A QUANTITATIVE THEORY OF THE PRECIPITIN REACTION

II. A Study of an Azoprotein-Antibody System*

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The precipitin reaction may be considered the resultant of a series of competing bimolecular reactions, the quantitative outcome of which depends on the relative proportions in which the components are mixed (1). In a typical instance, the reaction between Type III pneumococcus specific polysaccharide and homologous antibody produced in the horse, it was shown that the entire course of the reaction could be expressed by simple equations derived from the mass law (1, 2). These equations permitted the quantitative expression of the behavior of an unknown serum over the entire reaction range with the aid of a small number of micro analyses for nitrogen.

Since the system studied above concerns hapten and antibody, it was considered desirable to test the new relationships on precipitin reactions between true antigens and their antibodies. Furthermore, in order that antigen could be directly determined in all precipitates and differentiated from the antibody thrown down at the same time, it was found convenient to make use of R-salt-azo-biphenyl-azo-crystalline egg albumin, a dark red antigen the preparation and properties of which have already been described (3). With this product antigen and antibody could be separately determined in the specific precipitates by the methods given in Reference 3, advantages which

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were not provided when colorless antigens were used. Preliminary data obtained in this way were published some years ago (4).

It is shown in the present communication that the theory outlined above (1) is applicable to the azoprotein-antibody system[1] with very little modification. An empirical relation is also given which permits the calculation of the maximum amount of specifically precipitable antibody from the same analyses used to establish the equation for the reaction in the region of excess antibody. Questions arising in connection with the determination of the excess of antigen in inhibition zone supernatants have also been studied.

EXPERIMENTAL

Rabbit antisera were used in every instance and most of the sera were obtained[2] with the aid of multiple injections of 1 to 5 mg. of alum-precipitated dye (3). The sera were preserved with 0.01 per cent of merthiolate in order to avoid contamination during use.

The methods used for setting up the determinations and washing and analyzing the precipitates have been described in References 1 and 3. The colorimetric determination of the azoprotein in the specific precipitate appeared to be equally satisfactory whether or not colorless protein was added to the alkalinized standard. Occasionally the color of the dissolved precipitate failed to match the standard, in which case light intensities were approximately matched. The optimum amount of alkali for both precipitates and standards in the colorimetric comparisons was about 0.1 cc. of normal sodium hydroxide per 5 cc. After the color estimation the solution of the precipitate was quantitatively rinsed into a micro Kjeldahl flask (100 cc. flasks were used) for the determination of total nitrogen. Total nitrogen minus antigen nitrogen equals antibody nitrogen. In general, nitrogen estimations are reported as far as the third decimal place, the exact value of which is, however, uncertain except in the case of total dye nitrogen added.

In Table I are given comparative data obtained at 37°C., at 0°, and by allowing the tubes to stand at room temperature for 1 or 2 hours and then in the ice box. In the experiments which were carried out at low temperatures throughout, the tubes were allowed to stand for 48 hours, and were stirred after 24 hours in the same way as described in References 1 and 3.

In Table II are given data obtained at low temperatures throughout. The serum was chilled in ice water before mixing with 1.0 cc. of the appropriate concentration of the dye solution and the tubes were usually allowed to stand in the ice box for 2 days. It was found, however, that this was not long enough for

[1] Referred to subsequently as the dye-antidye system.

[2] This portion of the work was carried out by Mr. C. M. Soo Hoo.

experiments in the inhibition zone, in which a period of at least 4 days in the refrigerator was required before precipitation ceased. The results of this experiment are plotted in Text-fig. 1 as Lines A and B, and as Curve C, of which the last represents total nitrogen precipitated plotted against dye nitrogen precipitated.

Table III shows two parallel serial experiments on the supernatants from the first two pairs of tubes in the experiment summarized in Table II. Several serial experiments were run with larger amounts of other sera and also gave straight lines when the data were plotted as in Text-fig. 1.

In Table IV are given the maximum amounts of specifically precipitable nitrogen in many of the sera used in the present work. In each instance antigen and antibody were determined separately in the washed specific precipitate, and the figures given represent antibody nitrogen. The other data in Table IV are referred to in the discussion.

Table V gives data collected in the region of antigen excess, comprising the zone of maximum antibody precipitation and the inhibition zone.

The data summarized in Table VI deal with the determination of antigen in the supernatants of the inhibition zone by the specific precipitin method given in References 5 and 1. An aliquot portion of the supernatant was set up with fresh serum for which the total nitrogen curve, corresponding to C, Text-fig. 1, was known. Care was taken to choose a portion of the supernatant small enough to avoid complete exhaustion of the antibody added. The precipitate was analyzed for dye nitrogen and total nitrogen. The total nitrogen on the antibody curve corresponding to the dye nitrogen in the precipitate was then read from the curve and compared with the total nitrogen actually precipitated. It was thus possible to form some idea of the effect of the dissolved antibody in the inhibition zone supernatants on the analysis for antigen by this method. The results are discussed below.

Supplementary Analytical Data on R-Salt-Azo-Biphenyl-Azo-Crystalline Egg Albumin (Cf. 3)

The R-salt-azo-biphenyl-azo grouping, $C_{22}H_{15}O_7N_4S_2$, of formula weight 511.3, contains 12.54 per cent of S; egg albumin 1.57 per cent of S (6). The following micro analyses for S were run by Mr. William Saschek.

Azoprotein, dried to constant weight, 7.860, 11.815, 10.705, 11.130 mg.
$BaSO_4$, 1.75, 2.20, 2.18, 2.27 mg.
S, 3.06, 2.56, 2.80, 2.81 per cent, calculated to the ash-free basis.
Calculated for azoprotein with 8 disazo groups; S, 2.78 per cent.
with 9 disazo groups, S, 2.91 per cent.

8 disazo groups are also equivalent to the coupling of one disazo group with each tyrosine group in egg albumin, which contains about 4.1 per cent of tyrosine (7).

DISCUSSION

In the following discussion it is assumed that R-salt-azo-biphenyl-azo-crystalline egg albumin, fractionated according to Reference 3 and purified by ultrafiltration, can be treated as a single substance, also that the average behavior of the antidye is that of a single substance. The dye protein is almost entirely precipitable within very narrow limits of ammonium sulfate concentration, and practically all of the portion reactive in anti-egg albumin serum has been eliminated, but in spite of this the azoprotein may consist of a mixture of dyes, each containing

TABLE I

Precipitation of Antidye N at Various Temperatures

Dye N used	Dye N pptd.	Antibody N pptd.	A:D ratio	Dye N pptd.	Antibody N pptd.	A:D ratio	Difference in total N pptd. between 37° and 0°
mg.	*mg.*	*mg.*		*mg.*	*mg.*		*mg.*
	B 186 37° (2 hrs.)				B 186 0°		
0.014(6)	0.013	0.205	15.8	0.014(5)	0.223	15.4	0.02
0.073	0.062	0.636	10.3	0.068	0.742	10.9	0.11
	B 189 II 37° (2 hrs.)				B 189 II 0° (48 hrs.)		
0.093	0.069	0.659	9.6	0.077	0.673	8.7	0.02
	B 187 37° (2 hrs.)				B 187 20° and 0°*		
0.029	0.025	0.367	13.9	0.028	0.358	12.8	0.01
0.058	0.049	0.513	10.5	0.055	0.529	9.6	0.02
0.088	0.069	0.581	8.4	0.075	0.607	8.1	0.03

A = antidye N, D = dye N.

* At room temperature until precipitate began to separate, then in ice box 72 hours.

a different number of the R-salt-azo-biphenyl-azo groupings in the molecule. It is perhaps for this reason that in the region of excess antibody a given antidye serum precipitates only a certain proportion of the dye, characteristic for any serum, but varying from about 75 per cent to 95 per cent of the total added in different sera. From the sulfur content of the dye it would appear that the average number of disazo linkages in the product is eight or nine, figures consistent with Hooker and Boyd's finding that the larger atoxylazocasein molecule must contain at least thirteen azo groups in order to precipitate in the cross-reaction with atoxylazo egg white antiserum (8). It should be

recalled, also, that the egg albumin was not coupled with a maximal amount of the azo component (3), so that it is not surprising that the

TABLE II

Addition of Increasing Amounts of Dye to 4.0 Cc. of Pooled Sera B 186, at 0°

Dye N added	Dye N pptd.	Total N pptd.	Antibody N by difference	Ratio antibody N:dye N in ppt.	Tests on supernatant	Antibody N pptd., calcd. from equation [3]
mg.	*mg.*	*mg.*	*mg.*			*mg.*
0.007(3)	0.007(2)	0.130	0.123	17.1	Excess A	0.102
0.014(6)	0.014(5)	0.238	0.223	15.4	" "	0.201
0.037	0.035	0.494	0.459	13.1	" "	0.445
0.048	0.047	0.604	0.557	11.9	" "	0.567
0.073	0.068	0.810	0.742	10.9	" "	0.743
0.098	0.091	0.962	0.871	9.6	" "	0.881
0.110	0.106	1.052	0.946	8.9	No A or D	0.941
0.146	0.134	1.168	1.034	7.7	0.002 mg. dye N in excess*	0.988
0.183	0.162	1.242	1.080	6.7	Excess D	
0.219	0.191	1.320	1.129	5.9	" "	
0.256	0.207	1.318	1.111	5.4	" "	
0.280†	0.229	1.360	1.131	4.9	" "	
0.485	0.271	1.295	1.024	3.8	" "‡	
0.970	0.249	0.931	0.682	2.7	" "§	
1.455†	0.112	0.394	0.282	2.5	" "	
‡	0.074	0.924	0.850	11.5		
§	0.098	1.096	0.998	10.2		
**	0.072	1.000	0.928	12.9		

Mean percentage of dye precipitated up to region of excess antigen, 95 per cent.

Maximum specific precipitable antibody N, calculated from Line B, Text-fig. 1, 1.098 mg.

* Determined by precipitating 7.0 cc. of combined supernatants with 4.0 cc. B 186 and reading off dye from total N curve (*cf.* 5, 1).

† Not run in duplicate.

‡ 2.0 cc. portions run with 4.0 cc. B 186.

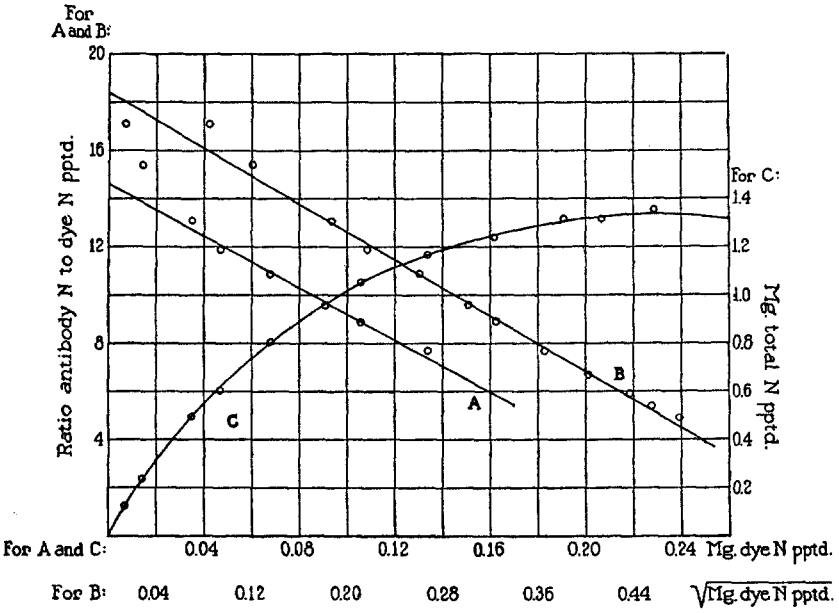
§ 0.70 cc. portions run with 4.0 cc. B 186, also ** 0.60 cc. portions.

amount combined corresponds roughly to the tyrosine content of the protein.

In planning the experiments it was necessary to ascertain the effect of changes in temperature on the amount of nitrogen specifically precipitable from antisera by the dye. Contrary to the effect noted in horse sera (9) there was in general only a slight difference in the amount and composition of the precipitate in the

experiments run at 37° and those carried out at 0°. It also appeared immaterial whether the runs were started at room temperature and continued at 0°, or were carried out entirely at low temperatures, as stated in Reference 3. The data on the influence of temperature are collected in Table I. The slight difference between the values at 37° and at 0°, roughly proportional to the quantity of precipitate, may be interpreted as an indication that these rabbit sera, unlike antipneumococcus horse sera, contain relatively little of an antibody fraction precipitable at 0° but not at 37°.

Experiments at pH 6.7 and 7.9 with antidye serum disclosed no differences, in agreement with Marrack and Smith (10), except possibly a slightly steeper



TEXT-FIG. 1

gradient in the inhibition zone at the higher pH, and are therefore not given in detail.

From the data summarized in Table II and given in graphic form in Text-fig. 1, it is evident that the quantitative theory of the precipitin reaction presented in Reference 1 is applicable to the present antigen-antibody system. The theory may be briefly restated as follows, but for the derivation and a detailed discussion the reader is referred to Reference 1.

The precipitin reaction between dye and antidye is considered as a series of bimolecular reactions which take place before precipitation occurs. The first step in the reaction between antidye (A) and dye protein (D) would then be

$$A + D \rightleftharpoons AD \quad \ldots\ldots [1]$$

This would represent the equivalence point compound in its simplest form, as composed of 1 unit of A and 1 unit of D, regardless of their actual molecular proportions. Since both A and D 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 (*cf.* 1). Thus the AD compound initially formed could react with other molecules of the same compound, or with A or D, whichever 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:

$$\begin{aligned} AD + A &\rightarrow AD \cdot A, \text{ and} \\ AD + AD &\rightarrow AD \cdot AD \end{aligned} \quad \ldots\ldots [2]$$

Since both A and D are multivalent with respect to each other the products of reaction [2] would combine chemically until aggregates large enough to separate from the solution are formed. If A and D are mixed in equivalent proportions the AD produced according to [1] would merely polymerize, and the equivalence point precipitate would be $(AD)_n$.

As in the reaction studied in Reference 1 the composition of the precipitate would thus depend on the relative proportions in which the reactants are mixed. The ratios given in Table II, and the colors of the precipitates, which range from pink to deep purplish red, are in accord with this view. Based on this concept of the reaction expressions [3] and [4] below may be derived with the aid of the mass law as in Reference 1. Since only a small proportion of the antibody was found to react to form compounds containing more A than twice the equivalence point ratio, the additional reactions which it was necessary

to consider in Reference 1 may be neglected. The equation, then, for reaction [2] in the region of excess antibody becomes:

$$\text{mg. antibody N precipitated} = 2\,RD - \frac{R^2D^2}{A} \quad \ldots\ldots\ldots\ldots\ldots\ldots [3]$$

in which R is taken as the ratio of antibody nitrogen to dye nitrogen at the point at which antigen first appears in excess,[3] D = the amount of dye nitrogen precipitated (= amount added × a fraction characteristic for the serum), and A = the amount of antibody N precipitated at the point at which antigen first appears in excess.

In the region of excess D up to the inhibition zone the equation becomes:

$$\text{mg. dye N precipitated} = 2\,R'A - \frac{(R')^2A^2}{D\text{ added}} \quad \ldots\ldots\ldots\ldots\ldots\ldots [4]$$

Since the maximum amount of specifically precipitable nitrogen is not obtained at the equivalence point, but in the region of excess antigen R′ is defined as $\frac{1}{R}$, or the ratio of dye nitrogen to antibody nitrogen in the precipitate at the point at which antigen first appears in excess, and A as the maximum specifically precipitable antibody nitrogen, D being the antigenic portion of the dye added (total × a fraction characteristic for the serum used). Duplicate analyses with a slight excess of dye are sufficient to establish A.

In the inhibition zone an expression of the type given in Reference 2[4] is found to apply:

$$\frac{D_{soln}}{A_{soln}} = K \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots [5]$$

In this expression D_{soln} is the amount of antigenic dye nitrogen in solution and A_{soln} is the difference between A as defined for expression [4] and the amount precipitated at the point under consideration in the inhibition zone. While K is a constant for any serum over the

[3] Agreement of [3] with the experimental data is better if R is taken as the ratio at this point instead of at the equivalence point. The value of R is then not very different from the empirically useful R″ (see below).

[4] Heidelberger and Kendall (2), page 820.

greater part of the inhibition zone, it can scarcely be a true dissociation constant, since it is shown in Table II that the composition of the precipitate varies in this zone as well. Here, too, the relative proportions of the components appear to be the determining factors, since a diminution of the concentration of D by dilution with saline is not followed by precipitation, even after several days in the cold.

From expression [3] may be derived the linear relation:

$$\frac{\text{Antibody N}}{\text{Dye N}} \text{ in the precipitate} = 2R - \frac{R^2}{A} D \quad \ldots\ldots\ldots\ldots\ldots\ldots [6],$$

and in the text-figure Line A is plotted in this way from the data in Table II up to the region of excess antigen. Thus for the pooled sera B 186, the intercept on the y-axis, $2R, = 14.6$, and the slope of the line, $\frac{R^2}{A}, = 54$, whence $R = 7.3$, $A = 0.988$. The experimentally found values are $R = 7.7$, at the antigen excess end of the equivalence zone, and $A = 1.034$. A comparison of the fourth and last columns of Table II shows close agreement between the experimentally determined values of antibody N precipitated and those calculated according to [3], except in the case of the first two points. Since [6] is a linear expression, the equation for the line may be approximately fixed for any serum by two determinations (in duplicate) of the amounts of antigen and antibody nitrogen precipitated in the region of excess antibody. Naturally, if more points are determined the accuracy is greater. More than one-third of the antibody should be precipitated, as the small portion present yielding compounds of ratio > 2 R would otherwise introduce a relatively large error.

Data are given in Table III for experiments in which dye was added serially in small portions to an excess of B 186 mixture, using, in the two cases given, the supernatants from the initial precipitations with 0.007 and 0.015 mg. of dye N in the experiment summarized in Table II. The A:D ratios lie on a line somewhat above Line A in Text-fig. 1, as would be expected from the Danysz effect. Hence the amount of antibody N precipitated was actually higher for a given amount of antigen added in serial portions than calculated from Line A (equation [6]). This is shown by comparison of Columns 3 and 5 of Table III. Other serial experiments on larger quantities of serum

were also shown to follow expression [6], but the data were insufficient to permit a comparison at more than one point with the antibody N precipitated by a single addition of antigen. In one such experiment with the serum of Rabbit R 18, it was found that after a single addition of dye, 0.25 mg. of antibody N and 0.024 mg. of dye N were precipitated per cc., while practically the same amount of antibody N, 0.24, and only 0.016 mg. per cc. of dye N were precipitated if the dye was added serially in six small portions. The Danysz effect is thus brought within the theory presented.

TABLE III

Serial Additions of Dye to Pooled Sera B 186, Calculated Back to 4.0 cc.

Total of successive dye N additions	Total dye N pptd.	Antibody N pptd.	Ratio A N:D N	Antibody N pptd., from equation [3]	
mg.	*mg.*	*mg.*		*mg.*	
0.014(6)	0.014(5)	0.223	15.4	0.201	
0.037	0.036	0.488	13.6	0.456	
0.059	0.057	0.694	12.2	0.656	
0.087	0.081	0.883	10.9	0.828	
0.116	0.092	0.939	10.2		Excess D; 0.19 mg antibody N not pptd.
0.007(3)	0.007(2)	0.123	17.1	0.102	
0.029	0.029	0.408*	14.1	0.378	
0.051	0.051	0.623	12.2	0.605	
0.079	0.077	0.842	10.9	0.804	
0.108	0.093	0.936	9.9		Excess D; 0.19 mg. antibody N not pptd.

* One determination lost.

Serial experiments at 0° in the dye-antidye system differed from those studied in Reference 1 in that a portion of the antibody was not precipitated, a circumstance which might contribute to the Danysz effect. Thus it is seen from Table III that about 17 per cent of the antibody present remained in solution, although this portion of the antibody was precipitated with the rest when enough dye was added in a single portion, as in Table II. It is possible that this part of the antibody contains too few specific groupings to build up A·D aggregates large enough to separate from solution unless these can combine

with A·D aggregates formed by the rest of the antibody. R 18, also, showed 13 per cent of antibody which was non-precipitable in a serial experiment, so that it is evident that even in serum from a single animal there are antibodies of different reactivities. The failure of the first two points in Text-fig. 1 to follow either linear relation has already given evidence that a portion of the antibody can react to form com-

TABLE IV

Maximum Specifically Precipitable Nitrogen in Antidye Sera and Composition of Dye-Antidye Precipitate in Equivalence Zone and at Maximum Precipitation

Serum or antibody	Maximum specifically precipitable N found per cc.	Maximum calcd. from equation [7]	Ratio at maximum (= R') calcd. from equation [7]	Ratio antibody N:dye N at beginning of equivalence zone	Equivalence point ratio	Ratio antibody N:dye N at end of zone (= R)	R calcd. from equation [6]
	mg.	*mg.*					
7.42	0.11			>13.2	(>11.6)	(10)	
8.01	0.32	0.31 (4 points)	6.7	(<12)	(<10.2)	8.4	7.8
8.08	0.14	0.14 (2 ")		>12.9	(>11.5)	(10)	
R 18	0.27	0.28 (2 ")		>10.4	(>9.2)	>8.0	
1.14	0.51	0.52 (2 ")				8.3	
1.15 + 1.46*	0.27	0.28 (3 ")	6.4			8.3	
CV18 serum	0.29	0.30 (2 ")				(7.0)	
CV18 globulin	0.19	0.19 (3 ")	6.0	(10)	(8.3)	6.6	6.9
B 186*	0.28	0.27 (12 ")	6.1	(9.4)	(8.6)	7.7	7.3
B 187*	0.16	0.16 (8 ")	6.8	(10.5)	(8.6)	6.6	8.1
3.76	0.26	0.26 (5 ")	6.1	9.9	(9.0)	8.1	7.3
Mean			6.4				

Values in parentheses indicate probable value deduced from nearest actual determination.

* Pooled sera.

pounds of greater A:D ratio than 2 R. It is not surprising, therefore, that there are experimental deviations from a theory based on the statistical treatment of the antibody as a unit.

In most instances insufficient experiments were run to fix the equivalence zone with great precision, but in the last four sera (two of which were mixtures) (see Table IV) it was quite accurately mapped out. These sera showed an average calculated equivalence point

of 8.6, with considerable variation in the extent of the zone. It must be pointed out, however, that at least two sera, 7.42 and 8.08, must have had equivalence point ratios exceeding 10 (see also Table IV). A more detailed discussion of questions related to the equivalence zone will be given in connection with data on the crystalline egg albumin-antibody system.

TABLE V

Comparison of Calculated and Found Values of Dye Precipitated in Region of Antigen Excess, B 186

Dye N added (95% antigenic)	Antigenic dye N added	Dye N pptd. (found)	Dye N pptd., from equation [4]	
			Using experimental values $A = 1.13$, $R' = \frac{1}{7.7}$	Using values calcd. from equation [7] $A = 1.10$, $R' = \frac{1}{6.1}$
mg.	*mg.*	*mg.*	*mg.*	*mg.*
0.146	0.139	0.134	0.139	0.127
0.183	0.174	0.162	0.170	0.174
0.219	0.208	0.191	0.190	0.205
0.256	0.243	0.207	0.205	0.227
0.280	0.266	0.229	0.213	0.239

Inhibition Zone

			Dye N in solution	Antibody N dissolved		K, equation [5]		Antibody N dissolved (calcd.)	
				A = 1.131	A = 1.098	A = 1.131	A = 1.098	K = 1.50	K = 1.59
0.485	0.461	0.271	0.190	0.107	0.074	1.78	2.57	0.127	0.120
0.970	0.921	0.249	0.672	0.449	0.416	1.50	1.62	0.448	0.423
1.455	1.383	0.112	1.271	0.849	0.816	1.50	1.56	0.847	0.799

Columns 1 and 3 are taken from Table II.

When the maximum amount of precipitable antibody nitrogen in an antidye serum has been determined, or else has been calculated according to the method given in the following paragraph, the amount of dye nitrogen precipitated in the region of excess antigen up to the inhibition zone may be computed for various additions of dye by means of equation [4]. Calculated and found values for pooled sera B 186 are compared in Table V, making use of the experimentally determined figures for A and R′ on the one hand, and the values calculated according to the next paragraph, on the other. It will be seen

that the agreement is good, particularly when the experimental values of A and R′ are used.

By means of an empirical relation the same two or three points determined by chemical analysis in the region of excess antibody may be used to calculate the maximum amount of antibody nitrogen precipitable from the serum if it is desired to omit separate analyses to determine this constant. It has been found that if $\frac{\text{Antibody N}}{\text{Dye N}}$ in the precipitate is plotted against the square root of the dye nitrogen precipitated an even closer approximation to a straight line is obtained than when the points determined are plotted according to equation [6]. For the six sera for which sufficient data are available this empirical relation has been found to reduce to the general form:

$$\frac{\text{Antibody N}}{\text{D}} \text{ in the precipitate} = 3\,R'' - 2\sqrt{\frac{(R'')^3 D}{A}} \quad \ldots\ldots\ldots\ldots\ldots\ldots [7]$$

in which 3 R″ is the intercept on the y-axis and $-2\sqrt{\frac{(R'')^3}{A}}$ is the slope of the line, D = amount of dye nitrogen precipitated, A = maximum precipitable antibody nitrogen, and R″ the A:D ratio at the maximum. For B 186 the equation for this line, drawn through the points experimentally determined for 4 cc. of serum (Line B, Text-fig. 1) is $\frac{\text{Antibody N}}{\text{Dye N}}$ in the precipitate $= 18.4 - 29\sqrt{D}$. Then antibody N precipitated $= 18.4\,D - 29\,D^{3/2}$, and when the first derivative, $18.4 - 43.5\,D^{1/2} = 0$, antibody N precipitated at the maximum, or A, = 1.10, a value in good agreement with that, 1.13, actually found. Found and calculated values for A are given in Table IV for ten sera, and it will be seen that the greatest deviation is 0.01 mg. of N per cc., a result well within the experimental error of the analytical methods used and more accurate than obtainable from [6] with only two sets of analyses. By this method, then, constants A and R″ characteristic of the serum are obtained, the former, at least, with a high degree of accuracy. The calculated values for R″ given in Table IV are quite uniform and in reasonable agreement with the experimental ratios, for it must be borne in mind that experimentally it is found that maximum precipitation often occurs over a fairly wide range of antigen ex-

cess, and unless a large number of analyses are made it is difficult to locate exactly the smallest amount of D yielding the A value. Thus, in B 186, maximum values for A were obtained at antibody N:D ratios from 5.9 to 4.9 (Table II), the former being in good agreement with the calculated value. The exact mapping out of this zone would scarcely be necessary for ordinary purposes, and equation [7] may be

TABLE VI

Analyses of Inhibition Zone Supernatants for Antigen

Antigenic dye N added	Antigenic dye N in 5.0 cc. supernatant	Antibody N in 5.0 cc. supernatant	Fraction of supernatant analyzed	Antigenic dye N in aliquot taken (from Column 2)	Dye N found colorimetrically in aliquot taken	Total N on Curve C, Text-fig. 1 corresponding to D found	Total N actually determined	Difference	Antibody N in aliquot of inhibition supernatant
mg.	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
					B 186				
0.461	0.190	0.107	0.4	0.076	0.074	0.850	0.92	0.07	0.04
0.921	0.672	0.449	0.14	0.094	0.098	1.00	1.10	0.10	0.06
0.921	0.672	0.449	0.12	0.081	0.072*	0.84	1.00	0.16	0.05
	B 187 (total N curve not given in Text-fig. 1)								
0.128 (88% of amount added)	0.016	0.012	0.8	0.013	Not run	0.210†	0.238	0.03	0.01
0.257 (88% of amount added)	0.147‡	0.150	0.2‡	0.029	0.025	0.295‡	0.350‡	0.055	0.03
0.411 (88% of amount added)	0.314	0.283	0.133	0.042	0.039	0.494	0.570	0.08	0.04
	B 189 (total N curve not given in Text-fig. 1)								
	In 2.5 cc. supernatant								
0.149	0.068	0.01	0.4	0.027	0.027	0.310	0.350	0.04	0.00
0.299	0.207	0.133	0.2	0.041	0.042	0.398	0.420	0.02	0.03

* Poor color match.

† Corresponding to calculated amount taken.

‡ Using B 189, which precipitated 80 per cent of dye added. 1.5 cc. portions B 189 used.

used as far as the inhibition zone when the minimum number of analyses seems desirable. The resulting error beyond the maximum did not exceed 10 per cent in the sera studied.

It remains, then, only to determine the amount of antibody and antigen nitrogen dissolved at a point well within the inhibition zone to obtain a value of K in equation [5], and when this is found, the

amount and composition of the precipitate in this range may be calculated. Here, too, it would be advantageous to perform analyses at two points. Inhibition zone data for B 186 are given in the second part of Table V. In the case of B 187 the values of K found from Columns 2 and 3 of Table VI according to equation [5] were 1.08 and 1.11.

Thus with a minimum of three sets of micro analyses for dye and antidye nitrogen precipitated, two in the region of excess antibody, and one in the inhibition zone, it is possible to derive expressions from which the amount of antibody nitrogen precipitated by any amount of antigen may be roughly calculated, as may also be the amount of antigen precipitated in the maximum antibody zone. With a larger number of analyses the entire behavior of the serum in the precipitin reaction may be predicted with a satisfactory approximation to the experimentally determined values.

The above considerations have been presented with the use of a single serum mixture, B 186, as the principal example. Less complete data are available with other sera, and in some of these agreement with the theory is even more satisfactory than in the case of B 186. In order to save space most of these data have been omitted, the more so as the utility of the quantitative precipitin theory in the case of a pure, crystalline, colorless antigen, egg albumin, and its homologous antibody has been demonstrated and the the data are being prepared for publication.

The dye-antidye system is thus chiefly of use in permitting a series of orientating experiments which would not have been possible in a system with a colorless antigen. As an additional example, the determination of the excess of antigen in the supernatant in the region between the equivalence zone and the inhibition zone according to Reference 1 or 5 offers no complications with either a colored or colorless antigen. In the latter case, the amount of antigen in solution in the inhibition zone can be determined from an aliquot portion of the supernatant, set up against fresh antibody in excess, only after the effect of the dissolved antigen-antibody compound present is first ascertained. Since there is no way of distinguishing between antigen nitrogen and antibody nitrogen in a colorless precipitate or supernatant it was necessary to study the question in the dye-antidye

system. The data so obtained are summarized in Table VI, in which the amount of antigenic dye N and antibody N in the supernatant in the case of B 186 are taken from Table V, using A = 1.13 as the maximum. It would seem that the entire amount of dissolved antigen-antibody compound present in the aliquot appears in the precipitate formed with fresh antibody in excess, a result which might have been predicted on the basis of the writers' theory. A small, but consistent discrepancy in these determinations is also evident, averaging 0.03 or 0.04 mg. of nitrogen. This appears even in the instance in which no appreciable amount of soluble AD compound had been formed, and may be due to the presence of a portion of the difficultly precipitable antibody mentioned in connection with the Danysz effect.

Since the writers' preliminary work (4) studies on azoprotein-antibody systems have been made by Marrack and Smith (10) and by Haurowitz and Breinl (11). Both groups confirmed the varying composition of the specific precipitate with varying proportions of antigen and antibody.

In conclusion it must again be emphasized that the present theory represents an attempt to deal formally with antigen and antibody on the basis that these behave statistically as multivalent, homogeneous substances. There is ample evidence that antibody, in the systems already studied, is a mixture of substances of different reactivities, so that the present quantitative theory is offered merely as a useful expedient, applicable to antisera as they occur, until such time as it may be possible to separate from the complex antibody mixture an antibody possessed of a single reactivity.

SUMMARY

1. A quantitative theory of the precipitin reaction based on the laws of classical chemistry has been tested on an azoprotein-antiprotein system and found to apply.

2. With its aid relationships may be deduced which permit the calculation of the behavior of an antidye serum over its entire range after a few quantitative chemical analyses have been made for antigen and antibody in the precipitate.

3. An empirical relation is also presented which further reduces the number of analyses necessary.

4. A study of supernatants in the inhibition zone has shown that the entire amount of dissolved antigen-antibody compound present is precipitated when supernatants are analyzed for antigen by the precipitin method.

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