molten at 155° for one minute, dissolved in 5 ml. of benzene, diluted with 5 vol. of petroleum ether and chromatographed (18 × 2.5 cm.).

7 yellow, irreversible
40 colorless
37 orange-red, natural methylbixin: 485, 454 mμ
4 pink, all-*trans:* 489, 457.5 mμ
4 orange, neo A: 485.5, 452 mμ
10 yellow-orange, neo B: 471, 444 mμ
2 almost colorless
50 orange, unchanged neo C: 480, 448.5 mμ
Filtrate: yellow (irreversible)

The colorimetric ratio was, natural: all-*trans:* neo A: neo B: unchanged neo C = 51:4:4:5:36.

(c) ***cis–trans* Isomerization of Neomethylbixin C by Iodine Catalysis at Room Temperature.**—Three milligrams of pigment was catalyzed in benzene with 20 μg. of iodine. After standing for thirty minutes the solution was developed with benzene–petroleum ether (1:5) on a column (18 × 1.9 cm.).

20 colorless
49 orange-red, all-*trans:* 489, 456.5 mμ
20 orange, neo A: 484, 452.5 mμ
2 yellow
23 yellow-orange, unchanged neo C: 478, 448 mμ

The colorimetric ratio was, all-*trans:* neo A: unchanged neo C = 63:25:12.

(d) **Photochemical *cis–trans* Isomerization of Neomethylbixin C.**—A solution of 2 mg. of pigment in 3 ml. of benzene was insolated for fifteen minutes and after dilution with 10 ml. of petroleum ether, chromatographed (18 × 1.9 cm.).

23 colorless
15 orange-red, natural methylbixin: 485, 453.5 mμ
11 colorless
4 pink, neo A: 484.5, 453 mμ
3 colorless
65 yellow-orange, unchanged neo C: 478.5, 448 mμ

The colorimetric ratio was, natural: neo A: unchanged neo C = 21:2:77.

Summary

Besides "labile methylbixin," now named "natural methylbixin," and "stable" termed "all-*trans*-methylbixin," two other stereoisomers, neo A and C, have been isolated in crystals; several minor members of this set were observed in solution. The mutual conversion of the stereoisomers can be carried out by means of thermal methods, iodine catalysis or insolation. Natural and all-*trans*-methylbixin are practically not interconvertible by refluxing or exposure to sunshine. In contrast, the reversible formation of neo C from natural methylbixin and of neo A from the all-*trans* form takes place easily under similar conditions. On the basis of spectroscopic data, especially "*cis*-peak" measurements, configurations are suggested for the four main observed methylbixins.

PASADENA, CALIFORNIA RECEIVED OCTOBER 25, 1943

[CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, No. 947]

The Serological Properties of Simple Substances. VI. The Precipitation of a Mixture of Two Specific Antisera by a Dihaptenic Substance Containing the Two Corresponding Haptenic Groups; Evidence for the Framework Theory of Serological Precipitation

BY LINUS PAULING, DAVID PRESSMAN AND DAN H. CAMPBELL

The framework theory (lattice theory) of serological precipitation and agglutination, first proposed by Marrack,[1] was shown by Marrack and by Heidelberger and Kendall[2] to account for many experimental observations. Because of its simplicity and its compatibility with the available information about intermolecular forces, this theory was incorporated in his general theory of the structure and process of formation of antibodies by one of the present authors.[3]

Strong support of the framework theory has been provided during the past two years by the results of extensive studies of the reactions of antibodies and simple substances,[4] based upon the observations by Landsteiner and Van der Scheer[5] of the precipitation of antibody by certain simple substances containing two haptenic groups. It was found[4] from experiments with about fifty substances that all of those (about twenty) containing two or more haptenic groups (azophenylarsonic acid groups) per molecule gave precipitates with antiserum homologous to this haptenic group, and that none of the monohaptenic substances gave a precipitate. This fact is most readily accounted for by the framework theory.

The argument might be made, however, that no more than one of the haptenic groups of a molecule of a polyhaptenic substance is involved in interaction with antibody molecules, and that the difference in precipitability of polyhaptenic and monohaptenic substances with antiserum is due to some difference in properties of these two classes of substances, such as a tendency to asso-

(1) J. R. Marrack, "The Chemistry of Antigens and Antibodies," Report No. 194 of the Medical Research Council, His Majesty's Stationery Office, London, 1934; Second Edition, Report No. 230, 1938.

(2) M. Heidelberger and F. E. Kendall, *J. Exptl. Med.*, **61**, 559, 563; **62**, 467, 697 (1935); M. Heidelberger, *Chem. Rev.*, **24**, 323 (1939).

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

(4) Linus Pauling, David Pressman, Dan H. Campbell, and collaborators, THIS JOURNAL, **64**, 2994, 3003, 3010, 3015 (1942); **65**, 728 (1943).

(5) K. Landsteiner and J. Van der Scheer, *Proc. Soc. Exptl. Biol. Med.*, **29**, 747 (1932).

ciate into colloidal particles.[6] In order to test this point, we have carried out a new experiment, the results of which show that each of the two haptenic groups of a dihaptenic substance enters into specific combination with antibody in the formation of the precipitate.

The experiment[7] was made with use of two different antisera, one of which (anti-R serum) was prepared by injecting rabbits with azoprotein containing R groups (*p*-azophenylarsonic acid groups) and the other (anti-X serum) by injecting with azoprotein containing X groups (*p*-azobenzoic acid groups). A substance was used as precipitating antigen which had one R group and one X group per molecule. It was found that this RX substance does not give a precipitate with either anti-R serum or anti-X serum alone, but does give a precipitate with a mixture of these two antisera. Similar results were obtained also with another RX substance.

This striking experimental result corresponds exactly to the behavior predicted by the framework theory: according to this theory the RX substance cannot give a precipitate with anti-R serum because its molecules contain only one R group, and are hence univalent with respect to anti-R antibodies and so are unable to form a framework with them; and similarly it can not give a precipitate with anti-X serum. (Indeed, it is predicted, and verified by experiment, that this effectively monohaptenic substance can inhibit the precipitation of either anti-R serum or anti-X serum by corresponding polyhaptenic simple substances or azoproteins.) But the RX substance is effectively dihaptenic and bivalent with respect to a mixture of anti-R serum and anti-X serum, since each molecule can form two bonds, one with an anti-R antibody molecule and one with an anti-X antibody molecule; and accordingly a specific framework precipitate, containing equal numbers of the two kinds of antibodies, can be formed with the mixed antiserum.

Let us now ask to what extent this experimental result supports the framework theory—whether it might not be equally compatible with some other theory. The answer is that our experiment shows that both of the two haptenic groups R and X of the RX molecule enter into specific combination with antibody, that the molecule of precipitating antigen is hence truly bivalent, and that this bivalence is necessary for precipitation. This, however, is common to two theories—the framework theory, which requires that the antibody molecules, as well as the antigen molecules, be bivalent or multivalent, and an alternative theory, which is based on the assumption that a complex of a multivalent antigen molecule and two or more univalent antibody molecules is for some reason or other insoluble, and constitutes the precipitate.[8]

Basis for decision between these theories is provided by the results of two other experiments, one relating to the antibody–antigen molecular ratio in the precipitate and the other to the solubility of the precipitate in excess antibody. Since we know that the RX precipitating antigen is truly bivalent, the effective valence of the antibody molecules can be calculated from the antibody–antigen molecular ratio. The value found by analysis for this ratio is 0.7, which corresponds to an average effective antibody valence of $2/0.7 = 2.8$; for univalent antibody the molecular ratio would be 2. (The assumption is made here that the antigen molecules are not associated into complexes, which might then form a precipitate with antibody with use of only some of the haptenic groups. Evidence against association of this sort is furnished by the failure of the RX substances to precipitate with anti-R serum or anti-X serum alone.)

The second experiment is based on the following argument. If antibody molecules are univalent, and the precipitate consists of RX antigen molecules each of which has two attached antibody molecules, increase in antibody concentration, with amount of antigen held constant, would necessarily increase the amount of precipitate; decrease in the amount of precipitate, which could occur only by formation of a soluble complex, would not occur because the antigen molecules in the precipitate would be already saturated with antibody, and so could not increase their valence, and the antibody molecules could not decrease their valence (below the value 1) and remain attached to antigen. But a framework precipitate with multivalent antibody could dissolve in excess antibody by formation of soluble complexes, the effective valence of the antibody molecules in these complexes being less than that in the precipitate (1 instead of 2 or 3), and the antibody–antigen ratio being greater than that for the precipitate; according to the principles of chemical equilibrium, increase in the antibody concentration would then lead to solution of the precipitate. The observation that increase in the amount of mixed antiserum results in pronounced decrease in the amount of precipitate formed with the RX substance accordingly eliminates the theory of univalent antibody and provides further proof of the framework theory.

A detailed discussion of these experiments and of other experiments with the RX substance (hapten inhibition, etc.) is given below, following the section on experimental methods and results.

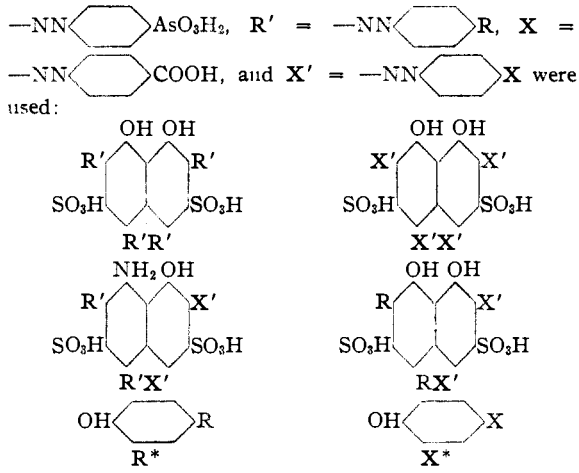
Experimental Methods and Results

The following substances containing groups R =

(6) K. Landsteiner, "The Specificity of Serological Reactions," Charles C. Thomas, Baltimore, Md., 1936, p. 120.

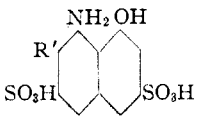
(7) A brief statement about this work has been published in *Science*, **98**, 263 (1943).

(8) The "occlusion" theory proposed by W. C. Boyd, *J. Exptl. Med.*, **75**, 407 (1942), is a theory of this sort. See also F. Haurowitz and P. Schwerin, *British J. Exptl. Path.*, **23**, 146 (1942).



The substances R'X' and RX' are the "RX substances" referred to in the preceding discussion.

Preparation.—The substances R'R' and R* have already been described,[4] and X'X' and X* are described in a paper to be published soon.[9] The substance R'X' was prepared by diazotizing 1.3 g. (0.0038 mole) of *p*-(*p*-aminophenylazo)-phenylarsonic acid, removing excess nitrite with urea, and coupling with 1.35 g. (0.0040 mole) of recrystallized "H-acid" in acetate buffer. The intermediate red compound was precipitated by addition of hydrochloric acid and washed twice with 500 ml. of water containing 10 ml. of 6 *N* hydrochloric acid and 3 g. of sodium chloride. The substance was then dissolved in sodium carbonate solution and added to a diazotized solution of 0.312 g. (0.0012 mole) of *p*-(*p*-aminophenylazo)-benzoic acid.[9] The product was precipitated with hydrochloric acid, dissolved in 500 ml. of sodium hydroxide solution, and reprecipitated with hydrochloric acid and sodium chloride and an equal volume of ethanol, leaving most of the remaining intermediate in solution. The process of solution and precipitation was repeated until the solution showed constant color, and sodium chloride was then removed from the product by extraction with 90% ethanol. Although the product was not crystallized, there is little doubt of its identity and purity; the method of preparation is based on the fact that at low *p*H substitution occurs in the 7 position of H-acid, ortho to an amino group, and not in the 2 position, ortho to a hydroxyl group, reaction at the latter position occurring in basic solution.



The RX' compound was made by diazotizing 4.0 g. (0.020 mole) of *p*-arsanilic acid and adding it very slowly to 6.0 g. (0.019 mole) of chromotropic acid in solution with sodium carbonate (final *p*H 9). After one hour a diazotized and neutralized solution of 1.3 g. (0.005 mole) of *p*-(*p*-aminophenylazo)-benzoic acid was added and the *p*H was adjusted to 9. The product was purified in the way described above; this process removes any RR compound which is formed.

Anti-R, anti-R', and anti-X sera were prepared as described elsewhere,[4,9] and the precipitation experiments were carried out in the usual way.[4] Several pools of antisera were tested; the same pools of anti-R and anti-X antisera were used for all of the quantitative experiments reported in this paper.

The substance RX' was found not to precipitate with anti-X serum, anti-R serum, or anti-R' serum, or with a mixture of anti-R' serum and anti-X serum; it formed heavy precipitates with a mixture of anti-R serum and anti-X serum.

(9) David Pressman, Stanley M. Swingle, Allan L. Grossberg and Linus Pauling, paper to be submitted for publication in THIS JOURNAL.

The substance R'X' gave no precipitate or only very slight precipitates with various pools of anti-X serum, anti-R serum, or anti-R' serum; heavy precipitates were formed with mixtures of anti-R serum and anti-X serum and with mixtures of anti-R' serum and anti-X serum. The quantitative experiments reported in Tables I to V were carried out with pools of sera which separately gave no precipitate with R'X'.

The failure of the substance RX' to precipitate with a mixture of anti-R' serum and anti-X serum is related to the fact[4] that anti-R' serum in general does not precipitate with polyhaptenic substances containing R groups.

It was found that the substances RX' and R'X' act as monohaptenic substances in inhibiting the specific precipitation of R'R' with anti-R' serum or anti-R serum and of X'X' with anti-X serum. Data showing the effect of haptens R* and X* on some precipitation reactions are given in Table II.

TABLE I

PRECIPITATION OF ANTIGENS R'R', X'X', R'R' + X'X', AND R'X' WITH MIXTURES OF ANTI-R SERUM AND ANTI-X SERUM

Antigen solution, 2 ml.; mixed antiserum, 1 ml.; 1 hour at room temperature and 2 nights in refrigerator; *p*H of supernates 8.1.

Ratio anti-R serum: anti-X serum[a]	Antigen	Moles of antigen × 10^9: 6	18	54	167	500
		Amount of precipitated antibody, μg.[b]				
93:7	R'R'	52	522	1134	721	162
	X'X'	0	0	0	0	0
	R'R' + X'X'[c]	14	136	913	1070	564
	R'X'	48	60	72	60	60
62:38	R'R'	44	(261)	(446)	(169)	25
	X'X'	4	22	80	50	23
	R'R' + X'X'[c]	8	104	439	481	236
	R'X'	22	158	792	821	623
17:83	R'R'	(12)	16	9	5	4
	X'X'	9	23	706	756	280
	R'R' + X'X'[c]	9	12	137	1024	906
	R'X'	12	16	25	66	90

[a] The ratio 62:38 was selected as that for which the optimum concentrations of the antigens R'R' and X'X' are the same; the other ratios are 8 and 1/8 times as great, respectively. [b] Average of triplicate analyses, with mean deviation ±2%; duplicate analyses in parentheses. [c] In equimolal amounts, with totals as given above.

Discussion of Results and Comparison with Theory

The qualitative behavior of the substances RX' and R'X' in precipitation and in hapten inhibition, as described above, is just that predicted by the framework theory. The quantitative data given in Tables I and II are also in accord with this theory. In particular, there is some verification of the prediction of the framework theory that the precipitate with R'X' should contain equal numbers of anti-R molecules and anti-X molecules; this would require that the amount of precipitate be small in case that either anti-R or anti-X serum be present in small amount, in agreement with observation.

It is also predicted, and verified by experiment, that precipitation of mixed antiserum with R'X' can be completely inhibited by the presence of either hapten R* or hapten X*, whereas precipitation with a mixture of R'R' and X'X' is only partially inhibited by either of these haptens.

TABLE II

INHIBITION BY HAPTENS R* AND X* OF PRECIPITATION OF ANTIGENS WITH MIXTURES OF ANTI-R SERUM AND ANTI-X SERUM

Antigen solution, 1 ml.; mixed antiserum, 1 ml.; hapten solution, 1 ml.; 1 hour at room temperature and 2 nights in refrigerator; *p*H of supernates 8.1.

Ratio anti-R serum: anti-X serum	Antigen[a]	Moles of hapten R* × 10^9					Moles of hapten X* × 10^9			
		0	37	111	333	1000	37	111	333	1000
		Amount of precipitated antibody, μg.[b]								
93:7	R'R' + X'X'[c]	985	441	102	20	0	956	949	944	1004
	R'X'	64	41	(21)	(10)	0	50	27	19	5
62:38	R'R'	321	85	19	5	0	315	311	305	292
	X'X'	(71)	(61)	(59)	57	56	(43)	(15)	0	0
	R'R' + X'X'[c]	344	132	61	52	51	316	342	302	300
	R'X'	827	527	290	88	15	553	219	48	15
17:83	R'R' + X'X'[c]	496	502	470	434	319	223	48	11	11
	R'X'	49	39	31	21	11	34	17	9	7

[a] Total moles of antigen used: R'R', 50×10^{-9}; X'X', 50×10^{-9}; R'R' + X'X', 100×10^{-9}; R'X', 100×10^{-9}. [b] Average of triplicate analyses, with mean deviation ±2%; duplicate analyses in parentheses. [c] In equimolal amounts.

TABLE III

ANTIBODY-ANTIGEN MOLECULAR RATIO IN PRECIPITATES FORMED BY R'X' ANTIGEN AND A MIXTURE OF ANTI-R SERUM AND ANTI-X SERUM

Ratio of antisera 62:38; equal volumes of antigen solution and mixed antiserum: 1.5 ml. of each for first series, 2.0 ml. for second; 1 hour at room temperature and 2 nights in refrigerator; *p*H of supernates 8.1.

Moles of antigen × 10^9	Amount of precipitated antibody[a]		Amount of precipitated antigen[a]		Molecular ratio, antibody/antigen[a]
	μg.	moles	μg.	moles	
100	1421	8.89×10^{-9}	10.8	11.7×10^{-9}	0.76
33	456	2.85	4.2	4.5	.63

[a] Average of triplicate analyses, mean deviation ±2% for antibody, ±2% for antigen, ±2% for ratio. Assumed molecular weight of antibody 160,000, of antigen 927.

TABLE IV

PRECIPITATION OF MIXTURES OF NORMAL SERUM AND ANTISERUM AT CONSTANT AMOUNT OF ANTIGEN

Antigen solution, 2 ml., containing either 5×10^{-9} moles of antigen R'R' (series A) or 6.7×10^{-9} moles of antigen R'X' (series B); mixture of antiserum and normal serum, 2 ml.; 1 hour at room temperature and 2 nights in refrigerator; *p*H of all supernates 8.1.

Antiserum concentration in mixture[a]	Amount of antibody precipitated, μg.[b]	
	Series A[c]	Series B[d]
1.00	39	39
0.67	35	106
.45	93	120
.30	128	109
.20	133	81
.13	120	47
.088	90	23
.059	51	6

[a] Anti-R serum (Series A) or 62:38 mixture of anti-R serum and anti-X serum (series B) diluted with normal rabbit serum to the extent indicated in this column. [b] Averages of triplicate analyses, with mean deviation ±3%. [c] Blank of anti-R serum and buffer, 5 μg.; blank of normal serum and antigen R'R', 5 μg. [d] Blank of 62:38 antiserum mixture, 6 μg.; blank of normal serum and antigen R'X', 5 μg.

An interesting feature of the data in Tables I and II is that the 62:38 mixture of antisera gives a larger amount of precipitate with the R'X' antigen than with R'R' and X'X' together. This is presumably related to the fact that the amount of precipitate formed with antigen R'R' (or X'X') alone falls off very rapidly with decrease in the fraction of anti-R (or anti-X) antiserum in the mixture; for example, the amount of precipitate formed by X'X' with the 62:38 mixture is only about one-ninth of that formed with the 17:83 mixture, although the first mixture contains nearly one-half as much anti-X antibody as the second. The amount of precipitate formed by antigens R'R' and X'X' together would be expected to be about equal to the sum of the amounts formed by these antigens separately, since the two precipitates are mutually independent. But a single precipitate is formed by R'X', containing equal amounts of the two kinds of antibody, and accordingly the amount of precipitate would be expected to be about equal to that formed by R'R' or X'X' with a mixture containing double the amount of anti-R serum or anti-X serum as is, indeed, observed.

It has been noted in general that simple antigens, in contradistinction to protein antigens, are far from completely precipitated by antisera, and that the fraction of the antigen remaining in solution at the optimum region increases on dilution of the antiserum with normal serum or heterologous antiserum; this effect, as discussed above, is to be noted in Table I. We have not yet carried out a sufficiently detailed experimental study of these phenomena to justify an extended discussion of them.

A mathematical discussion based on the simplified model treated previously[4,10] can be carried out for the RX precipitation reaction, leading to results in general correspondence with experiment. Let A represent the R'X' antigen, $B^{(1)}$ and $B^{(2)}$ the anti-R and anti-X antibodies, respectively, which are assumed to be bivalent and homogeneous, and $H^{(1)}$ and $H^{(2)}$ the haptens R* and X*, respectively. It is assumed that, in addition to the precipitate $A_2B^{(1)}B^{(2)}$, certain soluble complexes exist, and that their equilibrium constants of formation have the values given below; these values have been assigned on the basis of structural considerations, with inclusion of the entropy term arising from the symmetry numbers of the molecules.

$A + B^{(1)} = AB^{(1)}$	$2K_1$
$A + B^{(2)} = AB^{(2)}$	$2K_2$
$2A + B^{(1)} = AB^{(1)}A$	K_1^2
$2A + B^{(2)} = AB^{(2)}A$	K_2^2
$2A + B^{(1)} + B^{(2)} = AB^{(1)}AB^{(2)}$	$4K_1^2K_2''$
$2A + B^{(1)} + B^{(2)} = AB^{(2)}AB^{(1)}$	$4K_1''K_2^2$

(10) Linus Pauling, Dan H. Campbell, and David Pressman, *Physiol. Rev.*, **23**, 203 (1943).

$$\begin{aligned} H^{(1)} + B^{(1)} &= H^{(1)}B^{(1)} & 2K_1' \\ H^{(2)} + B^{(2)} &= H^{(2)}B^{(2)} & 2K_2' \\ 2H^{(1)} + B^{(1)} &= H^{(1)}B^{(1)}H^{(1)} & K_1'^2 \\ 2H^{(2)} + B^{(2)} &= H^{(2)}B^{(2)}H^{(2)} & K_2'^2 \\ A + B^{(1)} + H^{(1)} &= AB^{(1)}H^{(1)} & 2K_1K_1' \\ A + B^{(2)} + H^{(2)} &= AB^{(2)}H^{(2)} & 2K_2K_2' \end{aligned}$$

By straightforward solution of the equilibrium equations there is obtained for the case that no hapten is present a set of simultaneous equations

$$\beta_1^2(1 + K_1\alpha)^2 - \beta_1(B^{(1)}_{total} - B^{(2)}_{total}) - S(1 + K_2\alpha)^2/\alpha^2 = 0 \quad (1)$$

$$A_{total} - B^{(1)}_{total} - B^{(2)}_{total} - \alpha + \beta_1(1 - K_1^2\alpha^2) + S(1 - K_2^2\alpha^2)/\alpha^2\beta_1 = 0 \quad (2)$$

$$A_2B^{(1)}B^{(2)}(pp) = B^{(1)}_{total} - \beta_1(1 + K_1\alpha)^2 - s \quad (3)$$

These three equations are to be solved for three unknown quantities, the amount of precipitate $A_2B^{(1)}B^{(2)}(pp)$ and the two auxiliary variables α, the concentration of molecular species A in solution, and β_1, the concentration of molecular species $B^{(1)}$; the other quantities are the equilibrium constants K_1 and K_2, the solubility product $S = [A]^2[B^{(1)}][B^{(2)}]$ of the precipitate, and the solubility s of the precipitate as the complexes $AB^{(1)}AB^{(2)}$ and $AB'^{(2)}AB^{(1)}$, with $s = 4(K_1''K_2'^2 + K_1^2K_2'')S$. A convenient method of solving the equations is to leave A_{total} undetermined, and to introduce numerical values of the other parameters; introduction in Equation 1 of a trial value of α permits solution for β_1, after which A_{total} may be evaluated from Equation 2 and $A_2B^{(1)}B^{(2)}(pp)$ from Equation 3.

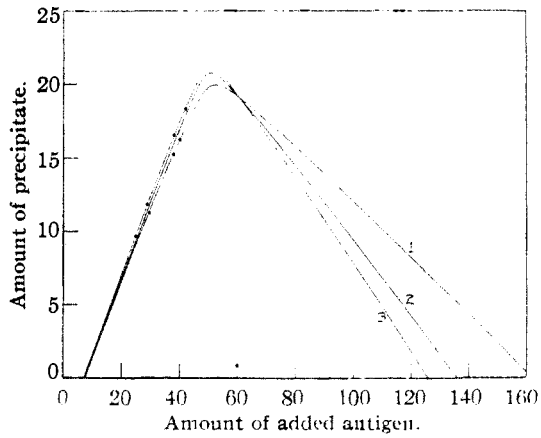


Fig. 1. -Curves showing results of theoretical calculations of amount of precipitate formed by antigen RX with an equimolal mixture of anti-R serum and anti-X serum, as function of amount of antigen: $B^{(1)}_{total} = B^{(2)}_{total} = 25$, $s = 2$, and $K_1 = K_1'' = 1$ for all curves; $K_2 = K_2'' = 0.2$ for curve 1, 1 for curve 2, and 5 for curve 3.

Curves showing dependence of amount of precipitate on amount of antigen with equal amounts of the two kinds of antibody present (Fig. 1) are similar to those for the precipitation of one kind of antibody by homologous dihaptenic antigen.[4] As the ratio of the amounts

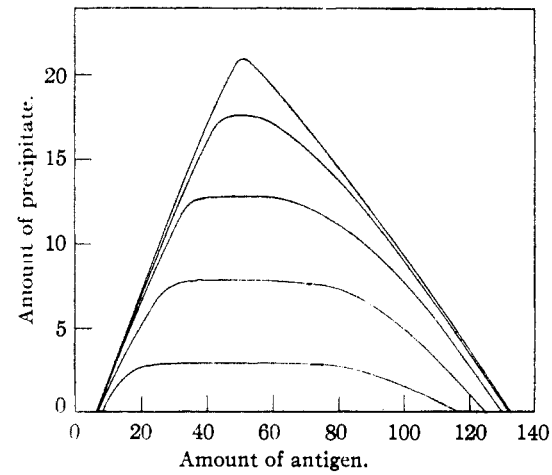


Fig. 2.—Curves showing calculated amount of precipitate as functions of amount of antigen RX for various relative amounts of anti-R and anti-X antibody. All curves are for $K_1 = K_1'' = K_2 = K_2'' = 1$ and $s = 2$. From top to bottom the curves correspond to the following pairs of values of $B^{(1)}_{total}$ and $B^{(2)}_{total}$: 25, 25; 20, 30; 15, 35; 10, 40; 5, 45.

of the two kinds of antibody is varied more and more from unity, the calculated curves of the amount of precipitate show broader and broader regions of approximate constancy (Fig. 2), resulting from the buffering action of the antibody present in excess, which combines with excess antigen to form a soluble complex, and thus interferes with the reaction of solution of the precipitate in excess of antigen; it is seen that the antigen concentration at which the amount of precipitate reaches its approximate maximum is determined by the amount of that kind of antibody which is present in smaller quantity, and that the antigen concentration at which the amount of precipitate begins to decrease is determined by the amount of the other kind of antibody. Experimental verification of these details of prediction cannot be expected until essentially homogeneous antibody solutions have been made by fractionation of antisera; however, the predicted general difference in nature is shown by the R'X' data given in Table I for the 93:7 mixture (broad region with nearly constant amount of precipitate) and the 62:38 mixture (rather narrow optimum region). Verification of the existence of the broad region of nearly constant amount of precipitate was obtained by the repetition of the experiment with the 93:7 mixture, with inclusion of points for smaller and larger amounts of the antigen R'X'; the amount of precipitated antibody was found to be constant (80 ± 10 μg.) over the range 18 to 500 $\times$ 10^{-9} mole of antigen, and to decrease for smaller and for larger amounts of antigen (34 μg. at 6.2 $\times$ 10^{-9} mole of antigen, 60 μg. at 1500 $\times$ 10^{-9}).

When hapten of one kind is also present in the system, the equilibrium expressions may be com-

bined to form the set of simultaneous equations 4 to 7.

$$\beta_1^2(1 + K_1\alpha + K_1'\gamma_1)^2 - \beta_1(B^{(1)}_{total} - B^{(2)}_{total}) - S(1 + K_2\alpha)^2/\alpha^2 = 0 \quad (4)$$

$$\beta_1^2\{(1 + K_1'\gamma_1)^2 - K_1^2\alpha^2\} + \beta_1\{A_{total} - B^{(1)}_{total} - B^{(2)}_{total} - \alpha\} + S(1 - \Lambda_2^2\alpha^2)/\alpha^2 = 0 \quad (5)$$

$$H^{(1)}_{total} = \gamma_1 + 2K_1'\beta_1\gamma_1 + 2K_1'^2\beta_1\gamma_1^2 + 2K_1K_1'\alpha\beta_1\gamma_1 \quad (6)$$

$$A_2B^{(1)}B^{(2)}(pp) = B^{(1)}_{total} - \beta_1(1 + K_1\alpha + K_1'\gamma_1)^2 - s \quad (7)$$

Here $H^{(1)}_{total}$ is the total amount of hapten (of the first kind) added and $\gamma_1 = [H^{(1)}]$ is another auxiliary variable. A similar set of equations can be derived for the case that hapten of the second kind is present. Solution of these equations may be made by selecting a value of γ_1 and finding by successive approximations the values of α and β_1 which satisfy Equations 4 and 5, and then determining $H^{(1)}_{total}$ and $A_2B^{(1)}B^{(2)}(pp)$ from Equations 6 and 7, respectively.

The calculated curves for hapten inhibition by either kind of hapten when equal amounts of antibodies of the two kinds are present are closely similar to those for the simpler systems previously treated.[4] However, an interesting effect is predicted to occur when one antiserum predominates in the mixture and a small amount of antigen is present: a normal inhibition curve, linear in amount of hapten added, is found from the equations for one hapten, whereas the other hapten, that homologous to the antibody present in the larger amount, is predicted to be ineffective in small amounts, and to produce inhibition of precipitation only after enough of the hapten has been added to neutralize the excess of antibody in solution; calculated curves are given in Fig. 3. To test this prediction experiments were carried out, in triplicate, for the 93:7 mixture with the R'X' antigen (antigen solution, 2 ml., containing 200 × 10^{-9} moles of R'X' antigen; mixed antiserum, 2 ml.; hapten solution, 2 ml.; one hour at room temperature and two nights in refrigerator; *p*H of supernates 8.1; mean deviation of triplicate analyses ± 2%); the results are shown in Fig. 4. It is seen that there is a small initial region for which inhibition by the R* hapten does not occur. (In the comparison of Figs. 3 and 4 it should be remembered that the curves for Fig. 3 are calculated for haptens of equal bond-strength constant K', whereas K' for hapten R* is considerably larger than that for X*.) It is likely that the predicted effect would be shown more strikingly by fractionated antibody solutions than by the heterogeneous antisera used in these experiments.

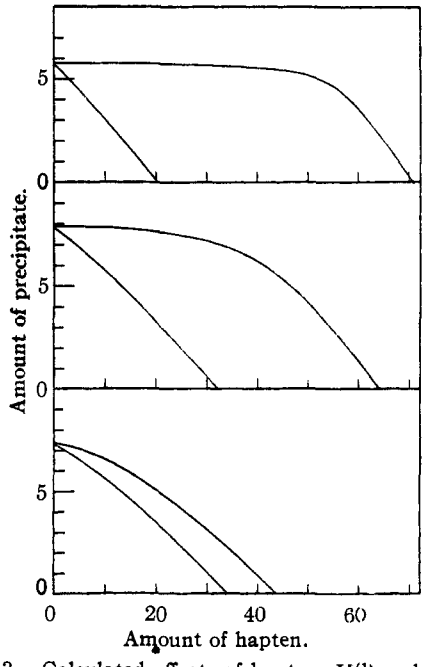


Fig. 3.—Calculated effects of haptens $H^{(1)}$ and $H^{(2)}$ on precipitation of antigen RX with an antiserum mixture containing four times as much anti-R antibody as anti-X antibody ($B^{(1)}_{total}$ = 10, $B^{(2)}_{total}$ = 40). From top to bottom the curves are for A_{total} = 20, 50, and 100; of each pair the curve at the left is for hapten $H^{(1)}$ and the other for hapten $H^{(2)}$.

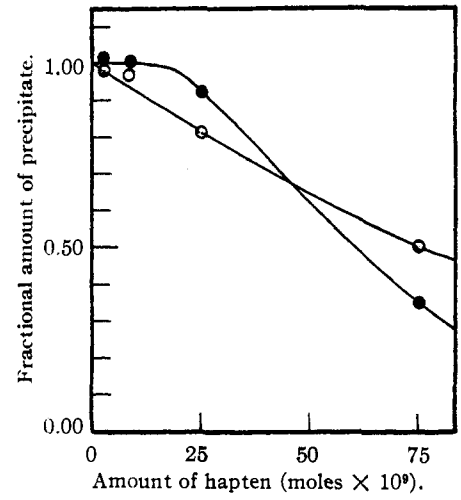


Fig. 4.—Experimental values of amount of precipitate formed by antigen R'X' with 93:7 mixture of anti-R serum and anti-X serum in presence of hapten R* (solid circles) or hapten X* (open circles).

Experimental data bearing on the valence of the antibody molecules are given in Tables III and IV. The observed antibody/antigen molecular ratio of about 0.7 in both the equivalence zone and the region of antibody excess (Table III) corresponds, with antigen known to be bivalent, to 2/0.7 = 2.8 for the average valence of the antibody molecules in the precipitate.

The second of our arguments for the multivalence of antibody molecules is based on the solubility of antigen–antibody precipitates in

excess antibody. This phenomenon has been reported for many antigen–antibody systems, especially with horse antibody. We have observed it to occur with rabbit antibody and simple polyhaptenic antigens; the results of an experiment of this sort, with anti-R serum and the substance R′R′, are given in Table IV. In this table there are given also data for a similar experiment with the substance R′X′ and a mixture of anti-R serum and anti-X serum; the observed decrease in the amount of precipitate with increase in amount of antiserum can be accounted for only with the theory of multivalent antibody.

It is known that excess of one component causes decrease in the rate of precipitation of antibody and antigen, and the question may be asked as to whether the effect of excess antibody reported in Table IV might be due to failure of the reaction to be completed in the time allowed. An experimental test of this possibility was made, with the results given in Table V. Tubes were set up corresponding to the first and fifth experiments of Series A of Table IV, representing, respectively, the region of antibody excess (Series C) and the region of optimum precipitation (Series D). These tubes were allowed to stand for times ranging from two to fifteen days, and then were analyzed. The values obtained after two days are close to the corresponding values of Table IV. In both series the amount of precipitate was observed to increase for six days, and then to remain essentially constant. The difference in amount of precipitate for Series C and Series D remained constant throughout the period; from these results we conclude that the effect of excess antibody in decreasing the amount of precipitate is real, and is not due to a difference in the rate of precipitation.

TABLE V

EFFECT OF TIME OF STANDING ON AMOUNT OF PRECIPITATE OBTAINED IN THE REGIONS OF ANTIBODY EXCESS AND OPTIMUM PRECIPITATION

Solution of antigen XXX, 2 ml. (5×10^{-9} mole); anti-R serum, 2 ml. for series C and 0.4 ml. plus 1.6 ml. of normal serum for series D; 1 hour at room temperature and indicated times in refrigerator.

Time of standing in refrigerator, days	Amount of antibody precipitated (μg.)[a] Series C	Series D
2	34	110
4	37	138
6	78	150
8	59	156
10	27	138
15	62	131

[a] Averages of triplicate analyses with mean deviation ±2 μg. Values are corrected by subtraction of the blanks for serum and buffer, which ranged between 14 and 23 μg. The large values of the blank in comparison with those of Table IV (5 μg.) may be due to change in the antiserum during the period of three months between the experiments.

Solubility of the precipitate in excess of antiserum has been reported for a few systems, such as diphtheria toxin and horse antitoxin, but not for antigen–antibody systems in general. Theoretical considerations[3,11] indicate that solubility in antibody excess should occur for antigens with small valence (such as the dihaptenic substances of Table IV) but not for antigens with large valence, which would only with difficulty be saturated with antibody to form a soluble complex.

This work was carried out with the aid of a grant from The Rockefeller Foundation. Dr. Stanley Swingle and Mr. Allan L. Grossberg helped with the experimental work.

Summary

It has been found by experiment that substances of the type RX, containing two different haptenic groups, do not form precipitates with either anti-R serum or anti-X serum alone, but do form precipitates with a mixture of the two specific antisera. This provides proof of the effective bivalence of the dihaptenic precipitating antigen, and thus furnishes further evidence for the framework theory of antigen–antibody precipitation. In these experiments the anti-R serum and anti-X serum were made by injecting rabbits with sheep serum coupled with diazotized *p*-arsanilic acid and diazotized *p*-aminobenzoic acid, respectively, and the RX substances used were 1-amino-2-*p*-(*p*-azophenylazo)-phenylarsonic acid-3,6-disulfonic acid-7-*p*-(*p*-azophenylazo)-benzoic acid-8-hydroxynaphthalene and 1,8-dihydroxy-2-*p*-azophenylarsonic acid-3,6-disulfonic acid-7-*p*-(*p*-azophenylazo)-benzoic acid-naphthalene.

The antibody–antigen molecular ratio in the precipitate was found by analysis to be 0.7, which, with antigen known to be bivalent, leads to the average valence 2.8 for the antibody molecules in the precipitate. Further evidence that the antibody valence is greater than 1 is given by the observation that the precipitate is soluble in excess of antiserum.

A simple physicochemical theory of the precipitation of RX antigen with mixed antiserum and of its inhibition by haptens is developed and compared with experiment.

PASADENA 4, CALIFORNIA RECEIVED SEPTEMBER 7, 1943

(11) A. D. Hershey, *J. Immunol.*, **42**, 455 (1941).