combined fractions were recrystallized from a minimum of alcohol and ethyl acetate and yielded 13 mg. of long thin needles which melted at 121.5–122.5° and had $[\alpha]^{26}D$ −23.5° (*c*, 0.89 in water).

Anal. Calcd. for $C_8H_{16}O_5S$: C, 42.85; H, 7.19; S, 14.27. Found: C, 42.58; H, 6.77; S, 13.50.

Reaction of L-Arabinose with Ethyl Mercaptan.—L-Arabinose (2 g.) was treated with ethyl mercaptan (8 ml.) and hydrochloric acid (8 ml.) as described for methyl α-D-mannopyranoside. The dried neutralized residue was acetylated with pyridine (20 ml.)-acetic anhydride (20 ml.), the crude acetylation product dissolved in absolute alcohol (3 ml.), and the solution was allowed to stand in the refrigerator overnight. The resulting crystals (2.35 g.) were filtered and washed with cold alcohol. After two recrystallizations from 95% alcohol the crystals melted at 79–80° $[\alpha]^{27}D$ −27.0° (*c*, 1.6 in chloroform). These data are in accord with those given for tetraacetyl-L-arabinose diethyl mercaptal by Wolfrom and Newlin.[26]

Anal. Calcd. for $C_{17}H_{28}O_8S_2$: C, 48.11; H, 6.65; S, 15.08. Found: C, 48.27; H, 6.65; S, 15.27.

Acknowledgment.—The authors wish to thank Dr. Oskar Wintersteiner for helpful discussions during the course of this work and Mr. Joseph F. Alicino, Miss Anne C. Crickenberger and Miss Ruth Karitzky for the microanalytical determinations.

(26) M. L. Wolfrom and M. R. Newlin, THIS JOURNAL, **52**, 3619 (1930).

Summary

α- and β-ethyl tetraacetyl-1-thio-D-mannopyranosides have been prepared by the prolonged action of ethyl mercaptan and hydrochloric acid on both D-mannose and methyl β-D-mannopyranoside. Methyl α-D-mannopyranoside yielded only the β-anomer. Ethyl 1-thio-β-D-mannopyranoside has been obtained in crystalline form.

Styracitol has been prepared from ethyl tetraacetyl-1-thio-β-D-mannopyranoside by desulfurization with Raney nickel.

α- and β-ethyl 1-thio-D-galactopyranosides and their tetraacetates have been prepared in crystalline form.

The action of ethyl mercaptan and hydrochloric acid on L-arabinose for eighteen hours led only to the diethyl mercaptal.

NEW BRUNSWICK, N. J. RECEIVED AUGUST 3, 1948

[CONTRIBUTION NO. 1135 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Reaction of Simple Antigens with Purified Antibody[1]

BY ARTHUR B. PARDEE[2] AND LINUS PAULING

Many biological reactions, including the interaction of antibodies and antigens, the effect of enzymes on their substrates, and the self-reproducing behavior of genes, are characterized by a high degree of specificity. The great problem of the nature of the forces responsible for this biological specificity is being attacked vigorously in many ways at the present time. During the past six years we have carried out a series of investigations[1] on the reactions of antisera with simple substances, extending and refining the work of Landsteiner.[3] The results obtained provide strong support for the concept that biological specificity is due to a detailed complementariness in surface configuration of the molecules involved (antigen and antibody) and that the forces which contribute to specific attraction of two molecules—van der Waals electronic forces, hydrogen bond forces, etc.—are in general short-range forces, effective over distances of a few Ångström units. The conclusion has been reached that the surface approximation of antibody and the haptenic groups of antigens is to within about 1 Å.

Much of our work has consisted of studies of the precipitation of an antiserum by a simple polyhaptenic substance. The observation that certain simple substances containing two or more haptenic groups would form precipitates with the homologous antiserum was made by Landsteiner and van der Scheer.[4] It was suggested by Landsteiner that the forces between dye molecules which favor the formation of colloidal solutions, that is, of polymerized aggregates, are responsible for the ready precipitability of these substances, many of which are dyes. We, however, have presented evidence that the presence of two or more haptenic groups in each molecule, making the formation of a framework possible, is responsible for their precipitability.

The suggestion that it is polymerization of these simple precipitating antigens that gives them their precipitating power has been revived by Boyd and Behnke,[5] who reported that they had found one of the simple antigens used by us to be highly (11-fold) polymerized in saline solution, and who stated that accordingly the results of our earlier investigations might not justify the

(1) The Serological Properties of Simple Substances. XIV. For number XIII of this series see D. Pressman, J. H. Bryden, and L. Pauling, THIS JOURNAL, **70**, 1352 (1948).

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(3) See K. Landsteiner, "The Specificity of Serological Reactions," Harvard University Press, Cambridge, Massachusetts, 1945.

(4) K. Landsteiner and J. van der Scheer, *Proc. Soc. Exptl. Biol. Med.*, **29**, 747 (1932); *J. Exptl. Med.*, **56**, 399 (1932); **57**, 633 (1933); **67**, 79 (1938).

(5) W. C. Boyd and J. Behnke, *Science*, **100**, 13 (1944). In this preliminary note about their work these authors wrote that details would be published elsewhere; their detailed paper has not yet appeared.

interpretation that we had given them. This criticism has been repeated by Kabat.[6]

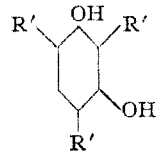
In the present paper we report the results of experiments on the interaction of simple precipitating antigens with purified antibody solutions. It has been found that the behavior of the simple antigens with purified antibody solutions is somewhat different from that with antiserum, the difference apparently being due to a rather strong non-specific combination of the dye molecules with the constituents of serum not present in purified antibody, presumably mainly albumin.[7,8] The effect of this combination seems to be to reduce the concentration of the free dye molecules to such an extent that in serum solution they react mainly in the monomeric form.

Experimental

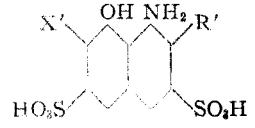
Synthetic Antigens.—The compounds used as synthetic antigens and their preparation have been described in previous papers except resorcinol-R$_3'$, which was prepared by Mr. A. L. Grossberg, by the method used for chloroglucinol-R$_3'$ (XI) with use of a different phenolic nucleus.[9,10]

R represents the *p*-azobenzenearsonic acid group, R′ the *p*-(*p*-azobenzeneazo)-benzenearsonic group, and X′ the *p*-(*p*-azobenzeneazo)-benzoic acid group.

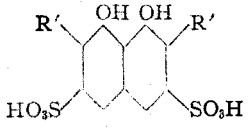
Resorcinol-R$_3'$ (R′)



H-acid-R′X′ (R′X′)



Chromatropic acid-R$_2'$ (XXX)



Antibodies and Other Substances.—The anti-R serum was obtained from rabbits by the method previously described.[9]

Specifically purified anti-R antibody (referred to in this paper as purified antibody) was prepared by Prof. D. H. Campbell.[11] Various preparations using resorcinol-R$_3'$ as precipitant were pooled and used for the succeeding experiments.

The pneumococcus type I antibody used in some of the experiments was a commercial preparation, Lederle Refined and Concentrated Rabbit Globulin. The purified polysaccharide was prepared by Dr. J. E. Cushing, Jr.

The rabbit serum albumin was prepared by Dr. George A. Feigen, by a single salt precipitation.

Salt and Buffer.—All experiments were done in 0.9% sodium chloride solution, without buffer present unless otherwise noted. When a buffer is referred to as *x*F it is meant that the sum of all forms of the buffering material is *x* formula weights per liter.

Precipitation Reaction; General Methods.—Antigen and antibody at approximately the final *p*H were mixed and allowed to stand for one hour at room temperature and then for two to five days at 5°. (It was found that resorcinol-R$_3'$ gave the same amount of precipitate in two hours as in three days, and that the maximum turbidity under the conditions used was reached in ten minutes at room temperature.) After having been washed,[9] the precipitates were analyzed for antigen by adding 2.5 ml. of 1 *N* sodium hydroxide, making up to 5.5 ml. in a centrifuge tube, and reading the light absorption on a Beckman spectrophotometer at an appropriate wave length and slit width. Compound resorcinol-R$_3'$ was read at 500 mμ and 0.03 mm. slit width, and antigens XXX and R′X′ at 600 mμ and 0.04 mm. slit width. The first two compounds showed no tendency to fade over a period of several days in alkali or over several months in approximately neutral solution, but R′X′ faded about 20% in a day in alkali. The compounds reacted slowly with the soft glass bottles. The error in the colorimetric procedure is estimated at ±3% or less for samples containing more than 4×10^{-9} moles of antigen, and is somewhat greater for smaller samples.

The same sample was then analyzed for protein by the modified Folin–Ciocalteu method.[12] A correction was made for the color of the antigen. To test the method ten triplicate analyses were run on purified antibody samples by both the modified Folin–Ciocalteu and the Nessler method by two analysts. The first method gave values which averaged 1.035 ± 0.035 times the second.

In some of the experiments with purified antibody the supernatant liquids were decanted from the centrifuged precipitates, both parts were analyzed, and the precipitates were corrected for the small amount of antigen and protein in the remaining supernatant liquid. In some of the later experiments only the precipitates were analyzed and the correction was calculated from the amount of reactants added.

The molecular weight of the antibody was taken as 160,000 for calculations.

Results

Precipitates were analyzed both with and without washing, as described in the previous section. The amounts of precipitate and the mole ratios in the precipitate for the two procedures are compared in Table I and also in Table II. It can be seen that for the antigen used (XXX) the amount of precipitate washed away is 30 to 45% of the original amount; the ratio of antigen to antibody in the precipitate remains about the same, however. Only 10 to 15% of the precipitate with antigen resorcinol-R$_3'$ (at *p*H 6.9) was lost on washing.

It was found that the amount of precipitate obtained with antigen XXX and purified antibody at *p*H 7.6 and 8.1 was only slightly dependent on the volume of the reaction mixture, the amount of precipitate varying over about a 20% range for any given amounts of antigen and antibody on three-fold dilution with buffer solution. The mole ratio in the precipitate was essentially independent of the volume of the mixture. In contrast, a three-fold increase in the volume of a system containing antiserum and a simple poly-

(6) E. A. Kabat, "Annual Review of Biochemistry," Annual Reviews, Inc., Stanford University, California, 1946, p. 528.

(7) B. D. Davis, *Am. Scientist*, **34**, 611 (1946).

(8) I. Klotz, THIS JOURNAL, **68**, 2299 (1946); **69**, 1609 (1947).

(9) L. Pauling, D. Pressman, D. H. Campbell, C. Ikeda, and M. Ikawa, *ibid.*, **64**, 2994 (1942).

(10) L. Pauling, D. Pressman, and D. H. Campbell, *ibid.*, **66**, 330 (1944).

(11) D. H. Campbell, R. H. Blaker, and A. B. Pardee, *ibid.*, **0**, (2496 1948).

(12) D. Pressman, *Ind. Eng. Chem., Anal. Ed.*, **15**, 357 (1943).

TABLE I

PRECIPITATION OF PURIFIED ANTIBODY BY ANTIGEN XXX

Varying amounts of antigen XXX in 2 ml. of saline were added to 2 ml. of saline containing 4.2×10^{-9} mole of antibody. Final *p*H 8.1, no buffer. Values are averages of triplicate analyses

Antigen added, moles $\times 10^9$	Precipitated antibody, moles $\times 10^{9a}$		Mole ratio antigen / antibody in ppt.[a]	
264	0.45	0.81	9.2	11.6
132	.53	.91	5.9	8.2
66	.62	1.03	4.9	5.5
33	.97	1.53	2.6	3.4
16	1.28	1.84	2.0	2.7
8	1.51	2.40	1.9	1.8
4	1.44	2.06	1.5	1.6
2	1.00	1.46	1.2	1.3
1	0.59	1.03	(1.0)	(0.9)

[a] The first column is for experiments in which the precipitate was washed in the conventional way; the second is for experiments in which the supernate was decanted from the precipitate and both were analyzed as described in the text.

TABLE II

PRECIPITATION OF PURIFIED ANTIBODY BY ANTIGEN XXX

Varying amounts of purified antibody in 2 ml. of saline were added to 4.1×10^{-9} mole of antigen XXX in 2 ml. of saline. Final *p*H 8.1, no buffer. Analyses in triplicate

Antibody added, moles $\times 10^9$	Precipitated antibody, moles $\times 10^{9a}$		Mole ratio antigen / antibody in ppt.[a]	
16.9	2.0	2.5	1.1	1.1
12.5	2.0	2.8	1.4	1.1
8.5	2.1	2.8	1.4	1.2
4.2	1.4	2.1	1.5	1.6
2.1	0.7	1.2	1.8	1.9
1.0	.2	0.4	(1.8)	(2.7)

[a] See note of Table I.

haptenic antigen[13] caused the amount of precipitate to decrease by 50%.

A number of experiments other than those reported in Table II were carried out in which varying amounts of purified antibody were added to a constant amount of antigen in a constant volume of solution. A 5 to 10% decrease in precipitated protein was noted at the largest amounts of antibody in every experiment. This effect of inhibition of precipitation by antibody excess is similar to but not nearly so marked as the effect in serum,[10] in which a four-fold increase in amount of antiserum above the optimum was found to decrease the amount of precipitate to a third of the maximum value.

Many precipitation experiments in which constant amounts of purified antibody were added to various amounts of antigen were carried out. The detailed results were in all cases similar to those reported in Table I, and their important features are summarized in Table III. Some experiments with added serum are also reported at the end of Table III for comparison. The first two columns give the antigen used and the *p*H of the system. A stands for antigen and B stands for antibody. Columns 3 to 5 have to do with conditions at the optimum, *i. e.*, the conditions which give the maximum amount of precipitate. Column 3 gives the per cent. of total antibody precipitated. The values in this column depend on the antigen, and, for resorcinol-R_3', decrease with increasing *p*H. In the presence of serum an optimum is obtained at about *p*H 9.0.[14] The presence of serum greatly reduces the amount of precipitable antibody, especially in the case of antigen R'X', which gives no precipitate in serum.

TABLE III

SUMMARY OF PRECIPITATION EXPERIMENTS WITH PURIFIED ANTIBODY

Antigen	*p*H	Per cent. of antibody pptd. at optimum	Mole ratio A/B in system at optimum	Mole ratio A/B in precipitate at optimum	A/B in preclpitate at A = 0
XXX	8.1	73	1.8	1.3	0.9
XXX	8.1	58	1.5	1.8	1.1
Resorcinol-R_3'	6.6	82	18	19	2.5
Resorcinol-R'	6.9	84	7.0	10	5.0
Resorcinol-R_3'	7.6[a]	76	5.1	5.0	1.1
Resorcinol-R_3'	7.9	68	5.7	4.5	2.0
Resorcinol-R_3'	8.7[b]	63	6.0	7.0	1.5
R'X'	7.9[b]	50	12	4.6	2.8
XXX (in serum)	8.1[b]	19	7	..	...
Resorcinol-R_3' (in serum)	8.5	36	5.1	2.2	2.0
R'X' (in serum)	8.3	0	..	..	...

[a] This experiment was started at *p*H 9.9; when no precipitate appeared, the mixtures were acidified to *p*H 7.6. [b] 0.01 F Veronal buffer present. Mr. Leonard Lerman found no non-specific precipitation between resorcinol-R'_3 and normal rabbit γ-globulin under conditions similar to the above.

Column 4 gives the number of moles of antigen required per mole of antibody to form the maximum amount of precipitate. The antigen resorcinol-R_3', which is more highly associated than the others,[15] requires a higher ratio, and the ratio is greater at lower *p*H. Antigen R'X' has a strikingly different ratio than the structurally similar compound XXX. The presence of serum has little effect on this ratio for resorcinol-R_3' but a large effect for XXX.

The fifth column gives the mole ratio of antigen to antibody in the precipitate at the optimum. The values are similar to those in column 4 in the absence of serum, and decrease with increasing *p*H. The antigen R'X' is an exception, requiring a much higher ratio in the system than in the precipitate. Experiments in serum also show a lower antigen–antibody ratio in the precipitate than in the system, probably because much of the antigen is non-specifically combined with serum proteins.

(13) L. Pauling, D. Pressman, D. H. Campbell, and C. Ikeda, THIS JOURNAL, 64, 3003 (1942).

(14) D. Pressman, unpublished work.

(15) See the following paper, THIS JOURNAL, 71, 148 (1949).

The sixth column gives the mole ratio of antigen to antibody in the precipitate extrapolated to zero antigen–antibody ratio. This value can be obtained from either an experiment with variable antigen and constant antibody or an experiment with constant antigen and variable antibody. The values for the two types of experiment are the same within experimental error for antigen resorcinol-R_3' and for XXX but are 4.0 and 2.8 for R'X'. The ratio is of interest because one would expect non-specific reactions of antigen with proteins, and also association, to be least important when there is a large excess of antibody in the system. Ideally the ratio should be unity according to the framework theory.[16] This is the value found for antigen XXX and approached by resorcinol-R_3' as the *p*H is increased. In the experiment which was started at *p*H 9.9 and finished at *p*H 7.6 it appeared as if the antigen combined as a small aggregate at the higher *p*H and remained unassociated as the *p*H was decreased. Earlier experiments by Pressman[14] showed that if resorcinol-R_3' were aged for even one hour at *p*H values below 9 before addition to antibody, the amount of precipitate was appreciably decreased below the optimum amount obtained with antigen kept above *p*H 9. R'X' has a higher limiting ratio than the structurally similar antigen XXX, indicating that R'X' is associated in the precipitate even at antigen–antibody ratios less than unity. If the values in the fifth and sixth columns of Table III agree, the non-specific effect in the purified antibody experiments is probably small. Experiments in serum all led to fairly low ratios.[10]

The shapes of the plots of amount of precipitate *vs.* amount of antigen added were generally similar. One quarter to one sixth of the optimum amount of antigen was required to give half the maximum amount of precipitate with purified antibody, and slightly more was required with antiserum. Generally about eight times the optimum amount of antigen was required to reduce the amount of precipitate to a half by antigen-excess inhibition. An exception was R'X', which required more than a fifteen-fold excess.

The Effect of Addition of Serum or Serum Albumin.—Normal rabbit serum was added to the purified antibody and precipitation tests were made, with the results reported in Table III. The effect of the serum was to reduce the amount of precipitate with antigens resorcinol-R_3' and XXX, and to prevent precipitation with antigen R'X'. Serum albumin had the same effect as normal rabbit serum: it completely inhibited precipitation with R'X', and reduced the amount of precipitate obtained with antigen XXX. The tests in serum albumin were made at *p*H 6.2.

(16) L. Pauling, THIS JOURNAL, **62**, 2643 (1940); L. Pauling, D. Pressman, and C. Ikeda, *ibid.*, **64**, 3010 (1942).

A Study of Non-specific Adsorption.—To see how much antigen was held non-specifically to the precipitates in the purified antibody experiments, precipitations were carried out between purified pneumococcus polysaccharide type I and a commercial globulin preparation from rabbit serum, containing about 10% antibody precipitable by the polysaccharide. In the first four tubes of the series reported in Table IV the compound resorcinol-R_3' was present at the time of precipitation of approximately optimal amounts of the reactants. In tubes 5 to 8 the precipitation was completed and then the supernatant liquid was replaced by solutions of resorcinol-R_3'. The results given in the table show that there is a very strong non-specific affinity of the precipitate for the simple antigen. That the antigen is not merely trapped in the precipitate is shown by the fact that the second four experiments gave ratios very similar to the first four. Although there is ten times as much protein in the solution as in the precipitate, the precipitate can hold 80% of the antigen. That the increase in precipitate with increasing amounts of antigen is a non-specific effect is shown by the ninth and tenth tubes of the table, in which no polysaccharide was present. Experiments with compound XXX and the pneumococcus polysaccharide system showed that the protein in the precipitate is more effective for non-specific combination than is the protein in solution for this compound also, and that the effect is not due to a difference in the type of protein, because in some experiments only a fraction of the antibody was precipitated and there was still preferential

TABLE IV

NON-SPECIFIC ADSORPTION OF COMPOUND RESORCINOL-R_3'

To 10^{-4} g. pneumococcus polysaccharide type I in 1 ml. saline there was added 1 ml. of saline containing 4.5 mg. of commercial anti-polysaccharide type I. The precipitation was done in the presence of 1 ml. of saline containing compound resorcinol-R_3' in tubes 1–4, and the supernate of the precipitate was replaced by 3 ml. of saline containing compound resorcinol-R_3' in tubes 5–8. Tubes 9 and 10 were controls, containing no polysaccharide. Final *p*H 8.0, no buffer, duplicate analyses

	Resorcinol-R_3' added moles × 10^9	Precipitated protein moles × 10^9	Mole ratio $\frac{R_3'}{\text{protein in precipitate}}$	% of total resorcinol-R_3' in precipitate
1	400	3.7	12.0	11
2	85	3.3	8.8	34
3	22	2.9	4.8	63
4	7	2.6	1.9	71
5	400	2.4	7.1	4
6	85	2.6	9.5	29
7	22	2.8	5.7	72
8	7	2.6	2.1	78
9	85	0.3	30	11
10	22	.2	14	14

A Referee suggests that it would be preferable to carry out this experiment with antibody prepared by more gentle methods because the Lederle product may have an altered affinity for haptens due to its method of preparation.

combination with the precipitate. Compound XXX was not bound as strongly as was resorcinol-R$'_3$. R'X' was bound to about the same extent as XXX.

These observations can perhaps best be explained by considering the precipitate to have a few locations where a dye-polymer molecule can attach itself very firmly, perhaps by combining with several protein molecules. Such sites would be absent in the protein in solution. Since only a few polymer molecules could go into these locations, the fraction of dye combined with the precipitate would be large when there was little dye in the system, and would become smaller when the dye was increased and the stronger locations were filled. Then the protein in the solution would compete successfully with the smaller amount of precipitate.

Hapten Inhibition.—Varying amounts of the strong hapten *p*-(*p*-hydroxybenzeneazo)-benzenearsonic acid were added to an optimal mixture of purified antibody and antigen XXX at *p*H 7.6 before precipitation had begun. This hapten, in 20-fold excess over the added antigen, was able to inhibit the precipitation almost completely, and gave a heterogeneity index[17] of $\sigma = 1.5$. A 50-fold excess of the weaker hapten benzenearsonic acid had almost no effect on the amount of precipitate obtained with purified antibody and resorcinol-R$'_3$. These results on hapten inhibition with purified antibody are closely similar to those obtained with antiserum.

Discussion of Results

In our earlier work on the molecular ratio of antigen to antibody in the precipitate formed by dye antigens with hapten-homologous antiserum, values of about 1.2 for this ratio were found. These values indicate a valence of approximately 2 (2.4) for antibody if the dyes are assumed to be bivalent. The equality of the values found for trihaptenic and tetrahaptenic dyes with those for dihaptenic dyes was explained as resulting from steric hindrance of the large antibody molecules, which prevents more than two of the haptenic groups of the small dye molecules from being effective. A constant antigen-antibody ratio was obtained (to within about $\pm 20\%$) even when the amount of dye added to a constant amount of antiserum was varied over a wide range, from antigen excess to antigen deficiency, and when the *p*H was varied from 7.6 to 9.2. Essentially the same results were obtained for seven dyes. The new results, obtained with different dyes and with purified antibody, are much different. A strong dependence of the ratio on the amount of antigen is observed, and the ratios are much higher than those found before, except in the antibody-excess region. The results found in the present investigation are of the nature to be expected in case that the dyes form aggregates that are taken up by the precipitate through non-specific adsorption. The amount of non-specific adsorption of resorcinol-R$'_3$ shown in Table IV is enough to account for the high values of the molecular ratio of dye and antibody reported in Tables I, II and III.

The limiting values of the molecular ratio extrapolated to zero dye concentration are approximately 1.0 for antigen XXX and somewhat larger for resorcinol-R$'_3$.

We believe that the difference between the results obtained with purified antibody and those obtained earlier with antiserum is due to the non-specific combination of the dye molecules with serum albumin or other constituents of serum not present in the purified antiserum,[7,8] and that in the presence of serum this effect causes the concentration of uncombined dye molecules to be so small as to keep the amount of aggregation down to a low value. It seems likely that the high values for the amount of dye in the precipitates formed with purified antibody are due largely to the inclusion of the aggregates in the precipitate through non-specific adsorption, and possibly also to some extent to their incorporation in the framework as effective precipitating antigens.

The results obtained with the antigen R'X' indicate that dimers or larger aggregates of this molecule are able to form a precipitate with anti-R serum, the precipitation being inhibited by the presence of serum. In our earlier investigation[10] we observed that a small amount of precipitate was formed by R'X' with some pools of anti-R serum; it is likely that these precipitates were due, not to cross-reaction, but to the presence of aggregates, these pools of antiserum perhaps containing smaller amounts of the complexing material than are usually present.

The conclusion that may be reached from the experimental results obtained is that the earlier work, carried out with serum, is presumably reliable, despite the tendency of the dyes to aggregate, because this tendency was counteracted by the presence of complexing materials in the serum. Moreover, the limiting molecular ratio found by extrapolation to antibody excess of the results obtained in the present investigation, with purified antibody, supports the molecular ratio of about 1 given by the earlier work. It would, however, be desirable, in order to carry conviction, for additional experiments to be made, with use of non-aggregating polyhaptenic precipitating antigens.

In the earlier studies it was found that non-aggregating polyhaptenic compounds (amides, rather than azo dyes) form precipitates with antisera, whereas the monohaptenic substances do not; however, analyses of the precipitates were not made because of the difficulties of determining the amount of the colorless precipitating antigens.

It may be pointed out that the quantitative hapten inhibition experiments reported in earlier

(17) L. Pauling, D. Pressman, and A. L. Grossberg, THIS JOURNAL, 66, 784 (1944).

papers of this series are not made unreliable by the phenomenon of aggregation of the precipitating antigen, for two reasons: first, the experiments were carried out in the presence of serum, which presumably inhibited the aggregation of the antigens; and second, the experiments all involve the comparison of the concentration of hapten required for a standard amount of inhibition (50%) with the concentration of a standard hapten producing the same effect, and this concentration ratio of the haptens, which do not themselves form aggregates, would be expected to be the same whether the test substance (the precipitating antigen) was aggregated or not.

The investigation reported in this paper was carried out with the aid of a grant from The Rockefeller Foundation. We wish to thank Professors Dan H. Campbell and Verner Schomaker, and Drs. David Pressman, Frank Lanni, J. E. Cushing, Jr., George Feigen and Stanley Swingle for advice in connection with the work and for providing some of the materials used. Mr. Dan Rice carried out many of the protein analyses.

Summary

Experiments have been carried out on the precipitation under various conditions of specifically purified anti-azobenzenearsonic acid antibodies by three azo dyes, serving as precipitating antigens. The non-specific combination of the dyes with pneumococcus polysaccharide: antipolysaccharide precipitates has also been investigated.

In solution these dyes exist as monomeric molecules and as aggregates. The molecular ratio of dye to antibody in the precipitate was found in general to be much larger than in earlier experiments involving antiserum, and to depend greatly upon conditions of the precipitation, whereas the earlier ratio (approximately 1.2) was essentially independent of these conditions. The dye is also carried down in non-specific serological precipitates (pneumococcus polysaccharide:antipolysaccharide antibody), apparently through non-specific adsorption or entrapment in the precipitate. The large molecular ratio in the precipitate formed with the homologous antibody is probably due mainly to the non-specific adsorption of dye aggregates. It is suggested that the results obtained with antiserum, which seem not to be affected by aggregation of the dye molecules, are to be explained as resulting from the combination of the dye molecules with constituents of serum, mainly albumin, which are not present in the purified antibody, this combination keeping the concentration of free antibody low, and preventing the formation of appreciable amounts of the aggregates.

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The Degree of Association of Some Simple Antigens

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In the preceding paper, which considers the effect of serum on the precipitin reaction between certain simple polyhaptenic precipitating antigens and antibodies, it was assumed that the antigens could be associated into aggregates. This paper presents measurements on some simple antigens which, because of their size and color, may be expected to be associated to a measurable extent.

In their treatment of the precipitin reaction between antisera and simple polyhaptenic substances (hereafter called simple antigens), Pauling and coworkers were aware of the possibility that their simple antigens might be associated, but did not consider association important and neglected it. This was done because some of the simple antigens used by them were colorless[1] and hence almost certainly not associated and because a simple substance containing one haptenic group against one antiserum and another against another antiserum did not precipitate against either antiserum separately, as it should if it were polymerized, but did precipitate with the mixed antisera.[2]

Landsteiner[3] was the first to suggest that the precipitating power of simple antigens might be connected with their association. He observed that some of the simple antigens have structures similar to those of associating dyes.[4,5] Many dyes are associated in solution; the forces involved have been explained on the basis of the color-producing resonance in the conjugated structure.

Boyd and Behnke[6] published a note stating that one large and highly colored simple antigen was polymerized 11-fold in a saline solution.

(1) L. Pauling, D. Pressman, D. H. Campbell, C. Ikeda, and M. Ikawa, THIS JOURNAL, 64, 2994 (1942).

(2) L. Pauling, D. Pressman, and D. H. Campbell, *ibid.*, 66, 330 (1944).

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

(4) C. Robinson, *Trans. Faraday Soc.*, 31, 245 (1935).

(5) E. Rabinowitsch and L. F. Epstein, THIS JOURNAL, 63, 69 (1941).

(6) W. C. Boyd and J. Behnke, *Science*, 100, 13 (1944).