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The Serological Properties of Simple Substances. III. The Composition of Precipitates of Antibodies and Polyhaptenic Simple Substances; the Valence of Antibodies

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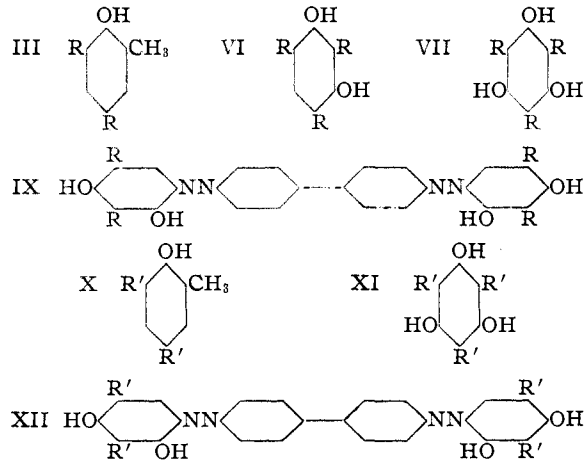
The antibody–antigen mole ratio of precipitates formed by interaction of antigens and homologous antisera provides information about the relative combining powers or "valences" of antigen and antibody molecules. For protein antigens it has been found by Heidelberger and co-workers[1] and by other investigators[2] that the antibody–antigen mole ratio is greater than unity, and increases from the antigen-excess region (where the values approach unity) through the equivalence zone to the antibody-excess region, the values for the last region being nearly twice those for the equivalence zone. These results, which show that the effective valence of the protein antigen molecules is greater than that of the antibody molecules, have been accounted for in a reasonable way by the framework theory and the postulate that antibodies which act as precipitins are bivalent.[3] Since the mole ratio gives only the relative valences of antigen and antibody, and information about the valence of protein antigens is in general not at hand, these data do not directly provide information about the valence of the antibodies; but similar data for simple antigens of known structure should give this information. In this paper, the third in a series of studies of the serological properties of simple substances,[4] we present and discuss the results of about 400 analyses of precipitates formed by antibodies and polyhaptenic substances.

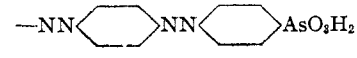
Experimental Methods.—The preparation of antigens and antisera and the techniques of precipitation and analysis have been described in the first paper of this series.

A series of tests was made to determine the extent to which the dyes used in the investigation might be non-specifically carried down with the specific precipitates. In each test 1 ml. of dye solution in borate buffer solution of *p*H 8 was added to 1 ml. of buffer solution containing 1 mg. ovalbumin; 1 ml. of antiovalbumin rabbit serum was then added, the tube was allowed to stand one hour at room temperature and overnight in the refrigerator, and the precipitate was then centrifuged down, washed three times with 10-ml. portions of saline solution, and analyzed for dye and protein. Six tests, with amount of dye increasing by threefold steps from 4 to 1000 μg., were made with each of the dyes III, VI, IX, X, XI, and XII. The amount of dye found in the precipitate was about 3 μg., exceeding this value only for the two largest amounts of added dye (1000 and 333 μg.). Since the amount of precipitated antibody in each test, about 6 mg., corresponds for specific precipitation to about 30 to 70 μg. of dye, a possible error of about 4 to 10% because of non-specific coprecipitation of dye is indicated.

Values of the Antibody-antigen Mole Ratio for Simple Antigens.—Many experiments were carried out in which equal volumes of antiserum were mixed with antigen solutions containing varying amounts of the dye. The following seven dyes were used



with R the short haptenic group —NN⟨⟩AsO_3H_2 and R′ the longer group



In most of the experiments no difference in

(1) M. Heidelberger, THIS JOURNAL, **60**, 242 (1938).

(2) See the summary of results given by J. R. Marrack, "The Chemistry of Antigens and Antibodies," His Majesty's Stationery Office, London, 1938, p. 161.

(3) L. Pauling, THIS JOURNAL, **62**, 2643 (1940).

(4) L. Pauling, *et al.*, *ibid.*, **64**, 2994 and 3003 (1942).

composition was found to within the estimated reliability of the results (about ±10%) for precipitates obtained in the entire range from antigen excess to antibody excess. Data for three series of tests are represented in Fig. 1. It is seen that in these three series, two of which cover the range from antigen excess (at the right) to antibody excess, there is evidenced no significant trend of the mole ratio.

In Tables I to VI there are given the averaged values of the mole ratio for the forty-three series of tests carried out, with a description of the conditions for each series. The mean deviation of the ratios from the average for each series is also given. The average of all tests for each antigen and the mean deviation from the average are shown at the bottom of the corresponding table. The mean deviations give an indication of the probable reliability of the results.

In three series the hydrogen-ion concentration was varied from about *p*H 7.8 to *p*H 9.2 for the supernate; no significant correlation with the mole ratio was detected, and the values were averaged for inclusion in the tables.

The mole ratio values given in Tables IV, V, and VI show especially pronounced variation. We have not detected any reliable correlation with the changed test conditions, nor found any explanation of the variation.

In a few series, notably the two for antigen IX, a pronounced decrease of the mole ratio with decrease in the amount of added antigen was found. This behavior, which is the opposite to that shown by protein antigens, is hard to understand; and we plan to study it further, checking especially the possibility of some so far undetected source of error.

Discussion.—The most extensive and consistent set of analyses, that for the trihaptenic antigen VI (Table I), gave the average value 0.85 ± 0.11 for the antibody-antigen mole ratio. The same average, 0.85, is obtained by adding to the 186 analyses for this antigen the 53 analyses made for the other trihaptenic antigens XI and VII (Tables II and III).

The interpretation of this number requires an assumption as to the number of haptenic groups of the dye which are effective in forming bonds with antibody molecules. If all three of the haptenic groups were thus effective, the valence of the antibody would be given by the observed ratio as 3/0.85 = 3.5. But the observations indicate

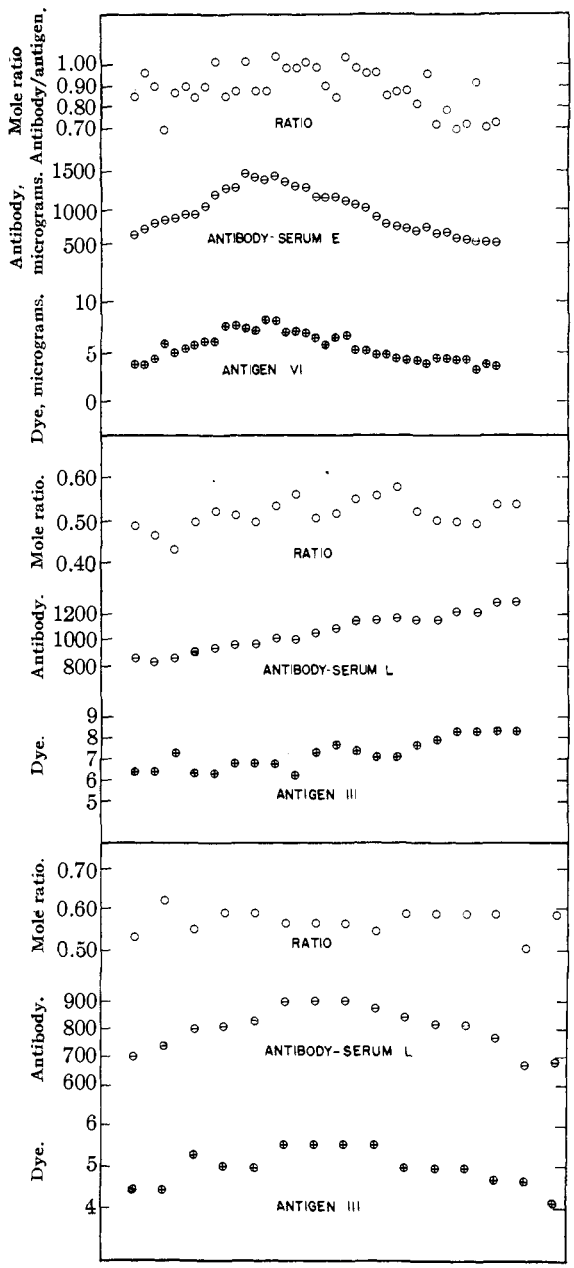


Fig. 1.—Values of (from bottom up) amount of antigen (micrograms) in precipitate, amount of antibody (micrograms) in precipitate, and antibody-antigen mole ratio. The three series of tests correspond to line 1 of Table I (top), line 6 of Table IV (middle), and line 7 of Table IV (bottom). The horizontal scale is the logarithm of the amount of antigen used, with the maximum at the left.

in several ways that the haptenic groups are not all effective. First, the average ratio from the 119 analyses for the dihaptenic dyes is 0.75, and that from the 44 analyses for the tetrahaptenic dyes is 0.83; and these numbers differ from the value for the trihaptenic dyes by much less than

TABLE I

ANTIBODY-ANTIGEN MOLE RATIO FOR TRIHAPTENIC ANTIGEN VI

Antiserum	Antigen solution[a]	Buffer	pH of supernate	Number of analyses	Mole ratio antibody/antigen
E, 3 ml.	3 ml., 75 to 13 μg.		8.1–8.2	37	0.88 ± 0.10
E, 3	3 ml., 110 to 14		8.2	25	.94 ± .09
H, 10	5 ml., 167 to 10	10 ml., pH 9.0	8.8	8	.88 ± .07
H, 7	5 ml., 75	7 ml., 7.0 to 10.0	7.8 to 9.2	7	.88 ± .06
H, 10	5 ml., 165 to 15	10 ml., 8.5	8.4	7	.79 ± .08
F, 7.5	7 ml., 105 to 21	20 ml., 9.0	8.8	4	.96 ± .01
F, 7.5	14 ml., 100 to 20	20 ml., 9.0	8.8	4	.93 ± .02
C, 2	2 ml., 100 to 12.5		8.4	8	.71 ± .08
C, 3[b]	3 ml., 75		8.1	24	.90 ± .05
E, 2[b]	2 ml., 50		8.1	5	.87 ± .07
E, 3[c]	3 ml., 38		8.0–8.3	14	.84 ± .04
E, 3[d]	3 ml., 75 to 22	3 ml., 7.0	7.6–7.7	8	.70 ± .04
E, 3[d]	3 ml., 75 to 22	3 ml. saline	8.1–8.2	7	.90 ± .07
E, 3[d]	3 ml., 75 to 22	3 ml., 8.0	8.1	8	.69 ± .05
E, 3[d]	3 ml., 75 to 22	3 ml., 9.0	8.8–8.9	8	.61 ± .06
R, 5	5 ml., 167 to 22		8.0	12	.71 ± .08
			Average of 186 analyses		**0.85 ± 0.11**

[a] In this and succeeding tables there are shown in this column the volume of solution and the amount of antigen contained in it. The notation "75 to 13 μg." in the first row, for example, indicates that these are the limiting values of the sequence of 37 differing by a constant factor (in this case 1/1.05). Unless otherwise indicated, the antigen solutions were made by dissolving the antigen in saline solution with sodium carbonate added to bring the pH to about 8.5. [b] From Table I of paper II. [c] From Table II of paper II. [d] From Table V of paper II.

TABLE II

ANTIBODY-ANTIGEN MOLE RATIO FOR TRIHAPTENIC ANTIGEN XI

Antiserum	Antigen solution	Buffer	pH of supernate	Number of analyses	Mole ratio antibody/antigen
J, 10 ml.	10 ml., 200 to 7.4 μg.	10 ml., pH 9.0	8.9	8	0.97 ± 0.05
J, 10	10 ml., 222 to 13	10, 9.0	8.9	9	.85 ± .13
J, 10	10 ml., 149 to 45	10, 9.0	8.9	4	.84 ± .09
J, 10	10 ml., 200 or 149	10, 7.0 to 10.0	7.7 to 9.4	8	.95 ± .10
C, 1	1 ml., 200 to 6.25		8.5	12	.87 ± .09
			Average of 41 analyses		**0.90 ± 0.07**

TABLE III

ANTIBODY-ANTIGEN MOLE RATIO FOR TRIHAPTENIC ANTIGEN VII

Antiserum	Antigen solution	Buffer	pH of supernate	Number of analyses	Mole ratio antibody/antigen
K, 3 ml.	3 ml., 67 to 6 μg.		7.8	6	0.72 ± 0.14
K, 3	3 ml., 67 to 9	3 ml., pH 9.2	8.8	6	.69 ± .11
			Average of 12 analyses		**0.71 ± 0.13**

TABLE IV

ANTIBODY-ANTIGEN MOLE RATIO FOR DIHAPTENIC ANTIGEN III

Antiserum	Antigen solution	Buffer	pH of supernate	Number of analyses	Mole ratio antibody/antigen
H, 20 ml.	10 ml., 360 to 105 μg.	20 ml., pH 8.5	8.5	4	0.99 ± 0.10
H, 10	5 ml., 167 to 33	10 ml., 9.0	8.8	5	1.19 ± .12
H, 10	10 ml., 222	10 ml., 7.0–9.5	7.7–9.1	5	0.85 ± .10
K, 5	5 ml., 67 to 11		7.9	11	.76 ± .04
K, 3	3 ml., 67 to 11[a]		8.8	11	.58 ± .05
L, 5	5 ml., 167 to 65		7.9	20	.51 ± .03
L, 5	5 ml., 167 to 65[a]		8.8	15	.57 ± .03
L, 5	10 ml., 125 and 55		7.9	2	.69 ± .01
L, 5	10 ml., 125 to 55[a]		8.8	3	.73 ± .02
M, 10	10 ml., 220 to 46		8.2	8	1.18 ± .16
N, 10	10 ml., 167	10 ml., 8.0	7.9	3	0.71 ± .02
O, 15	15 ml., 250	15 ml., 8.0	7.9	3	.60 ± .08
P, 15	15 ml., 375	15 ml., 8.0	7.9	2	.81 ± .08
Q, 5	5 ml., 158	5 ml., 8.0	7.9	3	.73 ± .00
			Average of 95 analyses		**0.73 ± 0.17**

[a] Antigen dissolved in buffer, pH 9.2.

TABLE V

ANTIBODY-ANTIGEN MOLE RATIO FOR DIHAPTENIC ANTIGEN X

Antiserum	Antigen solution	Buffer	*p*H of supernate	Number of analyses	Mole ratio antibody/antigen
F, 2.5 ml.[a]	5 ml., 100 μg.	7.5 ml., *p*H 9.0		17	0.93 ± 0.08
O, 2	2 ml., 200 to 19		9.0	7	.54 ± .09
			Average of 24 analyses		**0.82 ± 0.16**

[a] From Table III of paper II.

TABLE VI

ANTIBODY-ANTIGEN MOLE RATIO FOR TETRAHAPTENIC ANTIGENS IX AND XII

Antiserum	Antigen solution	*p*H of supernate	Number of analyses	Mole ratio antibody/antigen
C, 2 ml.	2 ml., 100 to 12.5 μg. antigen IX	8.4	8	1.20 ± 0.15
R, 5	5 ml., 167 to 22 μg. antigen IX	8.0	12	0.98 ± .25
C, 2	2 ml., 200 to 6.25 μg. antigen XII		12	.87 ± .06
R, 5	5 ml., 167 to 22 μg. antigen XII	8.0	12	.40 ± .06
		Average of 44 analyses		**0.83 ± 0.25**

the $33^1/_3\%$ which could be expected if all of the haptenic groups were effective, the antibody valence remaining constant. Second, if all three haptenic groups of a trihaptenic dye were able to form bonds with different antibody molecules a pronounced change in composition of the precipitate with change of the total antibody-antigen ratio for the system would be expected, and this is not observed. For bivalent antibody and trivalent antigen the expected values of the antibody-antigen ratio are 1.0 for antigen excess, 1.5 for the equivalence zone, and 2.0 for antibody excess.[3] Such change in composition is observed for protein antigens; but it did not appear, to within the estimated probable error of the work, in any of our series (illustrated by that for antigen VI shown in Fig. 1).

It is, moreover, not unreasonable that the number of antibody molecules to which a small antigen molecule can be bonded should be limited. If, as has been postulated,[3] the bond between dye and antibody is formed by the insertion of the haptenic group into a complementary cavity in the antibody molecule (Fig. 2), then it might well occur that two antibody molecules attached to a dye molecule would by steric interference prevent others from attaching themselves to the other haptenic groups of the dye molecule. If this were to hold for every dye molecule the antibody-antigen ratio for bivalent antibodies would be 1.

Even with the minimum effective antigen valence, 2, compatible with the framework theory, the valence of the antibody molecules must be taken greater than 2 to account for the observed values less than unity for the mole ratio. The possibility that dye not specifically bonded to tested as described above, and it was found that about 3 μg. of dye per 6000 μg. of precipitated antibody was carried down by ovalbumin-antiovalbumin precipitates. If a corresponding 10% correction were made, the antibody-antigen ratios (for specifically precipitated dye) would become 0.83, 0.93 and 0.91, which are still less than unity. It is, of course, possible that the non-specific precipitation of dye is somewhat greater, but it seems more probable that the extra dye molecules are held by specific hapten-antibody bonds, as discussed below.

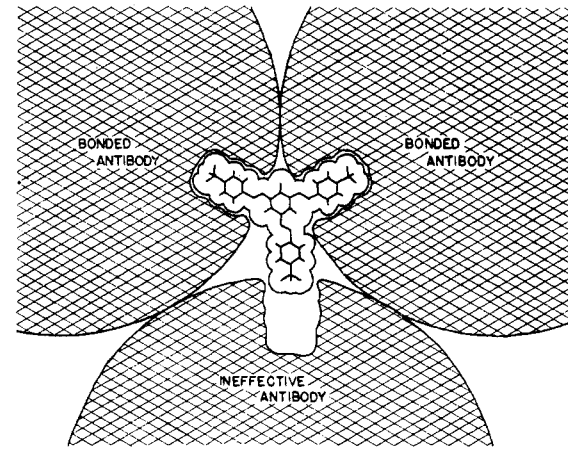


Fig. 2.—Scale drawing showing how steric interference of antibody molecules might prevent a trihaptenic dye from combining with more than two antibody molecules. The radius of curvature of the antibody molecules as drawn is 30 Å.

The calculation of the antibody valence on the basis of this assumption is not straightforward. If each dye molecule were to use two haptenic groups in bond formation the antibody valence

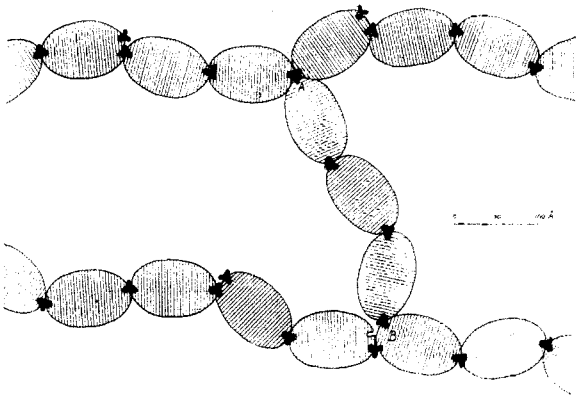


Fig. 3.—Scale drawing representing the structure of the precipitate formed by antibody and an antigen such as VI, $C_6H(OH)_2(NNC_6H_4AsO_3H_2)_3$, as indicated by the experimental results. Most of the antibody molecules are bivalent, and most of the antigen molecules use only two of their three haptenic groups for forming bonds with antibodies. Two long chains are shown, and a short connecting chain. This is attached at A to an antigen molecule bonded to three antibody molecules—a structural feature expected to be rare because of steric interference of the antibody molecules. The bond at B is to a trivalent antibody. There are four other trivalent antibodies, three with extra antigens attached, leading to the antibody-antigen ratio 0.85.

the precipitates with dihaptenic dyes, 2/0.85 = 2.3 for those with trihaptenic dyes, and 2/0.83 = 2.4 for those with tetrahaptenic dyes. If, on the other hand, extra dye molecules were held by a single bond to dye-antibody chains the increased antibody valence over 2 would need to be only one-half as great, and the antibody valence would be calculated to be 2.3, 2.2, and 2.2, respectively.

It seems not unlikely that some antibodies have more than two regions complementary to the haptenic group. Even if, as has been postulated,[3] only two parts of an antibody molecule assume configurations complementary to a portion of the surface of the immunizing antigen, this portion might include, in the case of an azoprotein, two or more haptenic groups, so that the resultant antibody molecule would be able to interact with three haptenic groups or more.

The picture of the antibody-dye precipitates indicated by our data is thus the following. Only two of the haptenic groups of a dye are ordinarily effective in reacting with antibody, others being presumably inhibited by steric interference of the attached antibody molecules. The effective valence of the antibody molecules is also usually 2, but some of these molecules, with three or more hapten-complementary regions, form bonds with additional dye molecules, causing the antibody-antigen mole ratio to fall below unity. Figure 3 illustrates various aspects of this picture.

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Summary

Many analyses of precipitates between dyes containing azophenylarsonic acid groups and hapten-homologous antisera have been made, leading to the average antibody-antigen mole ratios 0.75 for dihaptenic dyes, 0.85 for trihaptenic dyes, and 0.83 for tetrahaptenic dyes. The approximate equality of these values is interpreted as resulting from the limitation to 2 of the effective valence of polyhaptenic dyes by the steric interference of attached antibody molecules, and the approximation of the values to unity is taken to indicate bivalence of most of the antibody molecules.

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