# A QUANTITATIVE THEORY OF THE PRECIPITIN REACTION

## V. The Reaction between Crystalline Horse Serum Albumin and Antibody Formed in the Rabbit*

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In earlier papers there has been outlined a quantitative theory of the precipitin reaction and its application to hapten-antibody systems in horse and rabbit antisera (1, 2) and to antigen-antibody systems involving a dye protein (3) and crystalline egg albumin (4). It was shown that if the combination of antigen or hapten were considered to take place in a series of bimolecular competing reactions between multivalent antigen and antibody, simple equations expressing in several instances the entire course of the precipitin reaction could be derived from the law of mass action.

These equations were of the type

$$\text{Antibody N precipitated} = 2\ R\ \text{Sa N} - \frac{R^2}{A}(\text{Sa N})^2 \quad \ldots\ldots[1]$$

in which R is the ratio of antibody nitrogen to serum albumin[1] nitrogen at a reference point in the equivalence zone, Sa N is the amount of antigen nitrogen or hapten added, and A is the amount of antibody nitrogen precipitated at the reference point. By dividing through by Sa N the equation

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

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[1] Referred to throughout as Sa.

is derived. This is the equation of a straight line and permits evaluation of the constants 2R and $\frac{R^2}{A}$, hence also A, for any given serum.

An empirical relation obtained (3, 4) by plotting $\frac{\text{Antibody N}}{\text{Sa N}}$ in the precipitate against the square root of Sa N added yielded in some instances an even closer approximation to a straight line than that obtained by [2]. The equation of this line is

$$\frac{\text{Antibody N}}{\text{Sa N}} \text{ precipitated} = 3R'' - 2\sqrt{\frac{(R'')^3 (\text{Sa N})}{A}} \quad \ldots\ldots\ldots\ldots [3]$$

in which $3R''$ is the intercept on the y-axis and $-2\sqrt{\frac{(R'')^3 (\text{Sa N})}{A}}$ is the slope of the line, A = the maximum precipitable antibody nitrogen, and $R''$ = the antibody N:Sa N ratio at the maximum. For example, for serum $3.69_1$, if the equation of the best line obtained by plotting the ratios of antibody N: Sa N in the precipitates against the square root of added Sa N be multiplied by Sa N, the resulting equation describing the behavior of the serum is

$$\text{mg. antibody N precipitated} = 22.4 \text{ Sa N} - 45 (\text{Sa N})^{3/2} \quad \ldots\ldots\ldots\ldots [4]$$

The present investigation of the precipitin reaction between crystalline horse serum albumin[1] and the homologous antibody formed in the rabbit was undertaken in order to study the mechanism of a precipitin reaction involving a relatively easily purified serum constituent as antigen. It was thought that a quantitative study of this nature might serve as a starting point for the investigation of chemical and immunological relationships between corresponding proteins of different species. Taylor, Adair, and Adair (5) have also made observations on this reaction and found a zone in which both components appeared in the supernatant. This was ascribed to the presence of several components in the crystalline serum albumin, one of which was later shown by Goldsworthy and Rudd (6) to be globulin.

In the present work comparatively small injections of serum albumin were given and antisera were obtained which were relatively free from antiglobulin. In these sera neither antigen nor antibody could be detected in equivalence zone supernatants, as in the systems previously studied. An antiserum like those studied by Taylor, Adair, and Adair (5) was obtained from a rabbit which had been

subjected to prolonged immunization and it was shown that antiglobulin was present in large amounts.

A preparation of R-salt-azo-biphenyl-azo-crystalline serum albumin was also prepared according to (7). The reactions of this dye protein with homologous antisera and antisera to crystalline serum albumin are also included.

## EXPERIMENTAL

The crystalline serum albumin used in this communication was prepared according to (8) and was crystallized three times. For injection the material was precipitated with alum by adding 1 per cent alum and partially neutralizing with very dilute NaOH until precipitation was at a maximum. The suspensions were adjusted to a concentration of about 0.8 mg. of serum albumin per ml. in saline containing 1:10,000 merthiolate.[2] Rabbits were given intravenous injections of the suspension four times a week for 4 to 6 weeks, using a total of 20 to 40 mg. of protein per rabbit. Sera obtained after a second course were usually of higher antibody content. Appreciable amounts of antiglobulin were found only in the later bleedings of rabbit 3.68, which received 229 mg. of Sa.

Quantitative precipitin determinations were carried out as described in previous papers of the series. Nitrogen estimations on the washed specific precipitates were made by the micro Kjeldahl method. As noted in (4), it was occasionally necessary to centrifuge supernatants a second time, especially in the equivalence zone in which the precipitate tends to be loosely packed.

As in (4), it was assumed that supernatants which failed to react with anti-Sa serum in the excess antibody region and equivalence zone actually contained no Sa, since the test for Sa with homologous antibody is extremely delicate, and any soluble Sa-A compound formed would have to be extremely slightly dissociated to escape detection. Moreover, when soluble compounds are present, as in the inhibition zone, they are precipitated when fresh antibody is added. In the region of antibody excess and in the equivalence zone, therefore, N was calculated by subtracting the Sa N added from the total N found in the precipitate.

In Table I are given the effect of temperature and volume changes on the amount of specifically precipitable nitrogen. The solubility of the Sa specific precipitate is about 0.006 mg. N per ml., a value close to that of 0.005 mg. N per ml. found for the solubility of the egg albumin specific precipitate (4). Only very slightly more N was precipitated at 0°C. than at 37°.

Table II shows the result of adding increasing amounts of Sa to a constant volume of antiserum and also gives a comparison of the experimentally determined values with those calculated from equations

[2] Kindly presented by the manufacturers, Eli Lilly and Company, Indianapolis.

[1] and [4]. Since inhibition occurred with small increments of Sa after the maximum had been reached, it was not feasible to derive equations describing the limited region of maximum antibody precipitation.

In the region of slight antigen excess, Sa was determined by adding an aliquot portion of the supernatant to a measured volume of a calibrated serum, estimating the total N precipitated, and reading off the corresponding amount of Sa N from the total N curve of the

TABLE I

*Influence of Temperature and Volume on Nitrogen Precipitated by Serum Albumin from 1.0 Ml. of Antisera*

| Serum No. | Sa N added | Nitrogen precipitated at | | | | | | Tests on supernatants |
|---|---|---|---|---|---|---|---|---|
| | | 37° | | | 0° | | | |
| | | 2 ml. | 2.5 ml. | 8 ml. | 2.0 ml. | 2.5 ml. | 8 ml. | |
| | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | |
| $3.69_2$ | 0.05 | | | | 0.674 | | 0.628 | Excess A |
| | 0.25 | | | | 0.642 | | 0.584 | Excess Sa |
| $3.85_1$ | 0.03 | | 0.378 | | | 0.402 | | Excess A |
| | 0.04 | | 0.454 | | | 0.474 | | Trace A |
| | 0.15 | | | | | 0.472 | 0.436 | Excess Sa |
| $3.86_1$ | 0.05 | 0.930* | | 0.850 | 0.940† | | 0.888 | Excess A |

Including washings the solubility of the specific precipitate appears to be about 0.006 mg. N per ml. at 0°.

* A similar pair of tubes set up for 2 hours at 37° and overnight in the ice box gave 0.946 mg. N.

† A similar pair of tubes washed four times instead of twice with 2.0 ml. of chilled saline per washing gave 0.916 mg. N.

calibrated serum. The values in the second column of Table II were obtained by subtracting the Sa found in the supernatant from the total added.

In the inhibition zone a similar analysis for Sa N is made, but calculation of the amount of Sa N in the supernatant is complicated by the presence of dissolved antibody and the procedure is as follows (4):

Let A = the maximum antibody nitrogen found in the serum used, Sa = the amount of serum albumin nitrogen added, and N = the amount of nitrogen pre-

TABLE II

*Addition of Increasing Amounts of Serum Albumin to Constant Volume of Antiserum*

| Sa N added | Sa N precipitated | Total N precipitated | Antibody N by difference | Ratio antibody N: Sa N in precipitate | Antibody N precipitated, calculated from equation [1] | Antibody N precipitated, calculated from equation [4] | Tests on supernatants |
|---|---|---|---|---|---|---|---|
| *mg.* | *mg.* | *mg.* | *mg.* | | *mg.* | *mg.* | |
| Course 1. Rabbit $3.69_1$. 1.5 ml. serum used | | | | | | | |
| 0.025 | Total | 0.392 | 0.367 | 14.7 | 0.370 | 0.377 | Excess A |
| 0.038 | " | 0.554 | 0.516 | 13.6 | 0.515 | 0.512 | " " |
| 0.050 | " | 0.668 | 0.618 | 12.4 | 0.630 | 0.616 | " " |
| 0.080 | " | 0.864 | 0.784 | 9.8 | 0.796 | 0.775 | " " |
| 0.094 | " | 0.912 | 0.818 | 8.7 | 0.820 | 0.810 | " " |
| 0.100 | " | 0.904 | 0.804 | 8.0 | | 0.813 | Trace A and Sa* |
| 0.113 | " | 0.958† | 0.845 | 7.5 | | 0.818 | " " " " |
| 0.125 | " | 1.000 | 0.875 | 7.0 | | | " " " " |
| 0.150 | 0.144 | 1.010 | 0.866 | 6.0 | | | Excess Sa |
| 0.175 | 0.150 | 0.998 | 0.848 | 5.7 | | | " " |
| Equation [1]: mg. antibody N pptd. = $17.0\ \text{Sa N} - 88\ (\text{Sa N})^2$ | | | | | | | |
| Calculated from equation, Sa N max. = 0.0966 mg. | | | | | | | |
| Antibody N max. = 0.820 mg. | | | | | | | |
| Equation [4]: mg. antibody N pptd. = $22.4\ \text{Sa N} - 45\ (\text{Sa N})^{3/2}$ | | | | | | | |
| Calculated from equation, Sa N max. = 0.110 mg. | | | | | | | |
| Antibody N max. = 0.818 mg. | | | | | | | |
| Course 2. Rabbit $3.69_2$. 1.0 ml. serum used | | | | | | | |
| 0.020 | Total | 0.354 | 0.334 | 16.7 | 0.333 | 0.341 | Excess A |
| 0.040 | " | 0.604 | 0.564 | 14.1 | 0.561 | 0.552 | " " |
| 0.050 | " | 0.674 | 0.624 | 12.5 | 0.635 | 0.623 | " " |
| 0.060 | " | 0.726 | 0.668 | 11.1 | 0.683 | 0.677 | No A or Sa |
| 0.075 | " | 0.784 | 0.709 | 9.5 | 0.704 | 0.721 | " " " " |
| 0.100 | " | 0.820 | 0.720 | 7.2 | | 0.725 | Trace Sa? |
| 0.112(5) | " | 0.856 | 0.743 | 6.6 | | | No A or Sa |
| 0.125 | 0.120 | 0.858 | 0.738 | 6.2 | | | Excess Sa |
| Equation [1]: mg. antibody N pptd. = $19.3\ \text{Sa N} - 132\ (\text{Sa N})^2$ | | | | | | | |
| Calculated from equation, Sa N max. = 0.073 mg. | | | | | | | |
| Antibody N max. = 0.705 mg. | | | | | | | |
| Equation [4]: mg. antibody N pptd. = $25\ \text{Sa N} - 56\ (\text{Sa N})^{3/2}$ | | | | | | | |
| Calculated from equation, Sa N max. = 0.089 mg. | | | | | | | |
| Antibody N max. = 0.739 mg. | | | | | | | |

* ± after centrifuging.

† One determination lost.

TABLE II—*Concluded*

| Sa N added | Sa N precipitated | Total N precipitated | Antibody N by difference | Ratio antibody N:Sa N in precipitate | Antibody N precipitated, calculated from equation [1] | Antibody N precipitated, calculated from equation [4] | Tests on supernatants |
|---|---|---|---|---|---|---|---|
| *mg.* | *mg.* | *mg.* | *mg.* | | *mg.* | *mg.* | |
| | | | Rabbit 13.29. 1.0 ml. serum‡ used | | | | |
| 0.025 | Total | 0.362 | 0.337 | 13.5 | 0.329 | 0.342 | Excess A |
| 0.037(5) | " | 0.508 | 0.470 | 12.5 | 0.464 | 0.468 | " " |
| 0.050 | " | 0.624 | 0.574 | 11.5 | 0.580 | 0.576 | " " |
| 0.062(5) | " | 0.740 | 0.677 | 10.8 | 0.675 | 0.669 | " " |
| 0.087(5) | " | 0.906 | 0.818 | 9.4 | 0.812 | 0.806 | " " |
| 0.112(5) | " | 1.008 | 0.905 | 8.0 | | 0.894 | No A or Sa |
| 0.125 | " | 1.042 | 0.917 | 7.3 | | 0.924 | " " " " |
| 0.135 | " | 1.070 | 0.935 | 6.9 | | 0.935 | " " " " |
| 0.157(5) | 0.152(5) | 1.110 | 0.957 | 6.3 | | | Excess Sa |
| 0.200 | 0.158 | 1.070 | 0.912 | 5.8 | | | " " |

Equation [1]: mg. antibody N pptd. = $14.7\ \text{Sa N} - 62\ (\text{Sa N})^2$
Calculated from equation, Sa N max. = 0.1086 mg.
Antibody N max. = 0.871 mg.

Equation [4]: mg. antibody N pptd. = $18.7\ \text{Sa N} - 32\ (\text{Sa N})^{3/2}$
Calculated from equation, Sa N max. = 0.152 mg.
Antibody N max. = 0.944 mg.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | Serum M, used for determination of Sa in supernatants, 1.0 ml. | | | | |
| 0.016 | Total | 0.228 | | | | | Excess A |
| 0.032 | " | 0.390 | | | | | " " |
| 0.048 | " | 0.496 | | | | | " " |
| 0.064 | " | 0.594† | | | | | " " |
| 0.080 | " | 0.650 | | | | | No A or Sa |
| 0.080§ | " | 0.646 | | | | | " " " " |

‡ Absorbed with small additions of globulin until traces of globulin were present in excess.

§ 1 mg. R-salt-azo-biphenyl-azo-crystalline egg albumin added. The washed precipitate was colorless and contained no additional nitrogen.

cipitated at the point considered. Then the amount of specific nitrogen (antigen as well as antibody) in the supernatant is given by A + Sa − N, and all of this nitrogen would be precipitated in the analysis of the supernatant for Sa with excess antibody according to the quantitative theory elaborated in (1), and as was actually found in the dye-antidye system (3). The additional assumption is made that the entire precipitate obtained in this analysis is of uniform composition; in other words. that the dissolved Sa-A present can combine with A until

its composition is the same as that of the Sa-A formed by the free Sa present in its reaction with excess antibody.

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 + Sa − N) = antibody nitrogen precipitated from the serum used in the analysis. If the curve of antibody N precipitated by Sa from this serum be constructed, the amount of

TABLE III

*Composition of Precipitate in Region of Antigen Excess*

| Sa N added | Total N precipitated | Specific N in supernatant (A + Sa − N) | Fraction analyzed | Total N precipitated in analysis of fraction | Less specific N in fraction analyzed | Corresponding Sa N | Per cent Sa N 2nd precipitate* | Sa N in fraction analyzed | Sa N in entire supernatant | Sa N in precipitate | Antibody N in precipitate | Ratio antibody N:Sa N in precipitate |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *mg.* | *mg.* | *mg.* | | *mg.* | *mg.* | *mg.* | | *mg.* | *mg.* | *mg.* | *mg.* | |
| Serum $3.69_2$, maximum antibody N, 0.743 mg. per ml. | | | | | | | | | | | | |
| 0.150 | 0.800 | 0.093 | 0.50 | 0.194 | 0.147 | 0.010 | 6.4 | 0.0124 | 0.025 | 0.125 | 0.675 | 5.4 |
| 0.200 | 0.726 | 0.217 | 0.50 | 0.486 | 0.377 | 0.036 | 8.7 | 0.0423 | 0.085 | 0.115 | 0.611 | 5.3 |
| 0.250 | 0.642 | 0.351 | 0.50 | 0.660 | 0.484 | 0.056 | 10.4 | 0.0686 | 0.137 | 0.113 | 0.529 | 4.7 |
| 0.300 | 0.534 | 0.509 | 0.25 | 0.550 | 0.423 | 0.040 | 8.6 | 0.0473 | 0.188 | 0.112 | 0.422 | 3.8 |
| Serum 3.12, maximum antibody N, 0.449 mg. per ml. | | | | | | | | | | | | |
| 0.128 | 0.480 | 0.097 | 0.50 | 0.334 | 0.285 | 0.027 | 8.7 | 0.029 | 0.058 | 0.070 | 0.410 | 5.9 |
| 0.160 | 0.418 | 0.191 | 0.50 | 0.482 | 0.386 | 0.043 | 10.0 | 0.048 | 0.096 | 0.064 | 0.354 | 5.5 |
| 0.192 | 0.370 | 0.271 | 0.25 | 0.384 | 0.316 | 0.031 | 8.9 | 0.034 | 0.136 | 0.056 | 0.314 | 5.6 |
| 0.240 | 0.312 | 0.377 | 0.25 | 0.478 | 0.384 | 0 043 | 10.1 | 0.048 | 0.192 | 0 048 | 0.264 | 5.5 |

Serum M was used for all analyses for serum $3.69_2$ except for the last line, for which serum N was used.

Serum 3.13 was used for all supernatant analyses for serum 3.12.

* 100 times value in column 7 divided by sum of values in columns 6 and 7.

Sa corresponding to this quantity of antibody N may be read off. The percentage of Sa in this portion of the precipitate may then be calculated according to

$$\frac{\text{Sa N found} \times 100}{\text{Sa N found} + \text{antibody N found}} = \text{per cent Sa N.}$$

Since it was assumed that the entire precipitate contains this proportion of Sa, N′ × per cent Sa N thus found ÷ F = Sa N in total supernatant, and Sa N originally added minus this value = Sa N in the original precipitate.

The ratios of antibody N to Sa N in the first portion of the inhibition zone are shown in the last column of Table III, the calculations being those described above. This method was not applicable to the region

TABLE IV

*Serial Addition of Serum Albumin to Various Antisera, Calculated to Original Volume*

| Total Sa N added | Total N precipitated | Total antibody N precipitated | Ratio antibody N: Sa N in precipitate | Antibody N, calculated from equation [1] |
|---|---|---|---|---|
| *mg.* | *mg.* | *mg.* | | *mg.* |
| Serum 3.85; 10.0 ml. used | | | | |
| Equation [1]: mg. antibody N pptd. = 17.3 Sa N − 170 (Sa N)² for 1.5 ml. serum | | | | |
| 0.020 | 0.424 | 0.404 | 20.2 | |
| 0.041 | | 0.748 | 18.3 | |
| 0.063 | | 1.107 | 17.6 | |
| 0.086 | | 1.454 | 16.9 | 1.30 |
| Serum 3.11; 10.0 ml. used | | | | |
| Equation [1]: mg. antibody N pptd. = 12.4 Sa N − 76 (Sa N)² for 1.0 ml. serum | | | | |
| 0.080 | 1.180 | 1.100 | 13.8 | |
| 0.164 | | 2.012 | 12.3 | |
| 0.252 | | 2.817 | 11.2 | |
| 0.345 | | 3.540 | 10.3 | 3.37 |
| 0.442 | | 4.179 | 9.5 | 3.99 |
| Serum $3.69_3$; 5.0 ml. used | | | | |
| Equation [1]: mg. antibody N pptd. = 19.4 Sa N − 78 (Sa N)² for 1.0 ml. serum | | | | |
| 0.050 | 1.138 | 1.088 | 21.8 | |
| 0.105 | | 2.102 | 20.0 | |
| 0.166 | | 3.131 | 18.9 | |
| 0.233 | | 4.118 | 17.7 | 3.68 |
| 0.306 | | 5.023 | 16.4 | 4.47 |
| 0.387 | | 5.771 | 14.9 | 5.17 |
| 0.476 | | 6.12 | Excess Sa | |

5.0 ml. 0.476 supernatant + 1.0 ml. serum $3.69_3$ + 0.215 mg. Sa N..... 1.788

1.0 ml. $3.69_3$ + 0.215 mg. Sa N..... 1.624

Difference..... 0.164; calculated to original volume = 0.32 mg. N

Total antibody N: recovered 6.44 mg.; present originally 6.60 mg. Recovery, 97.6 per cent.

of marked inhibition since the experimental error is multiplied many times because of the small fraction of supernatant required for analysis with the relatively weak sera available.

Table IV shows the results of serial removal of the antibody calcu-

lated to the original volume in each step after the first. A relatively large quantity of each serum was treated repeatedly with a small fraction of the amount of Sa necessary to remove the antibody. In all three sera, the Danysz phenomenon is quite definite, as can be seen by comparison of the third and fifth columns of the table. It was also observed in serum $3.69_3$ that all of the antibody present could not be removed in a serial experiment. However, this residual antibody could be recovered by adding the supernatant to a mixture of fresh serum and Sa. A recovery of 97.6 per cent of the total was obtained, a value well within the limit of experimental error.

Table V indicates that removal of one-half of the antibody from a serum results in a different equation from that of the original serum diluted to the same antibody content as the absorbed serum.

Table VI is a compilation of the antibody N to Sa N ratios in the equivalence zones of the sera studied. The equivalence point ratios are the average of the values found at the antigen and antibody excess ends of the zone.

Table VII shows the results with the second bleeding of rabbit 3.68, which contained a considerable amount of antiglobulin. Although tests on the supernatants with more antibody showed the presence of excess Sa, positive tests were also obtained by the addition of Sa or serum globulin. Similarly, if the serum is precipitated with excess globulin, addition of more globulin or Sa to the supernatant gives definite reactions, showing that antibody is still present.

It was found (Table VIII) that the decreasing antibody content of resting rabbits, previously injected with Sa, was unaffected by the injection of another antigen such as Ea or typhoid vaccine, although a single injection of Sa into the same rabbit produced an immediate drop in antibody content followed by a definite increase.

*Preparation of R-Salt-Azo-Biphenyl-Azo-Serum Albumin.*[3]—(*Cf.* 7.) 0.46 gm. of benzidine was dissolved in 100 ml. of water containing 1.5 ml. of concentrated HCl. The solution was chilled to 7–8°C., 0.35 gm. of $NaNO_2$ was added, and the mixture stirred until no test for free nitrite was obtained with KI and starch. The solution was poured into 500 ml. of water containing 3 gm. of sodium acetate. 0.87 gm. of R-salt in 100 ml. of water and 20 ml. of 2 N $K_2CO_3$ were added. The mixture was allowed to stand in ice water for 1 hour before use.

[3] Referred to throughout as DSa (dye serum albumin).

TABLE V

*Comparison of Partially Absorbed Serum with Original at Same Antibody Content*

| Sa N added | Total N precipitated | Antibody N precipitated | Ratio antibody N:Sa N in precipitate |
|---|---|---|---|
| *mg.* | *mg.* | *mg.* | |
| Serum 3.85 diluted to same antibody content as 3.85B | | | |
| 0.01 | 0.166 | 0.156 | 15.6 |
| 0.02 | 0.300 | 0 280 | 14.0 |
| 0.03 | 0.388 | 0.358 | 11.9 |
| Equation [1]: mg. antibody N pptd. = 17.3 Sa N − 170 (Sa N)$^2$ | | | |
| Serum 3.85B after approximately one-half of antibody was removed serially in 4 absorptions | | | |
| 0 01 | 0.140 | 0.130 | 13.0 |
| 0.02 | 0.238 | 0.218 | 10.9 |
| 0.03 | 0.324 | 0.294 | 9.8 |
| Equation [1]: mg. antibody N pptd. = 14.5 Sa N − 164 (Sa N)$^2$ | | | |

TABLE VI

*Ratio of Antibody Nitrogen:Serum Albumin Nitrogen in Equivalence Zone*

| Serum No. | Ratio at antibody excess end of zone | Equivalence point ratio | Ratio at antigen excess end of zone | R calculated from equation [2] | R′ calculated from equation [3] |
|---|---|---|---|---|---|
| 3.13 | 6.5 | (6.3) | (6.1) | | |
| 3.12 | (9.1) | 8.6 | 8.1 | | |
| 12.47 | 7.8 | 7.1 | (6.3) | | |
| 13.29 | < 9.4; > 8* | | (6.5) | 7.4 | 6.2 |
| $3.68_1$ | (9) | (7.8) | (6.6) | | |
| $3.69_1$ | (8.6) | (7.4) | (6.2) | 8.5 | 7.4 |
| $3.69_2$ | < 12.5; > 11* | | (6.4) | 9.7 | 8.3 |
| 3.85 | (11.0) | (8.3) | (5.6) | | |
| Mean..... | 8.7 | 7.6 | 6.5 | | |

Equivalence point taken as arithmetical mean of the limiting zone ratios.
Values in parentheses calculated from nearest actual determination.
* Not used in calculating mean.

TABLE VII

*Behavior of Serum $3.68_2$. Rabbit Injected with 229 Mg. Serum Albumin*

| Antigen N added | Total N precipitated | Supernatant plus | | |
|---|---|---|---|---|
| | | Sa | Antibody | Sg* |
| *mg.* | *mg.* | | | |
| 0.04 Sa | 0.30 | +(++) | +± | +(+++) |
| 0.05 Sa | 0.34 | +(++) | ++ | +±(+++) |
| 0.075 Sa | 0.35 | +(++) | +++ | +(+++) |
| 0.42 Sg* | 0.41 | +++ | +++ | +++ |

Readings in parentheses taken after centrifugation.
* Sg = serum globulin.

To 1.03 gm. of crystalline serum albumin in 250 ml. of solution, 10 ml. of 2 N $K_2CO_3$ were added and 150 ml. of the diazo solution were allowed to run in dropwise with constant stirring. During the addition it was necessary to add 2 N $K_2CO_3$ several times, a total of 35 ml. being used. At this point addition of diazo solution was stopped since a preliminary test on a small portion showed that an insoluble compound was formed at the equivalent of 200 ml. of diazo solution. After coupling was complete the solution was chilled and acidified with acetic acid to pH 4.7 to precipitate most of the dye protein. The precipitate was suspended in about 150 ml. of cold water and 5 per cent $Na_2CO_3$ was added until solution was as nearly complete as possible. The solution was centrifuged in the

TABLE VIII

*Effect of Heterologous Protein Injection on Antibody Content*

| Rabbit No. | Date | Antibody N per ml. | |
|---|---|---|---|
| | *1935* | *mg.* | |
| $3.69_1$ | Feb. 13 | 0.58 | |
| $3.69_2$ | Mar. 19 | 0.74 | Allowed to rest |
| | Apr. 15 | 0.28 | |
| | " 22 | 0.18 | Then injected intravenously with 10 mg. Ea* |
| | " 24 | 0.15 | |
| | " 27 | 0.15 | No precipitate with Ea or anti-Ea |
| | May 2 | 0.11 | |
| | " 6 | 0.11 | Then injected with 10 mg. Sa |
| | " 8 | 0.05 | With Ea: −(+ after centrifugation) |
| | " 13 | 0.17 | 0.21 mg. on May 18, 1935; 0.22 on May 22, 1935 |
| $3.85_1$ | Apr. 17 | 0.72 | Allowed to rest |
| | May 15 | 0.24 | Then injected with typhoid vaccine (4.5 billion organisms) |
| | " 18 | 0.14 | 0.09 mg. on May 22, 1935 |

* Egg albumin.

cold[4] and the residue discarded. Precipitation with acetic acid left a colorless supernatant which, on neutralization, gave no reaction with anti-Sa. Solution and reprecipitation were repeated twice. In an effort to fractionate so as to obtain a portion which did not react with anti-Sa, the precipitate was dissolved as before in a volume of 130 ml., and 65 ml. of saturated $(NH_4)_2SO_4$ solution were added. The precipitate was centrifuged in the cold (fraction 1). To the supernatant 33 ml. additional $(NH_4)_2SO_4$ solution were added and the precipitate

[4] Using a refrigerated centrifuge manufactured by the International Equipment Company, Boston, Massachusetts.

(fraction 2) was centrifuged off. The supernatant was acidified with acetic acid, yielding fraction 3. When these fractions were redissolved fraction 1 seemed to give the weakest reaction with anti-Sa. It was therefore given two reprecipitations at one-third saturation with $(NH_4)_2SO_4$, after which the supernatant was quite light in color. The precipitate was centrifuged off sharply and suspended several times in cold redistilled acetone until the solvent remained colorless. The residue, which was now insoluble in water, was redissolved in water + $K_2CO_3$, neutralized, and filtered through a Chamberland L2 filter. Part of the material was precipitated with alum for use in injecting rabbits and part was ultrafiltered in the cold through a parlodion membrane until the filtrate was colorless, in order to remove a considerable amount of dye which was not combined with protein. The ultrafiltered material still precipitated anti-Sa but failed to precipitate at any dilution with an antiserum to R-salt-azo-biphenyl-azo-egg albumin (7). It also failed to inhibit precipitation in the latter serum by the egg albumin dye.

The serum of rabbits immunized with DSa reacted with Sa as well as with DSa and the dye-antidye reaction was not inhibited by R-salt.

An attempt was made to free fraction 2 of reactivity with anti-Sa by chromatographic adsorption, but no fractionation could be observed.

*Analytical Data on R-Salt-Azo-Biphenyl-Azo-Crystalline Serum Albumin.*—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; crystalline serum albumin 1.73 per cent of S (9). An analysis by Mr. William Saschek of 8.296 mg. ash-free azo protein gave 1.74 mg. $BaSO_4$, or 2.88 per cent S.

| | |
|---|---|
| Calculated for 15 disazo groups per molecule | 2.84% S |
| Calculated for 16 disazo groups per molecule | 2.91% S |

Sixteen tyrosine groups per serum albumin molecule would give a tyrosine content of 4.3 per cent as compared with the value of 4.7 per cent found by the Folin-Marenzi method (10), indicating approximately one disazo group coupled with each tyrosine group present, just as in the case of the egg albumin dye studied (3).

In determining dye N in the precipitate in the DSa experiments, the washed specific precipitate was dissolved with a definite amount of alkali (3), made up to a known volume, and compared in a colorimeter with a DSa solution of known N content to which a corresponding amount of alkali had been added. After the readings had been made the contents of the colorimeter cup were quantitatively transferred to a micro Kjehldahl flask and analyzed for total N. Antibody N was calculated by subtracting the dye N from the total N found.

Table IX shows the result of adding increasing amounts of DSa N and Sa N to a DSa antiserum. It will be seen that Sa failed to remove all of the antibody and that more could be removed from the supernatant by DSa. Both in the unabsorbed serum and in the serum

TABLE IX

*Antibody Nitrogen Precipitated from Antidye Serum by R-Salt-Azo-Biphenyl-Azo-Serum Albumin and by Serum Albumin*

| Antigen N added | Antigen N in precipitate given by DSa | Per cent dye N precipitated | Total N precipitated | Antibody N precipitated | Ratio antibody N:DSa N in precipitate | Tests on supernatants | Antigen N in precipitate given by Sa | Total N precipitated | Antibody N precipitated | Ratio antibody N:Sa N in precipitate | Tests on supernatants | Antigen N in precipitate given by DSa | Per cent dye N precipitated | Total N precipitated | Antibody N precipitated | Ratio antibody N:DSa N in precipitate | Tests on supernatants |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *mg.* | *mg.* | | *mg.* | *mg.* | | | *mg.* | *mg.* | *mg.* | | | *mg.* | | *mg.* | *mg.* | | |
| | 3.0 ml. antiserum 4.35 to R-salt-azo-biphenyl-azo-serum albumin | | | | | | | | | | | 3.0 ml. 4.35 absorbed with Sa | | | | | |
| 0.009(8) | | | | | | | | | | | | 0.008 | 82 | 0.092 | 0.084 | 10.5 | Excess A |
| 0.019(6) | | | | | | | | | | | | 0.016 | 82 | 0.176 | 0.160 | 10.0 | No A or DSa |
| 0.027 | | | | | | | Total | 0.234 | 0.207 | 7.7 | Excess A, no Sa | | | | | | |
| 0.028 | 0.025 | 89 | 0.340 | 0.315 | 12.6 | Excess A | | | | | | | | | | | |
| 0.037 | 0.032 | 86 | 0.430 | 0.398 | 12.4 | " " | | | | | | | | | | | |
| 0.039 | | | | | | | | | | | | 0.026 | 67 | 0.252 | 0.226* | 8.7 | Excess DSa |
| 0.049 | | | | | | | | | | | | 0.033 | 67 | 0.260 | 0.227* | 6.9 | " " |
| 0.054 | | | | | | | Total | 0.372 | 0.318 | 5.9 | Excess A, trace Sa | | | | | | |
| 0.056 | 0.047 | 84 | 0.554 | 0.507 | 10.8 | Excess A | | | | | | | | | | | |
| 0.074 | 0.062 | 84 | 0.690 | 0.628 | 10.1 | " " | | | | | | | | | | | |
| 0.081 | | | | | | | | 0.434 | | | Trace A, excess Sa | | | | | | |
| 0.084 | 0.068 | 81 | 0.726† | 0.658 | 9.7 | No A or DSa | | | | | | | | | | | |
| 0.108 | | | | | | | | 0.412 | | | No A, excess Sa | | | | | | |
| 0.111 | 0.086 | 78 | 0.820 | 0.734 | 8.5 | Excess DSa | | | | | | | | | | | |

* The figures show that antibody still remained in the absorbed serum. 60 per cent of the residual antibody could be recovered by adding a portion to a mixture of DSa and unabsorbed serum 4.35, but no additional antibody was taken out by a mixture of Sa and anti-Sa.

† Single determination on 1.0 ml. serum, calculated to 3.0 ml.

absorbed with Sa the same percentage of the added dye was found in the precipitate in the region of antibody excess. No evidence of a zone with both antigen and antibody in the supernatant was observed.

Table X gives a comparison of the amount of antibody N precipitated by DSa and Sa from an anti-Sa serum. At the maximum both antigens precipitated the same amount of antibody from the serum, and Fig. 1 shows that the same curve and equation result in both cases by plotting the antibody N or the ratios of antibody N to antigen

TABLE X

*Precipitation of Anti-Serum Albumin by Dye Serum Albumin and Serum Albumin*

| Antigen N added | Antigen N precipitated (DSa N) | Per cent antigen N precipitated | Total N precipitated | Antibody N precipitated | Ratio antibody N:DSa N in precipitate | Tests on supernatants | Antigen N precipitated (Sa N) | Total N precipitated | Antibody N precipitated | Ratio antibody N:Sa N in precipitate | Tests on supernatants |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *mg.* | *mg.* | | *mg.* | *mg.* | | | *mg.* | *mg.* | *mg.* | | |
| | | | | Serum 13.28 | | | | | | | |
| | | | 1.0 ml. used with DSa | | | | | 1.0 ml. used with Sa | | | |
| 0.025 | 0.023 | 92 | 0.366 | 0.343 | 14.9 | Excess A | | | | | |
| 0.027 | | | | | | | Total | 0.478 | 0.451 | 16.7 | Excess A |
| 0.049 | 0.039 | 80 | 0.620 | 0.581 | 14.9 | Excess A | | | | | |
| 0.054 | | | | | | | Total | 0.776 | 0.722 | 13.4 | Excess A |
| 0.074 | 0.060 | 81 | 0.794 | 0.734 | 12.2 | Excess A | | | | | |
| 0.081 | | | | | | | Total | 0.946* | 0.865 | 10.7 | Excess A |
| 0.098 | 0.088 | 90 | 0.898 | 0.810 | 9.2 | No A or DSa | | | | | |
| 0.108 | | | | | | | Total | 1.046 | 0.938 | 8.7 | Trace A |
| 0.123 | 0.105 | 85 | 1.020 | 0.915 | 8.7 | Trace DSa | | | | | |
| 0.147 | 0.124 | 84 | 1.122 | 0.998 | 8.1 | Excess DSa | | | | | |
| 0.152 | | | | | | | (0.151) | 1.138 | 0.987 | | Trace Sa |

* One determination only.

N in the precipitate against the amount of antigen N in the precipitate.

## DISCUSSION

The present quantitative study of the precipitin reaction differs from the systems previously studied (1–4) in that evidence exists that the antigen, crystalline horse serum albumin, consists of several components. Although the protein has been crystallized and shown

to have a uniform molecular weight (11) Soerensen has been able to isolate a number of fractions differing in solubility (12*a*) and Hewitt has separated fractions of widely different carbohydrate content (12*b*). If the immunological properties of these fractions differ, antisera produced by injection of the serum albumin should consist of a more complex mixture of antibodies than would be produced by injection of a single antigen.

By the injection of smaller amounts of serum albumin than used by other investigators (5), it was found possible to obtain potent antisera which behaved similarly to those yielded by egg albumin (4) and thus rendered feasible a similar quantitative study.

From Table II it will be seen that the equations [1] to [4] (see introduction) which accounted quantitatively for other precipitating systems are equally applicable to the Sa system. The experimentally determined values for antibody N precipitated are in close agreement with those calculated from equation [1] up to the beginning of the equivalence zone and with values calculated from equation [4] up to the maximum. In the derivation of equations [1] and [2] it was found that the volume factors cancelled and that the composition of the precipitate depended on the proportions in which the components were mixed (1). The data given in Table I indicate that the same applies in the Sa system, subject to a solubility correction similar to that obtaining for the egg albumin system (4). Tests on reaction supernatants showed in most of the sera a wide equivalence zone in which neither Sa nor antibody was present, evidence that the Sa was immunologically homogeneous.

From the above data the reaction between Sa and antibody may also be considered to take place in a series of bimolecular competing reactions between multivalent antigen and antibody before precipitation begins. The first step in the reaction would be

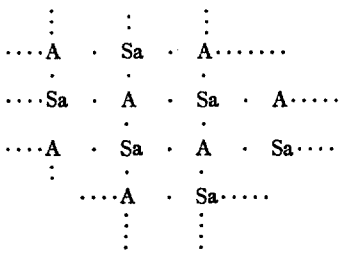
$$A + Sa \rightleftharpoons A \cdot Sa$$

in which A and Sa represent antibody and serum albumin molecules, respectively. This would be followed, for example, in the region of excess antibody by

$$A \cdot Sa + A \rightleftharpoons ASa \cdot A \quad \text{and}$$

$$A \cdot Sa + A \cdot Sa \rightleftharpoons ASa \cdot ASa$$

This process would continue, leading to the formation of larger and larger aggregates until these finally precipitated from solution. Such aggregates might be represented two-dimensionally as follows:




and would resemble those pictured by Marrack (13). It is possible that the insolubility of the precipitate is conditioned not only by the size of the ultimate aggregates but also by a reduction in affinity for water due to a juxtaposition of oppositely charged ionized groupings.

Since inhibition begins with a relatively slight excess of Sa, it was not possible to apply the equations derived for the maximum precipitation zone in the Type III specific polysaccharide-antibody system (1, 2). An attempt to calculate the composition of the precipitate in the immediately succeeding inhibition zone is given in Table III. The method was not applicable near the region of complete inhibition since the weak antisera available necessitated the use of small aliquot fractions in this region, introducing large errors. In one serum, 3.12, the composition of the precipitate remained fairly constant over the region studied, but in another serum a considerable variation in composition was observed, the proportion of Sa increasing with increasing additions of Sa.

The serial experiments shown in Table IV offer further evidence for the presence of only a single antigenic component, since Sa appears in the supernatant only after all the antibody precipitable under these conditions has been removed. In serum $3.69_3$, 7 per cent of the antibody was found to be non-precipitable by serial additions of Sa. As in the egg albumin system (4) much of this could be recovered by adding the supernatant to a mixture of antiserum and Sa and determining the additional antibody N precipitated over that given by the Sa and serum alone. A recovery of 97.6 per cent of the total antibody

N could be obtained in this way. The serially non-precipitable antibody may be considered to have a different reactivity from the rest of the antibody, perhaps, as postulated in (4), containing only a single immunologically reactive grouping per molecule. If this were true such antibody could not build up large aggregates by chemical interaction but could attach itself to free Sa groupings on large aggregates already in process of formation. In the serial experiments the Danysz phenomenon is quite definite, as noted in Table IV, and as is required by the theory (*cf.*, for example, (14)).

Table V shows that removal of a portion of the antibody results in a different equation for the residual serum than shown by the original at the same antibody content. This may be taken as additional evidence that antibody to a single substance consists of a mixture of antibodies of different reactivities (*cf.*, for example, (4)).

Table VI indicates the variation in the antibody N:Sa N ratios at either end of the equivalence zone in the sera studied. The values calculated for R and R″ in equations [1] and [3] are seen to lie in the equivalence zone. The data show considerable variation in the extent of the equivalence zone in different sera and in different bleedings of the same rabbit, indicating that the zone is characterized by no constant ratio and that no point within the zone would properly be called the equivalence point. A more detailed discussion is given in (4).

Unlike the sera studied in this communication, the anti-Sa sera of Taylor, Adair, and Adair (5) exhibited a marked zone in which both antigen and antibody appeared in the supernatant. This would indicate that the material injected was a mixture of different antigens and that the sera contained a complex mixture of antibodies. The finding of Goldsworthy and Rudd (6) that recrystallized serum albumin contained appreciable amounts of globulin offered an interpretation of these results, for it was possible to show that the serum of a rabbit which had received 229 mg. of crystalline horse serum albumin contained both antialbumin and antiglobulin. In this serum (Table VII) addition of excess Sa (as determined by a test on the supernatant with antibody) left in the supernatant antibody which reacted with serum globulin as well as with Sa (which presumably contained a small amount of globulin). Similarly, addition of excess

globulin left in the supernatant antibody which could be precipitated with either Sa or globulin (which presumably contained a small amount of Sa). The marked zone in which both antigen and antibody appeared in the supernatant is evident from Table VII and resembles the zones observed by Taylor, Adair, and Adair (5). An earlier sample of serum from the same rabbit, after injection of only 41 mg. of Sa, contained only antialbumin and showed an equivalence zone in which neither component appeared in the supernatant. Since Taylor, Adair, and Adair injected about 180 mg. of Sa for each course (15), it is probable that sufficient globulin was injected as impurity to stimulate marked antibody formation.

Table VIII indicates a failure to detect any anamnestic rise in the antibody content of the sera of two resting rabbits injected with heterologous antigens, although in the one instance tested a single injection of the homologous antigen produced a definite increase in antibody content. A similar lack of anamnestic effect has been observed in a rabbit with circulating anti-egg albumin (4).

Table IX summarizes the results obtained with an antiserum to R-salt-azo-biphenyl-azo-crystalline serum albumin (DSa). It will be noted that in the region of excess antibody the percentage of dye N precipitated to dye N added was constant within experimental error. With larger amounts of DSa a sharp equivalence zone appeared, characterized by negative tests with anti-Sa serum, as well. DSa may therefore be considered as a single immunological entity although antisera to DSa contain a mixture of antibodies, as do antisera to other single antigens. Crystalline serum albumin failed to remove all of the antibody N present in the anti-DSa serum and more could be taken out of the supernatant with DSa. The same percentage of DSa reacted in the region of antibody excess with the unabsorbed serum as with the serum absorbed with Sa. After the maximum amount of antibody was removed from the antidye serum first with Sa and then with DSa, a residual portion was still present. This could be removed by a mixture of DSa and unabsorbed DSa antiserum, but was not taken out by an Sa-anti-Sa specific precipitate, indicating that the residual antibody contained antidye groupings rather than anti-Sa groupings.

It is apparent from Table X and Fig. 1 that the same serological

reaction takes place in anti-Sa serum whether or not the Sa molecule is coupled with the dye. Not only do both DSa and Sa remove all of the antibody from an anti-Sa serum, but the same curve or equation results in each case when antibody N or antibody N:antigen N ratios

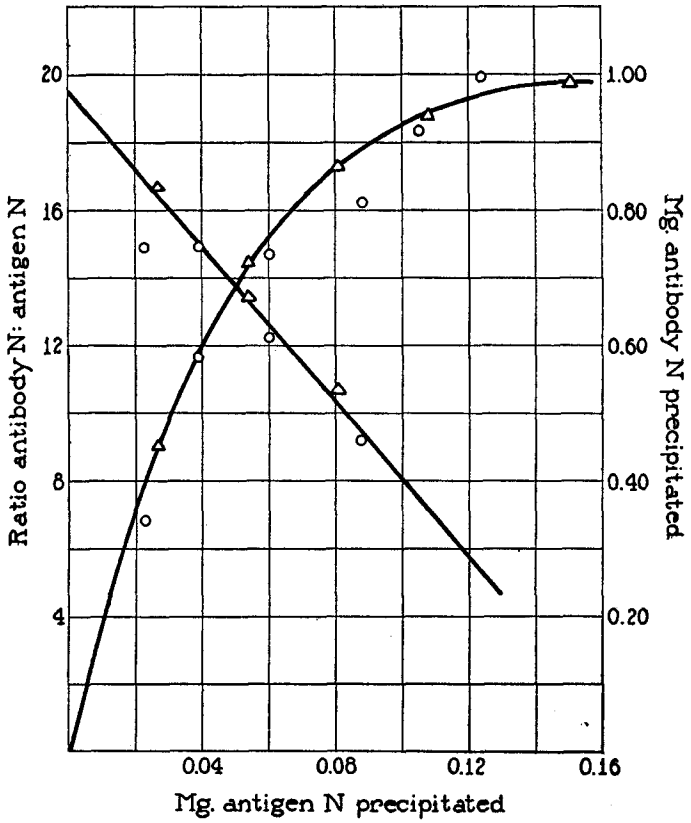



FIG. 1. Precipitation of anti-Sa by DSa and Sa.
○, DSa-anti-Sa reaction.
△, Sa-anti-Sa reaction.

are plotted against the amount of antigen in the precipitate. Similar results were obtained with another anti-Sa serum. That the reactivity with anti-Sa is not entirely due to a possible admixture with Sa is indicated by these data, by the results in anti-DSa serum, and by the behavior of the dye on purification. After removal of all antibody

reactive with Sa from the anti-DSa serum, 82 per cent of the DSa added to the remaining antibody was precipitated, indicating that even if unchanged Sa were assumed to be present in the dye, the amount could not exceed 20 per cent. Actually it must have been much less since part, at least, of the non-precipitable N in the dye was derived from highly colored azo compounds.

Although coupled with the same azo component, the serum albumin dye thus differs markedly in its antigenic properties from the corresponding egg albumin dye (3, 7, 16). While the latter could be fractionated so that it no longer reacted with most anti-egg albumin sera, antisera to the dye exhibited a peculiar cross reactivity with egg albumin (Ea). This reactivity was quantitatively charted (16) and shown to be of a totally different type from either the homologous Ea-anti-Ea reaction or the dye-antidye reaction. In the case of the DSa, however, anti-Sa reacts quantitatively identically with both DSa and Sa, whereas only a fraction of the anti-DSa reacts with Sa. In both dye proteins the number of disazo groups introduced corresponded roughly to the number of tyrosine groups in the antigen, so that the hapten was probably attached to the protein through these groups (*cf.* Landsteiner (17); (14)), or through histidine. Whatever the points of combination, it is evident that these groupings are only very slightly concerned with the serological specificity of Sa, since their modification produces so slight a change as to be undetectable in anti-Sa serum, even by the sensitive quantitative methods used. The serological specificity of Sa would therefore seem to be determined by groupings of amino acids other than tyrosine and perhaps histidine, while that of Ea would appear to involve molecular groupings including these two amino acids. The structural patterns of the two proteins therefore seem to differ very widely.

Although antisera to the DSa and DEa contained antibodies characteristic of the altered chemical structure, these sera gave only traces of precipitate when tested with the heterologous dye antigen, nor did they show inhibition with R-salt. While such sera have been encountered by Landsteiner (17) they have not hitherto been studied.

With the data now available it is possible to make rough calculations of the equivalent composition of the specific precipitates throughout the reaction range. If one accepts the value of the molecular

weight of Sa as 67,000 (11) and that of antibody (A) in the rabbit as roughly 150,000 (18) the ratio of the weight of A to that of Sa is 2.24. This may also be taken as the ratio of A N to Sa N, since the percentage of N in the two substances is probably not very different.

In the region of extreme antibody excess the average value of 2R, the maximum calculated A N:Sa N ratio, was 13.5 for nine sera. Dividing this by the A:Sa molecular weight ratio, 2.24, gives 6, indicating the equivalent composition of the precipitate at the extreme antibody excess end of the reaction range to be roughly $SaA_6$. At the antibody excess end of the equivalence zone the mean A N:Sa N ratio was 8.7, corresponding roughly to the equivalent composition $SaA_4$. At the antigen excess end of the zone, with the ratio 6.5, the composition would be roughly $SaA_3$. The specific precipitate in the inhibition zone would approximate the empirical composition $SaA_2$ and the soluble compound or compounds in the inhibition zone could then probably be represented by SaA (*cf.* 19). There would thus be a sixfold range of combining proportions possible between Sa and rabbit antibody.

The above empirical formulas are not offered in the sense that compounds of definite composition are indicated. They are merely approximations of the equivalent composition of the specific precipitate at definite points or regions in the reaction range. The maximum equivalent combining ratio, 6:1, would appear sufficiently small to justify the classical chemical treatment given, even though the formulas derived are not necessarily those of single chemical individuals.

## SUMMARY

1. The reaction between crystalline horse serum albumin and homologous antibody in rabbit sera is quantitatively accounted for by expressions similar to those derived from the law of mass action for other immune precipitating systems.

2. The reaction of an azo dye prepared from crystalline serum albumin by coupling with diazotized R-salt-azo benzidine was also studied with homologous antibody and anti-serum albumin.

3. Quantitative data obtained on cross reactions with the two antigens differ markedly from data on the corresponding reactions in the egg albumin system and indicate that tyrosine and perhaps histidine,

while important in determining the serological specificity of egg albumin, have little connection with the specificity of serum albumin.

4. Calculations are made of the equivalent composition of the specific precipitate at various reference points in the reaction range.

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