0.195	(0.048)	0.414	(0.366)	(7.6)			" "
0.307	(0.004)	0.106					" "
0.490		0.042					
Maximum Ea N, A N in ppt. according to equation [3], 0.095, 0.752; according to equation [6], 0.129, 0.836							
<b>Course II</b>							
Mg antibody N pptd. = 20.4 Ea N - 96 (Ea N) <sup>2</sup>							
Mg. antibody N pptd. = 26.1 Ea N - 48.1 (Ea N) <sup>3/2</sup>							
0.0155	Total*	0.306	0.290	18.7	0.293	0.312	Excess A
0.050	"	0.844	0.794	15.9	0.780	0.766	" "
0.088	"	1.144	1.056	12.0	1.053	1.039	" "
0.098	"	1.180	1.082	11.0	1.077	1.081	No A or Ea
0.118	"	1.214	1.096	9.3		1.127	" " " "
0.127	"	1.278	1.151	9.1		1.137	< 0.001 Ea N
0.135	"	1.280	1.145	8.5		1.138	< 0.001 Ea N
0.143	0.142	1.288	1.146	8.1		1.133	Excess Ea, analyses in Table III
0.195	0.123	1.024	0.901	7.3			" "
0.490	(0.018)	0.154	(0.136)	(7.5)			" "
0.490	(0.034)§	0.154	(0.120)	(3.5)			" "
Maximum Ea N, A N in ppt. according to equation [3], 0.106; 1.083; according to equation [6], 0.131, 1.139							

\* Assumed.

‡ 3.87 II, 1:1, used.

† At pH 6.36.

§ 3.87 I, 1:1, used.

TABLE II (concluded)

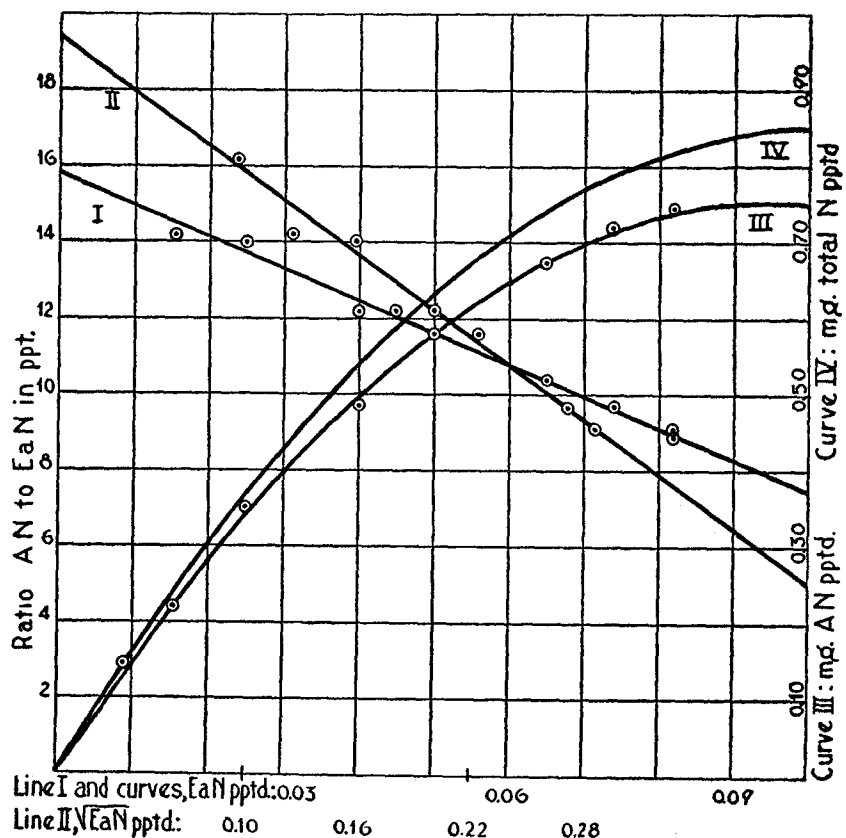
Ea N added	Ea N pptd.	Total N pptd.	Antibody N by difference	Ratio antibody N: Ea N in ppt.	Antibody N pptd., calcd. from eq. [3]	Antibody N pptd., calcd. from eq. [6]	Tests on supernatant
mg.	mg.	mg.	mg.		mg.	mg.	
Course III							
Mg. antibody N pptd. = 24.8 Ea N - 111 (Ea N) <sup>2</sup>							
Mg. antibody N pptd. = 31.8 Ea N - 56.6 (Ea N) <sup>3/2</sup>							
0.0296	Total*	0.665	0.635	21.5	0.622	0.652	Excess A
0.049	"	1.005	0.956	19.5	0.949	0.944	" "
0.079	"	1.320	1.241	15.7	1.267	1.256	Traces A, Ea (?)
0.082	"	1.370	1.288	15.7	1.288	1.279	Excess A
0.088	"	1.422	1.334	15.2	1.324	1.319	Slight excess A
0.098	"	1.468	1.370	14.0	1.364	1.379	No A or Ea
0.122	"	1.570	1.448	11.9	1.373	1.466	" " " "
0.140	"	1.592	1.452	10.4		1.487	" " " "
0.157	"	1.606	1.449	9.2		1.474	" " " "
0.195	0.194	1.650	1.456	7.5		1.330	Excess Ea, analyses in Table III
0.234	0.202	1.542	1.340	6.6			" "
0.296		1.025					
Maximum Ea N, A N in ppt. according to equation [3], 0.112, 1.393; according to equation [6], 0.140, 1.483							
0.140	} 0.140 + 3 ml. 0.140 supernatant	1.542	1.402				
		1.538	1.398				
0.157 + 1.0 ml. supernatant from 3.87 III serial experiment (Table V)	} Found above	1.866	1.709				
			1.449				
			0.36				

|| 1 determination discarded.

natant from tubes in which maximum precipitation has occurred fails to increase the amount of Ea-A normally precipitated. When it becomes necessary to analyze supernatants containing more Ea, as in the inhibition zone, the presence of dissolved Ea-A introduces complications. These were discussed in Reference 2, and it is accordingly assumed that in the analysis of inhibition zone supernatants for Ea, as well as in the dye-antidye system, all of the dissolved antibody in the

supernatant is precipitated together with that derived from the serum used for the analysis.

The following method of calculation, based on this assumption, gives the same result as the simpler method given at the beginning of the preceding paragraph in the region of antigen excess up to the inhibition zone, and the differences from it are small up to the range in which inhibition is pronounced. In this range the simpler



TEXT-Fig. 1

method soon leads to values of  $Ea$  in the supernatant higher than the total added, whereas the method given below gives this result only when inhibition is practically complete, in which case it is necessary to use so small an aliquot that any analytical errors would be greatly multiplied in the final result.

Let  $A$  = the maximum antibody nitrogen found in the serum used,  $Ea$  = the amount of egg albumin nitrogen added, and  $N$  = the amount of nitrogen precipi-

tated at the point considered. Then the amount of specific nitrogen (antigen as well as antibody) in the supernatant is given by  $A + Ea - N$ , and all of this nitrogen would be precipitated in the analysis of the supernatant for Ea with excess antibody according to the quantitative theory elaborated in Reference 1, and as was actually found in the dye-antidye system (2). The additional assumption is made that the entire precipitate obtained in this analysis is of uniform composition; in other words, that the dissolved Ea-A present can combine with A until its composition is the same as that of the Ea-A formed by the free Ea present in its reaction with excess antibody.<sup>3</sup>

If  $N'$  = the nitrogen precipitated in the analysis of the supernatant and  $F$  = the fraction of the supernatant used in the analysis,  $N' - F(A + Ea - N)$  = antibody nitrogen precipitated from the serum used in the analysis. If the curve of antibody N precipitated by Ea from this serum be constructed (as, for example, Curve III, Text-fig. 1), the amount of Ea corresponding to this quantity of antibody N may be read off. The percentage of Ea in this portion of the precipitate may then be calculated according to  $\frac{Ea\ N\ found \times 100}{Ea\ N\ found + antibody\ N\ found} = \text{per cent Ea N}$ . Since it was assumed that the entire precipitate contains this proportion of Ea,  $N' \times \text{per cent Ea N}$  thus found  $\div F = Ea\ N$  in total supernatant, and  $Ea\ N$  originally added *minus* this value =  $Ea\ N$  in the original precipitate.

For example (Tables II and III), 0.124 mg. Ea N precipitated 0.637 mg. N from 1.0 ml. diluted serum 3.87 I,  $A = 0.750$  mg. Then  $0.750 + 0.124 - 0.637 = 0.237$  mg. specific N in supernatant. When 0.75 of the supernatant (actually 1.5 ml.) was set up with 1.0 ml. of the same diluted serum 0.412 mg. N ( $N'$ ) was precipitated. Then  $0.412 - [0.75 \times 0.237] = 0.234$  mg. antibody N pptd. from the serum used for the analysis. From Curve III in Text-fig. 1 it is seen that this corresponds to 0.017 mg. Ea N, and  $\frac{0.017 \times 100}{0.017 + 0.234} = 6.8$  per cent. 6.8 per cent of  $N'$ , or  $0.412 \times 0.068 = 0.028$ , and this divided by 0.75 = 0.037 mg. Ea N in the entire supernatant.  $0.124 - 0.037 = 0.087$  mg. Ea N in the original precipitate.

The calculations made as above in the region of excess antigen are given in Table III, in which the data in the first three columns are taken from Table II. The amounts of Ea N precipitated, given in the last column of Table III, and the resulting ratios in the original precipitates are included in Columns 2 and 5 of Table II. Values in parentheses are considered uncertain for reasons given in the discussion.

Table IV is compiled from data given by Culbertson (5) in his Table 3, and from one each of the constant antigen and constant antibody experiments given by Taylor, Adair, and Adair (6), and a comparison is given of the experimental values and those calculated according to [3] and [6] below.

<sup>3</sup> Experiments have shown that the Ea-A precipitate at the antigen-excess end of the equivalence zone can combine with antibody added after precipitation is complete.



Table V shows the result of serial additions of small amounts of egg albumin to several anti-egg albumin sera (*cf.* 1). The data on 10.0 ml. of undiluted serum 3.87 II show a pronounced, but relatively small Danysz effect, while those on the diluted serum, 2.24A, do not show this effect, possibly on account of the small amount of antibody present. A serial experiment on 5.0 ml. of undiluted serum 3.87 III showed that 0.460 mg. Ea N, added in four portions, pre-

TABLE III  
*Calculation of Ea N in Precipitate in Region of Antigen Excess*

Ea N added	Total N pptd.	Specific N in supernatant (A + Ea - N)	Fraction analyzed	Total N pptd. in analysis of fraction	Less specific N in fraction analyzed	Corresponding Ea N	Per cent Ea N in 2nd ppt.*	Ea N in fraction analyzed	Ea N in entire supernatant	Ea N in ppt.
mg.	mg.	mg.		mg.	mg.	mg.		mg.	mg.	mg.
Serum 3.87 I, 1:1, maximum antibody N pptd., 0.750 mg.										
0.090	0.826	0.014	1.75†	0.078	0.053	0.0035	6.2	0.0048	0.003	0.087
0.098	0.820	0.028	0.75	0.106	0.085	0.0057	6.3	0.0067	0.009	0.089
0.124	0.730	0.144	0.75	0.412	0.304	0.0219	6.7	0.0276	0.037	0.087
0.135	0.610	0.275	0.50	0.596‡	0.458	0.0257	5.3	0.0316	0.063	0.072
0.195	0.414	0.531	0.34	0.676	0.495	0.0395	7.4	0.0500	0.147	0.048
0.307	0.106	0.951	0.167	0.674	0.515	0.0418	7.51	0.0506	0.303	0.004
Serum 3.87 II, 1:1, maximum antibody N pptd., 1.151 mg.										
0.143	1.288	0.006	1.75†	0.046	0.035	0.0017	4.6	0.0021	0.001	0.142
0.195	1.024	0.322	0.50	0.664	0.503	0.0285	5.4	0.0359	0.072	0.123
0.490	0.154	1.487	0.167	1.180	0.932	0.0667	6.68	0.0788	0.472	0.018
0.490	0.154	1.487	0.125	0.738§	0.552	0.0462	7.72	0.057	0.456	0.034
Serum 3.87 III, 1:1, maximum antibody N pptd., 1.456 mg.										
0.195	1.650	0.005	1.50†	0.026	0.018	0.001		0.001	0.001	0.194
0.234	1.542	0.148	0.75	0.566	0.455	0.0205	4.3	0.0243	0.032	0.202

\* 100 times value in Column 7 divided by sum of values in Columns 6 and 7.

† Not run in duplicate.

‡ 3.87 II, 1:1, used.

§ 3.87 I, 1:1, used.

cipitated 9.4 mg. A N, calculated to 5.0 cc. From the equation for 3.87 III, the same relative amount of Ea N, 0.046 mg., added at once to 1.0 ml. of 1:1 serum, is found to precipitate 0.91 mg. A N, or 9.1 mg. from 5.0 ml. undiluted serum—again a definite but small Danysz effect. When the serial experiment on this serum was carried to completion it was found that only 11.34 mg. A N could be precipitated, as against 14.55 mg. which should have been found if all the Ea had been added at once. Thus 22 per cent of the

TABLE IV

*Experiments of Other Authors Calculated According to Equations [3] and [6]*

Amt. Ea N added (pptd.)	Total N pptd.	Antibody N pptd.	Ratio A N: Ea N in ppt.	Antibody N pptd., calcd. from equation [3]	Antibody N pptd., calcd. from equation [6]
mg.	mg.	mg.		mg.	mg.
Culbertson (1935) Table 3. Mg. antibody N pptd. = $17.6 \text{ Ea N} - 64 (\text{Ea N})^2$ Mg. antibody N pptd. = $22.3 \text{ Ea N} - 34.8 (\text{Ea N})^{3/2}$					
0.023	0.423	0.400	17.4	0.371	0.391
0.045	0.678	0.633	14.1	0.662	0.670
0.068	0.968	0.900	13.2	0.901	0.899
0.080	1.079	0.999	12.5	0.998	0.996
0.097	1.207	1.110	11.4	1.105	1.110
0.114	1.249 (Antigen excess)				
0.143	1.320 ( " " , maximum total N)				
Maximum Ea N, A N in ppt. according to equation [3], 0.138, 1.210; according to equation [6], 0.182, 1.355					
Taylor, Adair, and Adair (1934) Table II, Serum 1754C					
Mg. antibody N pptd. = $18.2 \text{ Ea N} - 94 (\text{Ea N})^2$ Mg. antibody N pptd. = $22.3 \text{ Ea N} - 40.5 (\text{Ea N})^{3/2}$					
0.0149	0.250*	0.235	15.8	0.250	0.258
0.0297	0.506*	0.476	16.0	0.458	0.454
0.045	0.677	0.632	14.0	0.629	0.616
0.059	0.813	0.754	12.8	0.747	0.736
0.074	0.914	0.840	11.4	0.832	0.835
0.089†	0.974	0.885	9.9	0.875	0.907
Maximum Ea N, A N in ppt. according to equation [3], 0.097, 0.881; according to equation [6], 0.135, 1.003					
Taylor, Adair, and Adair (1934) Table III, Serum 1754D					
Mg. antibody N pptd. = $16.4 \text{ Ea N} - 54 (\text{Ea N})^2$ Mg. antibody N pptd. = $20.6 \text{ Ea N} - 30.6 (\text{Ea N})^{3/2}$					
<i>per ml. serum</i>	<i>per ml. serum</i>	<i>per ml. serum</i>		<i>per ml. serum</i>	<i>per ml. serum</i>
0.046	0.684	0.638	13.9	0.640	0.646
0.051	0.743	0.692	13.6	0.697	0.698
0.056	0.799	0.743	13.3	0.748	0.747
0.062	0.883	0.821	13.2	0.810	0.804
0.070	0.949	0.879	12.6	0.883	0.874
0.080	1.028	0.948	11.9	0.966	0.956
0.093	1.096	1.003‡	10.8	1.058	
0.111	1.258	1.147	10.3	1.156	1.153
0.139†	1.451	1.312	9.4	1.237	1.276
Maximum Ea N, A N calcd. from equation [3], 0.152, 1.246; from equation [6], 0.202, 1.384					

\* Mean of two determinations.

† Trace of antigen in excess.

‡ From weight of precipitate; omitted in calculation of line according to equation [6].

antibody is not precipitable unless other antibody is present, recalling the behavior of certain antidyse sera (2). The concluding data of Table II show, however, that the entire amount of this antibody is carried down if added to a fresh portion of serum and egg albumin. These findings are again referred to in the discussion.

TABLE V

*Serial Addition of Egg Albumin to Various Sera, Calculated to Original Volume*

Total of successive Ea N additions	Total N pptd.	Total antibody N pptd.	Ratio A N : Ea N in ppt.	Total antibody N pptd. if added in 1 portion (Equation [3], Table II)
mg.	mg.	mg.		mg.
3.87 II, undiluted, 10.0 ml.				
0.015s	0.388 (0.438*)	0.372 (0.422*)	24 (27*)	
0.032		0.775	24.2	
0.049		1.130	23.1	
0.067		1.528	22.8	
0.128		2.81	22.0	2.53
0.441		9.03	20.5	8.06
0.770		13.90	18.1	12.86
Mg. antibody N pptd. = 23.4 Ea N - 6.9 (Ea N) <sup>2</sup>				
Mg. antibody N pptd. = 24.9 Ea N - 7.9 (Ea N) <sup>2/2</sup>				
Serum 2.24 A, diluted to approximately same A content as 2.24B, 4.0 ml.				
0.015s	0.324	0.308	19.9	
0.033		0.576	17.5	
0.052s		0.789	15.0	
Mg. antibody N pptd. = 22 Ea N - 133 (Ea N) <sup>2</sup>				
Serum 2.24B, partially exhausted, 4.0 ml.				
0.015s	0.248	0.232	15.0	
0.033		0.464	14.1	
0.052s		0.687	12.1	
Mg. antibody N pptd. = 16.8 Ea N - 90 (Ea N) <sup>2</sup>				

\* Assuming solubility of precipitate in serum to be as in saline, approximately 0.005 mg. N per ml. This correction would need to be made only on the first precipitation, since the serum would then be saturated with Ea-A compound.

Text-fig. 2 is derived by putting  $A = 1.00$  in the equations according to [4] in Table II and the portion of Table IV dealing with Taylor, Adair, and Adair's figures. Thus the straight lines in the figure represent a comparison of the sera at a common concentration of 1.00 mg. of antibody N per ml.

The pH of serum 3.87 I was 7.8. 10.0 ml. were diluted to 20.0 ml. with saline and a drop of glacial acetic acid. The pH of the resulting dilution was 6.36.<sup>4</sup> Set

<sup>4</sup> Determined electrometrically by Mr. F. Rosebury, of the Department of Biological Chemistry.

up as in Table II with a number of the same dilutions of Ea, the more acid serum gave identical amounts of N, within the limits of error of the method, as at pH 7.8, even in the inhibition zone (*cf.* 2). Since Marrack and Smith (12) have also failed to observe differences due to pH changes within almost the same limits, the details of the experiment are not given.

In Table VI is contained a compilation of the writers' data relative to the equivalence zone and the equivalence point in the Ea-A system, as well as the found and calculated ratios at the point of maximum antibody precipitation.

TABLE VI  
*Ratios of Antibody N: Egg Albumin N in Equivalence Zone and at Maximum Antibody Precipitation*

Serum	Ratio at antibody excess end of zone	Calculated equivalence point ratio	Ratio at antigen excess end of zone	Ratio at first maximum pptn. point	Calcd. ratio at maximum, equation [6]
1.31			9.2	9.2	7.2
1.35	9.4	9.0	8.6		
1.36	9.3				6.2
1.68	11.5		>7.6		
2.24	16.1	14.0	11.8	10.5	9.8
2.32	9.1				
3.87 I	10.4	9.8	9.1	9.1	6.6
3.87 II	(11.5)	9.8	8.1	9.1	8.7
3.87 III	(15.0)	11.3	7.5	11.9	10.2

Values in parentheses are estimated from nearest actual determination.

#### DISCUSSION

That crystalline egg albumin is a single definite chemical individual appears to be established with as great certainty as possible in the case of a protein. When injected into rabbits it nevertheless gives rise to antibodies of differing reactivity, as has already been noted by Hooker and Boyd (13), and as is shown in the present communication. In spite of this it is assumed in the following discussion, as in References 1 and 2, that the average behavior of the antibody is that of a single substance, and that it may accordingly be treated mathematically as such.

Another assumption, which was made in Reference 1 but not found applicable in Reference 2, was that in the region of excess antibody all of the hapten (antigen) added was precipitated since none could be

detected in the supernatant by the exceedingly delicate serological test. The same assumption, which is not considered valid by Taylor, Adair, and Adair (6), is made in the egg albumin (Ea)-antibody (A) system, for the following reasons.

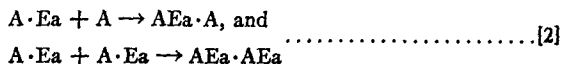
(a) The antigen is a single definite chemical individual. (b) The test for Ea with homologous antibody is sensitive to an antigen dilution of at least 1:200,000, and the dissociation constant of a soluble Ea-A complex would have to be exceedingly small if the Ea in it were to escape detection. (c) When soluble Ea-A compounds are known to be present, as in the inhibition zone, these are precipitated when fresh antibody is added. (d) In a serial experiment, such as the first in Table V, antigen appears in excess as soon as all but the last traces of antibody precipitable in this way have been thrown down. The serially non-precipitable antibody can thus scarcely be concerned in the disappearance of Ea into an undissociated, soluble Ea-A complex, the more so as it is quantitatively precipitated when added to fresh antibody and Ea (see last experiment in Table II). A third type of antibody would then have to be postulated. (e) If it is assumed that all of the Ea added in the region of excess antibody is precipitated, the resulting A N:Ea N ratios in the precipitate and the range of these ratios with varying Ea-A proportions are found to be of the same magnitude as was observed in the dye-antidye system (2) in which antigen and antibody in the precipitate were each directly determined.

As in the instances studied in the first two papers of this series (1, 2) it is considered that the precipitin reaction between crystalline egg albumin and antibody occurs in steps in a series of bimolecular reactions which take place before precipitation begins.

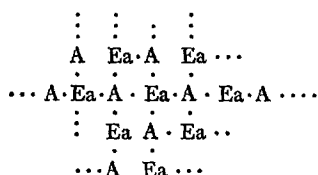
The first step in the reaction would be



This would represent the equivalence point compound in its simplest form, as composed of 1 unit of A and 1 unit of Ea, regardless of their actual molecular proportions. A molecular formula would be  $A_m Ea_n$ , and this compound would be arrived at through a series of bimolecular reactions. Since both A and Ea are proteins, and the opportunity is given for the immunologically reactive groupings to recur a number of times, the reactants may be considered multivalent with respect to each other. Thus the A·Ea compound initially formed could react with other molecules of the same compound, or with A or Ea, if either is present in excess. In the region of excess antibody the second step of the reaction would then consist of the two competing bimolecular reactions, in which dissociation is assumed to be negligible:



Since both A and Ea may be considered multivalent with respect to each other, the products of reaction [2] would combine chemically until aggregates large enough to separate from solution are formed. This might be represented two-dimensionally as follows:



in which the end-result would be much the same as that pictured by Marrack (14).

In the special case in which A and Ea are mixed in equivalent proportions the A·Ea (or A<sub>m</sub>Ea<sub>n</sub>) produced according to [1] would merely polymerize, and the equivalence point precipitate would be (A·Ea)<sub>x</sub>, or (A<sub>m</sub>Ea<sub>n</sub>)<sub>x</sub>.

As in the reactions studied in References 1 and 2 the composition of the precipitate would thus depend on the relative proportions in which the reactants are mixed. As in Reference 2, only a small proportion of the antibody was found to react to form compounds containing more A than twice the equivalence point ratio, so that the additional reactions which it was necessary to consider in Reference 1 may be neglected. Based on the above concept of the reaction the following expression for the region of excess antibody may be derived with the aid of the mass law as in Reference 1:

$$\text{mg. antibody N precipitated} = 2R(\text{Ea N}) - \frac{R^2(\text{Ea N})^2}{A} \dots\dots\dots[3]$$

in which R = the ratio of antibody nitrogen to egg albumin nitrogen in the precipitate at the point at which antigen first appears in excess; Ea N = egg albumin nitrogen precipitated, and A = the amount of antibody N precipitated at the point at which antigen first appears in excess. Only in serum 3.87 III was R in better agreement with the equivalence point than the point chosen (*cf.* also Table VI).

The Ea-A system differs from the dye-antidye and S III-antibody systems already studied in that maximum precipitation of antibody is reached at a point very close to that at which antigen first appears in excess, after which inhibition of specific precipitation begins very rapidly. The range of maximum precipitation in the region of antigen excess is thus so small that the use of an expression such as [4]

in Reference 2 was found unnecessary in the sera studied. Since anti-Ea sera are thus extremely sensitive to an excess of antigen it is necessary to use great care that only a very slight excess of Ea be present in analyzing for maximum antibody precipitation. The amount of Ea to be used may be determined by a rapid preliminary experiment on 0.5 ml. of serum at 37°, in which 0.1 mg. portions of Ea (or less in the case of weak antisera) are added until no further precipitation occurs.

No attempt is made in the present communication to formulate the reaction in the inhibition zone on account of the uncertainties of analysis discussed below. As in the dye-antidye system the relative proportions of the components appear to be the determining factors since precipitation does not occur in the supernatant when the concentration of Ea is reduced by dilution with saline.

From Table I it is seen that precipitation in the Ea-A system is complete in 1 hour at 37°C., and that the amount of nitrogen precipitated is only slightly, if at all less, than at 0° for 48 hours. In one experiment in the inhibition zone, however, with 0.305 mg. Ea N, 0.19 mg. N was precipitated at 37°, 0.12 mg. at 20° for 2 hours and overnight in the cold, and only 0.11 mg. at 0° for 4 days, a result consistent with increased dissociation of the soluble Ea-A compound at the higher temperatures. Except in the inhibition zone, then, there are no marked differences except in rate when the reaction is carried out at 37°, at 0°, or, as in ordinary immunological practice, at 37° for 2 hours and overnight in the cold. Numerous data obtained at 37° are accordingly omitted. Since certain sera show slightly more precipitable nitrogen at 0° for 48 hours than under other conditions this procedure was adopted in the present work, except that experiments in the inhibition zone were allowed to stand for 4 days, with thorough mixing each day. It will also be noted from Table I that the solubility of the specific precipitate up to the region of slight antigen excess is about 0.005 mg. per ml. at 0°, a value somewhat lower than that given by Marrack and Smith (12) for the pseudoglobulin-antibody precipitate. If this value be accepted it would be necessary to correct all values for antibody nitrogen given in this paper by addition of 0.02 or 0.03 mg. of N. Since, however, actual experimental data are presented the correction is indicated only in the case of a serial experiment with 10.0 ml. of serum (Table V), in which enough Ea-A compound is dissolved in the first supernatant (0.05 mg. N, calculated) to make the initial precipitate smaller in amount than the second.

The linear relation

$$\frac{\text{Antibody N}}{\text{Ea N}} \text{ in the precipitate} = 2R - \frac{R^2}{A} (\text{EaN}) \dots\dots\dots[4]$$

follows from equation [3]. By plotting the values of the ratio  $\frac{\text{Antibody N}}{\text{Ea N}}$  against Ea N precipitated a straight line results from which the values of the constants in equations [4] and [3] may be obtained. Thus, in Text-fig. 1, Line I is plotted in this way from data in Table II up to the region of excess antigen. For serum 3.87 I, 1:1, the intercept on the y-axis,  $2R$ , = 15.8, and the slope of the line,  $-\frac{R^2}{A}$ , = -83, whence  $R = 7.9$ ,  $A = 0.752$ . The experimentally found values are  $R = 9.1$  and  $A = 0.748$  at the antigen excess end of the equivalence zone. Equation [3] for serum 3.87 I, 1:1, is thus  $N = 15.8 \text{ Ea N} - 83 (\text{Ea N})^2$ , and is represented by Curve III in Text-fig. 1, on which the circles show the experimentally determined points. A comparison of the fourth and sixth columns of Table II shows, in general, close agreement between the experimentally determined values of antibody N precipitated and those calculated according to [3] for the three sera given in the table. Curve IV represents the corresponding total nitrogen curve,  $N = 16.8 \text{ Ea N} - 83 (\text{Ea N})^2$  for serum 3.87 I, 1:1.

Since [4] is a linear expression, the equation for the line may be approximately fixed for any serum by determination (in duplicate) of the amounts of antibody nitrogen precipitated at two points in the region of excess antibody. The greater the number of points determined the greater would be the accuracy attained. As recommended in References 1 and 2, more than one-third of the antibody should be precipitated in order to minimize the error due to the small portion of antibody yielding compounds of ratio  $> 2R$ .

In Reference 2 it was shown that if  $\frac{\text{Antibody N}}{\text{Dye N}}$  in the precipitate were plotted against the square root of dye N precipitated an even closer approximation to a straight line was obtained than by means of an equation of the type of [4].

Reduced to the same form as [7] in Reference 2, this gives

$$\frac{\text{Antibody N}}{\text{Ea N}} \text{ in the precipitate} = 3R'' - 2 \sqrt{\frac{(R'')^2 (\text{Ea N})}{A}} \dots \dots \dots [5]$$

in which  $3R''$  is the intercept on the y-axis and  $-2 \sqrt{\frac{(R'')^2 (\text{Ea N})}{A}}$  is the slope of the line,  $A = \text{maximum precipitable antibody N}$ , and  $R'' = \text{Antibody N}:\text{Ea N}$



ratio at the maximum. For serum 3.87 I, 1:1, the best equation for this line, (Line II, Text-fig. 1), arrived at by application of the method of least squares to the experimental data, is  $\frac{\text{Antibody N}}{\text{Ea N}}$  in the precipitate =  $19.4 - 36 \sqrt{(\text{Ea N})}$ .

Then

$$\text{mg. antibody N precipitated} = 19.4 \text{ Ea N} - 36(\text{EaN})^{3/2} \dots\dots\dots [6]$$

and when the first derivative,  $19.4 - 54 (\text{Ea N})^{1/2} = 0$ , antibody N precipitated at the maximum, or A, = 0.836, a value somewhat higher than that actually found, 0.748. R'', also, = 6.5, which is too low. It will be noted, however, from the maxima and from the values calculated according to equation [6] given in Column 7 of Table II, that this relation gives better agreement with the experimental values for serum 3.87 II than does equation [3], while in the case of serum 3.87 III both methods of calculation fit the data well.

In Table IV are given data from papers by Culbertson (5) and Taylor, Adair, and Adair (6) as far as the region of antigen excess, and it is seen that the Ea-A reaction in the sera studied by these workers may also be quantitatively expressed up to this region according to equation [3] which follows from the writers' theory of the reaction, or by the empirical relation [6].

Of the eleven sets of data given by Taylor, Adair, and Adair, only two fail to conform to equation [3] and three to equation [6]. Both of these equations may be used for total N precipitated, in which case the coefficient of the first term is increased by 1, since the total N:Ea N ratio is 1 greater than the A N:Ea N ratio. This was done in calculating Curve IV, Text-fig. 1. In comparing the values calculated according to [3] or [6] with Taylor, Adair, and Adair's data it should also be borne in mind that the first point of maximum antibody precipitation (with increasing amounts of antigen) need not coincide with the point of maximum total nitrogen, since antibody in this range is capable of combining with still more antigen, as shown by the decreasing ratios for A N:Ea N in the precipitate in this range and in the initial portion of the inhibition zone.

It is felt that the inhibition zone data in the Ea-A system offer too many uncertainties to warrant treatment of this portion of the reaction range as in References 1 and 2. The method of calculation of the composition of the precipitate given in the Experimental part (pages 715 and 716) appears to be the most reasonable, but it is not considered entirely trustworthy since it tends to show somewhat

higher amounts of Ea in the supernatants near the region of total inhibition than were actually added. There is an uncertainty as to what correction to apply in order to avoid this.

If, for example, one deducts from the nitrogen precipitated in the analysis of the supernatants the average discrepancy noted in Reference 2, 0.03 mg. N for precipitates of 0.300 mg. N and over, one obtains decreasing ratios for A N:Ea N precipitated in the inhibition zone which agree well with comparable, directly determined ratios in the inhibition zone of the dye-antidye system. However, it is not known whether a like discrepancy, to be corrected for, exists in the Ea-A system, and indeed, the next to last experiment in Table II would seem to argue against such a correction. The uncorrected ratios appear to become constant before inhibition is greatly advanced, and while this result is not improbable, the precipitates become more and more gelatinous as the amounts of Ea added are increased and inhibition becomes more complete, so that a change in composition seems indicated if A in this region may be considered as a single substance. It is hoped to eliminate these uncertainties in work now under way.

The serial experiments shown in Table V, as well as others which are not reported, also appear to conform to equations [3] and [6]. In addition they furnish evidence against the homogeneity of anti-egg albumin. Thus serum 2.24 B, exhausted by Ea additions to approximately the same A content as diluted serum 2.24 A, yielded an equation with entirely different constants (Table V).<sup>5</sup> In general, the Danysz effect is much smaller than would have been expected by analogy with References 1 and 2. In spite of this a fraction of the antibody in sera 2.24 and 3.87 III, the only ones studied from this standpoint, was found to be non-precipitable when the Ea was added in serial portions. This residual antibody, amounting to 22 per cent in the case of serum 3.87 III, might be considered to be of lower reactivity owing to the small number, possibly only one, of immunologically reactive groupings in an otherwise unaltered globulin molecule. Antibody of this type could scarcely build up the large A·Ea aggregates postulated by the writers and by Mar-rack as necessary before precipitation occurs. However, when multi-valent (or average) antibody is present any A·Ea compound formed

<sup>5</sup> Similarly, the supernatant from the serial experiment on serum 3.87 II, containing approximately the same amount of precipitin N as 3.87 I, gave a linear equation almost coinciding with the line for 3.87 I, Text-fig. 1. It will be noted, however, from Text-fig. 2 that the equations for the whole sera do not coincide at equal A content.

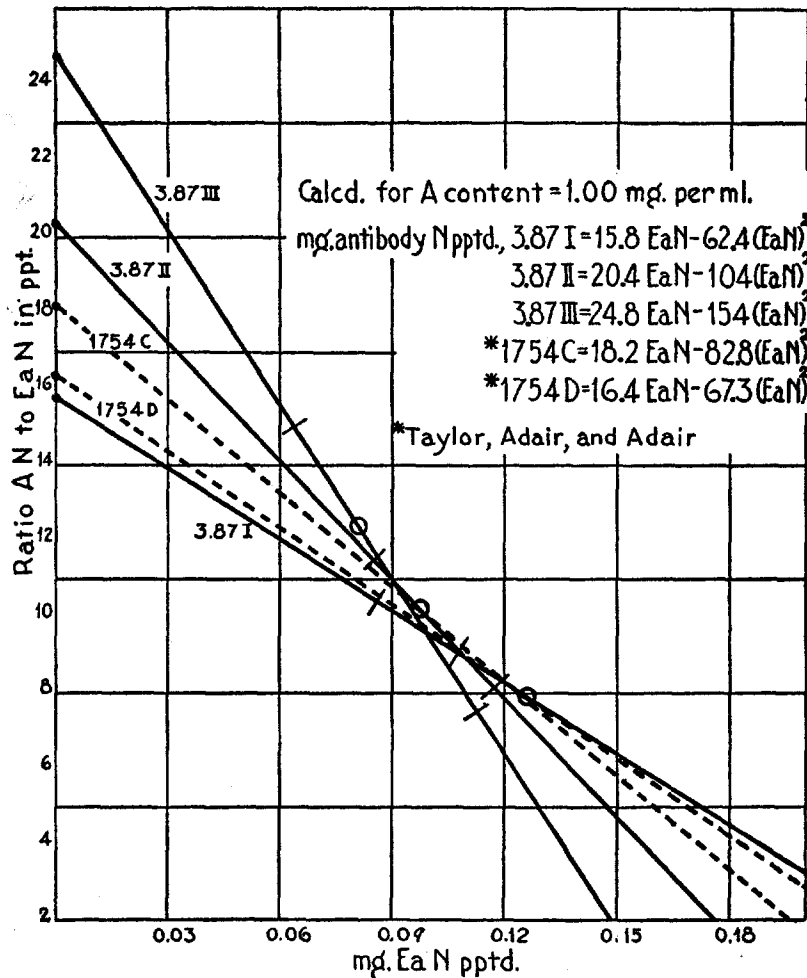
by the residual antibody would participate in the building up of aggregates through the multivalent Ea present and the maximum titer would be attained if sufficient Ea were added, as is actually found. Moreover, if this interpretation be correct, it would be expected that if such low-grade or residual antibody were added in suitable amount to serum before precipitation with a given quantity of Ea, the entire amount of residual antibody would be found added to the precipitable nitrogen obtainable from the serum and Ea alone. That this actually occurs, is seen in the last experiment given in Table II and on pages 717 and 719. The calculated residual antibody N in 1.0 ml. of the serial supernatant, allowing for the successive dilutions necessary, was 0.36 mg., and precisely this amount was found over the normally precipitated N when 1.0 ml. of supernatant was added to serum and Ea.

Thus by means of the quantitative method it has been possible to show, in an antiserum to a single protein antigen, the presence of a low-grade fraction of the total antibody, non-precipitable by the Ea when isolated, but precipitated with the remainder of the antibody under ordinary conditions. A simple explanation of the behavior of this antibody is also offered.

In Text-fig. 2 the lines given by equation [4] for the bleedings from Courses I, II, and III of Rabbit 3.87, (solid lines) and from the third and fourth bleedings of Rabbit 1754 (broken lines, Taylor, Adair, and Adair) are compared at 1.00 mg. of antibody N per ml.

It will be noted that all points up to the calculated maximum (indicated by the circle) on the line for 3.87 III lie above those of 3.87 II, which in turn lie above those of 3.87 I. The same result holds if the found maxima are taken as points of reference. Thus the antibody, considered as a single reactive unit, has become capable of combining with Ea to form compounds of higher A N: Ea N ratio in each successive bleeding. Two readily occurring explanations of this are: that antibody of higher and higher molecular weight, with the same combining capacity, is formed as the immunization proceeds, or that in the later stages antibody is formed which is reactive with a larger number of chemically distinct groupings on the Ea molecule than was the antibody produced in the earlier stages of immuniza-

tion. The first alternative is believed the less probable, for in such a mixture the antibody of higher molecular weight would probably react more slowly than the smaller molecules, and the pre-



TEXT-FIG. 2

cipitates formed with small amounts of Ea would have lower A N: Ea N ratios than those formed with larger amounts of Ea.<sup>6</sup> The

<sup>6</sup> This is contrary to the experimental findings, including those summarized in footnote 5.

second explanation is also in accord with the broadening of the scope of cross-reactions so often observed on continued immunization (for references, *cf.* (15)). At any point in the region of antibody excess a given amount of antibody 3.87 I combines with more Ea than does 3.87 II, and this, in turn combines with more than does 3.87 III: another way of expressing the relation noted above. This does not mean, however, that the same relative efficiency obtains at other regions in the reaction range; thus, at the antigen excess end of the equivalence zone, the last point at which all of the Ea added is precipitated, 1.00 mg. of antibody I, II, and III combines with 0.110, 0.123, and 0.133 mg. of Ea N, respectively. It is evident from these considerations and the results of References 1 and 2 that, in general, a given amount of antibody combines with the largest amount of antigen, or shows its greatest efficiency, toward the region of antigen excess. Since excess of antibody is approached in therapy through the region of antigen excess, it is possible that a number of small injections of serum over a short period might have a greater effect than a single large injection.

In the third (C) and fourth (D) bleedings of Rabbit 1754 reported by Taylor, Adair, and Adair and shown as the dotted lines in Text-fig. 2, it would appear that the antibody produced by this animal at the third bleeding was capable of reacting with more groupings on the Ea molecule than was that of the fourth bleeding, although the difference was not large. Attempts will be made to study these relationships in a series of animals from the first appearance of precipitin through a long series of courses.

In Text-fig. 2 the perpendicular lines mark the extent of the equivalence zone in each case, as also shown in Table VI. It will be noted that this zone was most limited in the earliest serum, was somewhat broader in II, although the equivalence point (taken as the mean of the ratios at the ends of the zone) remained the same, and was of such extent in III that the calculated equivalence point, though higher, scarcely has any real meaning. In this serum, tests for A and Ea in the supernatant were negative over a twofold range of concentration of Ea.

The broadening of the equivalence zone on continued immunization is also in accord with the conception that antibody formed in the later stages is reactive with an increased number of chemically distinct groupings on the Ea molecule.

As had been found in References 1 and 2 the data in Table VI indicate that it is a matter of considerable difficulty to fix accurately the limits of the equivalence zone so that the equivalence point may be even approximately calculated. In most of the anti-Ea sera studied by the writers, by Culbertson (4, 5), and by Taylor, Adair, and Adair (6), the equivalence zones extended over a considerable range, and the extremes of the approximate equivalence points differed by as much as 50 per cent of the lower values. Consequently, this point can scarcely be said to be "constant" as considered by the other workers and by Hooker and Boyd (7). While the average equivalence point ratio appears to be fairly characteristic for any antigen-antibody system, as shown by Hooker and Boyd, the variations of the equivalence point ratio in any one system are so great, even in different bleedings from the same animal, as to render it likely that this ratio is governed by other factors as well as the relative molecular weights of the reactants. It would appear to the writers that available evidence favors the view that combining ratios over the entire range of the precipitin reaction depend on the relative numbers of reactive groupings in the antigen and antibody molecules as well as on the molecular weights.

The parallel study of the constant-antigen and constant-antibody series made by Taylor, Adair, and Adair (6) indicates, as these workers and others have pointed out, the probable correspondence of the constant-antibody flocculation optimum with the equivalence point; the chemical significance of the constant-antigen optimum is not clear. Although titrations made in these two ways show optimal ratios with the quotient 1.6, experiments of both types yield similar relations when calculated according to equations [3] or [6], as shown in Table IV.

In conclusion it is again pointed out that evidence is presented in References 1 and 2 and the present paper that antibody is not homogeneous. Much of this evidence, it is believed, could not have been secured by the use of any but an accurate, quantitative method. At any rate it is apparent that a theory based on the statistical behavior of antibody as a single substance can serve merely as a temporary expedient, useful in its application to antisera as they

occur, and until such time as it may be possible to isolate antibody possessed of a single reactivity.

## SUMMARY

1. A quantitative theory of the precipitin reaction based on the laws of classical chemistry has now been found applicable to the crystalline egg albumin-antibody system. Equations derived from the theory permit the calculation of the behavior of an anti-egg albumin serum over most of the reaction range after a few quantitative analyses have been made for the nitrogen precipitated. Data of other workers also conform to the proposed equations.

2. The empirical relation, shown to have advantages in the dye-antidye system, may also be used for the Ea-A reaction.

3. Serum from the same animal after successive courses exhibits progressive changes which have been described graphically and quantitatively. These changes are believed to consist in the formation of more and more antibody capable of reacting with a larger number of chemically different groupings in the antigen molecule.

4. Evidence is presented that anti-egg albumin is not homogeneous, and that even after prolonged immunization the antiserum contains much low-grade antibody, incapable of forming precipitates unless more reactive precipitin is present.

5. Factors affecting the equivalence point ratio are discussed.

In conclusion the writers wish to express their thanks for assistance given by Dr. Torsten Teorell.

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