Precipitin and Hapten Inhibition Reactions in Serological Systems Involving Simple Chemical Substances Of Known Constitution

Thesis by

Allan Louis Grossberg

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I thank Dr. Stanley Swingle and Mr. John T. Maynard for the privilege of collaborating with them on portions of the work reported.

Preface

The studies reported below were done as part of the systematic program of immunochemical investigation being carried out at the Gates and Crellin Laboratories of the California Institute of Technology. They are concerned mainly with investigations of systems first developed for study at the start of the program in 1940.

Studies employing the precipitin and hapten inhibition reactions are useful in giving insight both into the structure of the protein antibody molecule and into the mechanism of the antibody-antigen precipitin reaction.

Cetain interpretations of the results of such studies herein mentioned are due to others, as will be made clear. However, certain interpretations will be advanced below for which the author assumes complete responibility, and will be designated as such.

The experimental data consist largely of the results of analyses of several thousand antigen-antibody precipitates by a method reported by Dr. David Pressman (ref. 5) and perfected by him with some assistance from others, including the author. Some of the data have been included in papers already published or about to be published (ref. 4 VII) and much of the interpretation of the structure of reactants and the mechanisms involved has advanced far beyond the stage existing at the time these studies were carried out. These later and presumably more correct interpretations, which are concerned with a heterogeneous antiserum, are indicated and included in this thesis because they represent the more quantitatively

correct and most advanced ideas which have been developed on the basis of the data included. Much contained in these interpretations depends, however, on further work done by others (ref. 4 VII).

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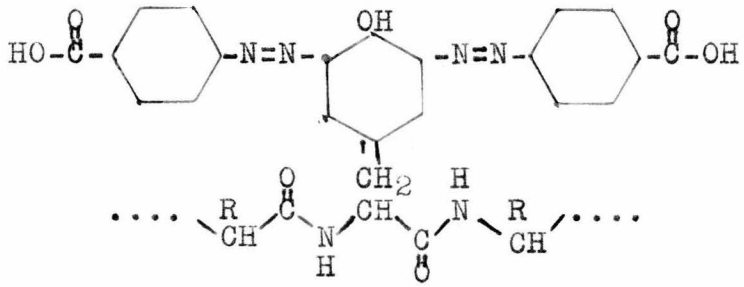
Introduction

The use of simple chemical substances of known constitution as precipitating antigens and of azoproteins as immunizing antigens was first perfected through the pioneering work of Dr. Karl Landsteiner and his associates (ref. 1). Although some previous work had been done, they were the first to demonstrate conclusively that simple chemical molecules, coupled to proteins, could be used to give rise to antibodies specifically directed against the coupled molecular structure when these treated proteins were injected into experimental animals. Careful work revealed that the presence of such antibodies could be demonstrated by using as test antigens proteins of widely different character from that of the injected antigen, to which were coupled the molecule under consideration. Thus, for example, it was found that the azo group provided the most convenient method of coupling structures to proteins. The coupling is evidently due to the reaction of a diazonium salt with the tyrosine and histidine groups contained in most proteins, and perhaps with some other amino acids (ref. 1, p. 103), giving rise to structures such as tyrosine-(bis)azobenzoic acid, if the diazonium salt of p-aminobenzoic acid is used:



(R = other amino acid "tails" in the peptide chain)

(Note: Although the above structure contains two azo groups coupled to the typrosine molecule, this is not meant to imply that every tyrosine or other molecule capable of coupling with more than one group, has two groups in the actual azoprotein.)

If then, the above mentioned diazonium salt is coupled to horse serum proteins and the resulting azoprotein injected into rabbits, after a sufficient time it can be demonstrated not only that the serum from such rabbits will give a precipitate with the azoprotein used for injecting (the immunizing antigen) but also that the serum will give a precipitate with an azoprotein prepared by coupling the same diazonium salt to another widely different protein, e.g., ovalbumin (the test antigen). It can further be demonstrated that untreated ovalbumin will give no reaction with this immune serum. The significance of these observations lies in the fact that thereby is demonstrated the existence in the rabbit serum of precipitating substances (antibodies) which react specifically with the coupled group, the azobenzoic acid group. The rabbit is thus said to have been immunized specifically against the structural group, azobenzoic acid, and its immune serum is said to contain antibodies specifically directed against this group.

It should be mentioned that there are other ways of demonstrating the presence of antibodies than by the precipitin reaction here involved. These other methods, such as complement fixation, demonstration of hypersensitivity (anaphylactic shock or smooth muscle contraction <u>in vitro</u>), etc., are discussed in any standard work on immunology. With the use of these other specific reactions it can also be demonstrated that azoproteins do cause the production of

antibodies which are specific for the coupled group involved.

As a further extension of this type of work, it was also discovered by Dr. Landsteiner and his associates that azodyes of a simple nature would give specific precipitates with antisera from, and elicit anaphylactic shock in, experimental animals injected with an azoprotein containing the dye-haptenic group. Thus the azodye resorcinol-disazo-p-suberanilic acid gave a precipitate with antisera from animals injected with an azoprotein containing the azo-p-suberanilic acid group. The tentative explanation given by Dr. Landsteiner [1] for the

[1] Ref. 1, p. 120

reason such a substance forms specific precipitates was that its relative insolubility, combined with its tendency to enter the colloidal state, is conducive to the formation of specific precipitates. However, work done in these laboratories, as will be explained further below, indicates that a more specific structural explanation can be invoked.

None-the-less, using these kinds of materials and techniques, Dr. Landsteiner and his associates were able to add much to the knowledge of the specificity of immunological reactions. They were able to conclude, in general, that the specificity of the antigen depends both on the nature of the ionic groups involved and on the structure of the antigen as a whole. Thus, for example, they were able to demonstrate that substituents on the benzene ring cause the substituted azobenzoic acid antigens to react less strongly with the antibody against the unsubstituted acid than does an antigen containing the homologous haptenic group. They also demonstrated that the nature of the acid group involved is of "decisive influence." For example, sera against benzene sulfonic acid

reacted with several substituted benzene sulfonic acid antigens, but very weakly or not at all with benzoic acid antigens, while phenylarsonic acid antigens gave rise to antisera which gave precipitates with the phenylarsonic acid antigens alone.

Landsteiner and his co-workers also used the hapten inhibition technique to further their studies. A hapten, in this sense, is considered to be a compound or substance of relatively low molecular weight which specifically inhibits the precipitation of antibody by antigen when present in a solution containing the two, but which does not, by itself, give a precipitate with the antibody. Their studies using this technique were mainly qualitative in nature, but did indicate that there was a difference in the ability of different, related haptens to inhibit a given antibody-antigen predipitation.

Among other workers who, in recent years, have contributed to this field might be mentioned Dr. Felix Haurowitz and his associates, who have published a number of papers[2a,b,c] in

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[2](a) F. Haurowitz and F. Breinl, Z. Physiol. Chem., 214, 111 (1933)
(b) F. Haurowitz et. al., J. Immunol., 40, 391 (1941)
(c) F. Haurowitz, ibid., 43, 331 (1942)
(d) F. Haurowitz, F. Kraus and F. Marx, Z. Physiol. Chem., 245, 23 (1936)
(e) F. Haurowitz and P. Schwerin, Brit. J. Exptl. Path., 23, 146 (1942)

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which careful studies of serological reactions involving numerous chemically altered proteins are presented. In 1936 Haurowitz, Kraus and Marx[2d] pointed out the possibility of the combination of antigen and antibody being due to molecular forces brought into play at close range. These workers are also attempting to understand the nature of the mechanism of the precipitin reaction.[2e]

An excellent summary of much of the work along these lines,

up to 1939, can be found in reference 2, chapter III.

In 1940 Professor Linus Pauling published a theory of the structure and method of formation of antibodies (ref. 3) in which, among other things, it was suggested that the precipitin reaction depended on the presence of two or more haptenic groups in the antigen molecule which allow a divalent antibody molecule to form a precipitate by building up an aggregate held together by the specific interactions involved. Subsequent experimental testing of this idea (ref. 4 I) indicated its correctness, in that of twenty-seven simple substances containing the phenylarsonic acid group as haptenic group, the twenty which contained two or more haptenic groups in the molecule were found to give precipitates with antisera prepared by injecting rabbits with sheep serum azophenylarsonic acid, while the seven compounds containing only one haptenic group in the molecule did not give precipitates with the same sera. This was taken as evidence in support of the framework theory of serological precipitates.[3]

[3]Ref. 2; ref. 3; M. Heidelberger, Chem. Rev., 24, 323 (1939); Bact. Rev., 3, 49 (1939).

In extending their studies on serological reactions, Professor Pauling and his co-workers developed a simple quantitative theory of hapten inhibition[4] in which the reciprocal

[4]Ref. 4 II

of the slope of the curve plotting amount of precipitate formed against amount of hapten present was given by the equation:[5]

$$-\frac{d\underline{H}}{d\underline{AB}(pp)} = \frac{\underline{C}}{\underline{K}'} + \underline{C}' \qquad (1)$$

[5]Ref. 4 IV

In equation (1) $\underline{K}'$ is the equilibrium constant for combination of hapten and antibody and $\underline{C}$ and $\underline{C}'$ are constants involving the solubility of the precipitate and the equilibrium constant for the combination of a haptenic group on the antigen with antibody, and are thus constant for a given antigen-antibody system. It was suggested[5] that $\underline{C}'$ be neglected as an approximation and thus $\underline{K}'$ be taken as proportional to the negative slope, $\frac{-d\underline{AB}(pp).}{d\underline{H}}$ It was found, however,[4,5] that experimental points satisfied the straight line relationship required by equation (1) only in the region of very slight inhibition, and that at higher hapten concentrations the inhibition was less than predicted. At the time it was suggested that this was due to the heterogeneity of antisera, which presumably contain antibodies of varying degrees of affinity for the antigen, in accord with the postulated mechanism of antibody formation (ref. 3).

I.

The Precipitation of Polyhaptenic Substances and Antiserum Homologous to the p-(p-Azophenylazo)-phenylarsonic Acid Group and its Inhibition by Haptens

The published paper which follows describes investigations the experimental work of which the author carried out in collaboration with Mr. John T. Maynard. The interpretations of the data which are given were developed largely by the senior author and Professor Pauling.

[Reprinted from the Journal of the American Chemical Society, **65**, 728 (1943).]

[CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, No. 916]

The Serological Properties of Simple Substances. V. The Precipitation of Polyhaptenic Simple Substances and Antiserum Homologous to the *p*-(*p*-Azophenylazo)-phenylarsonic Acid Group and its Inhibition by Haptens

BY DAVID PRESSMAN, JOHN T. MAYNARD, ALLAN L. GROSSBERG, AND LINUS PAULING

To extend our studies of the reactions of antibodies with simple substances,[1,2,3,4] which so far have been concerned with antisera made by inoculating rabbits with sheep serum coupled with diazotized *p*-arsanilic acid (we hereafter refer to these antisera as anti-R sera), we have now investigated the reactions of antisera made by inoculation with sheep serum coupled with diazotized *p*-(*p*-aminophenylazo)phenylarsonic acid (anti-R′ sera). These investigations, the results of which are reported in the present paper, included primarily quantitative studies of the precipitation of anti-R′ serum with simple substances containing two or more haptenic groups and the inhibition of these precipitation reactions by haptens; some work was also done on the precipitation reactions of anti-R′ and anti-R sera with ovalbumin coupled with diazotized arsanilic acid (R-ovalbumin) and with ovalbumin coupled with diazotized *p*-(*p*-aminophenylazo)phenylarsonic acid (R′-ovalbumin).

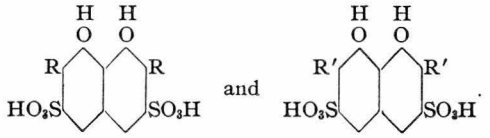
Experimental Methods

Protein Antigens.—The antigen used for inoculations was prepared by coupling diazotized *p*-(*p*-aminophenylazo)phenylarsonic acid with crude sheep serum globulin at *p*H 9. The azoprotein was purified by the method of Landsteiner and van der Scheer.[5] It was found on analysis to contain 2.1% arsenic. The crude serum globulin was precipitated from sheep serum by the addition of an equal volume of saturated ammonium sulfate solution.

The protein test antigens were prepared similarly with use of purified ovalbumin. The number of haptenic groups per molecule (with assumed molecular weight 40,000 for ovalbumin) was calculated from the results of analyses for arsenic[6] to be 8.8 for R-ovalbumin and 8.4 for R′-ovalbumin.

Preparation of Antisera.—The antisera were prepared in rabbits as described previously[1] for the preparation of anti-R sera. The sera from several bleedings after several different courses of injections were pooled according to titer to three pools, A, B and C.

Simple Antigens and Haptens.—The simple substances used have been described previously[1,4,7] except for the substances



The R substance was prepared by Mr. Carol Ikeda by adding diazotized arsanilic acid in 25% excess to chromotropic acid in sodium carbonate solution. The product was purified by repeated solution in sodium hydroxide solution and precipitation with hydrochloric acid, followed by recrystallization from 50% ethanol solution. The R′ analog was made by Mr. David H. Brown by adding diazotized *p*-(*p*-aminophenylazo)phenylarsonic acid in 50% excess to chromotropic acid in sodium carbonate solution containing pyridine. The product was purified by precipitation with hydrochloric acid and salt and solution in sodium hydroxide, followed by dialysis against water through cellophane sausage casing until the rate of dialysis of colored substance became negligible. This removed substances of smaller molecular dimension than the desired product, which does not pass through the membrane readily. The product was acidified and then precipitated with alcohol and washed with alcohol. These substances were analyzed chromatographically by Mr. Arthur Pardee as discussed previously.[1] The R substance was found to be essentially pure while the R′ substance contained 5 to 10% of a colored impurity. Carbon and hydrogen analyses indicated that the substances were sodium salts.

Reaction of Antiserum with Antigen and Hapten.—The reactants were mixed and permitted to stand one hour at room temperature and overnight in a refrigerator at 3°. The precipitates were then washed 3 times with 10-ml. portions of 0.9% saline solution, and the amounts of protein were determined by use of the Folin–Ciocalteu[8] reagent by a method to be described elsewhere.

The borate buffers were prepared by adding 0.16 *N* sodium hydroxide solution to 0.2 *M* boric acid in 0.9% sodium chloride solution. All dilutions of antigen and hapten were made with buffer of *p*H 8.0 unless otherwise noted.

Precipitation Reactions of Anti-R′ Serum and Simple Substances.—Precipitation tests first were made with the compounds VI, IX, XI, XIV, XX, XXIII, XXX, and XXXI.

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

(2) L. Pauling, D. Pressman, D. H. Campbell, and C. Ikeda, *ibid.*, **64**, 3003 (1942).

(3) L. Pauling, D. Pressman, and C. Ikeda, *ibid.*, **64**, 3010 (1942).

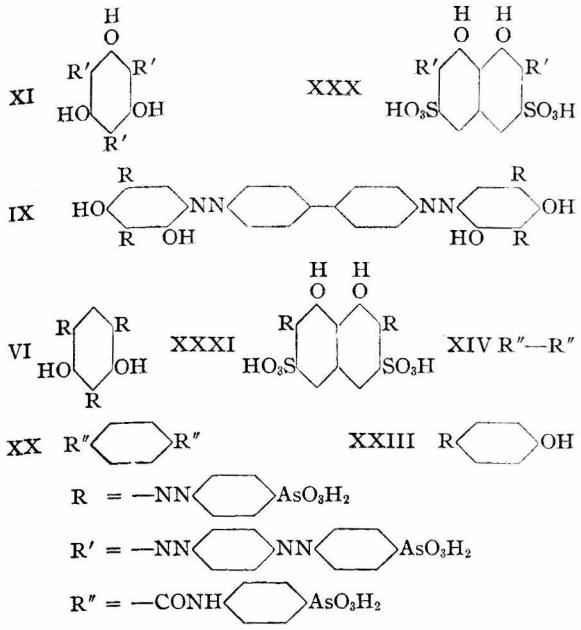
(4) D. Pressman, D. H. Brown, and L. Pauling, *ibid.*, **64**, 3015 (1942).

(5) K. Landsteiner and J. van der Scheer, *J. Exp. Med.*, **55**, 781 (1932).

(6) By the method of E. B. Sandell, *Ind. Eng. Chem., Anal. Ed.*, **14**, 82 (1942).

(7) D. Pressman and D. H. Brown, THIS JOURNAL, **64**, 540 (1942).

(8) O. Folin and V. Ciocalteu, *J. Biol. Chem.*, **73**, 627 (1927).



Equal volumes of anti-R′ serum A and of antigen solution of concentrations varying from 200×10^{-6} to 12.5×10^{-6} *M* in two-fold dilutions were mixed. The experiments were run in triplicate, with use of 1 ml. each of antigen solution and antiserum for antigens XI and XXX and 3 ml. for all the others except antigen IX, for which only 0.5 ml. was used and only a single tube was set up at each concentration. Precipitates were obtained only with the antigens XI, XXX, and IX. A normal precipitation reaction, showing an optimum zone, was observed. The results are given in Table I.

TABLE I

PRECIPITATION OF ANTI-R′ SERUM A AND SIMPLE ANTIGENS

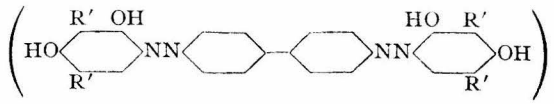
Antigen solution and antiserum, 1 ml. each. *p*H of supernates 8.1 for XI and XXX, 8.2 for IX. Control, antiserum and buffer, 5 μg.

	Moles of antigen added × 10^9				
	12.5	25	50	100	200
Antigen	Amount of antibody precipitated (μg.)[a]				
XI	233	612	(1172)	1055	(695)
XXX	(367)	(778)	1070	1053	748
IX[b]	[46]	[102]	[12]		

[a] The values given without parentheses are averages of triplicate analyses, with mean deviation of ±4%; those in parentheses are averages of duplicate analyses, and those in square brackets are single analyses only. [b] Run was made using 0.5-ml. portions. Reported values are for 1-ml. portions.

No precipitate would be expected with the monohaptenic substance XXIII. It is very interesting that no precipitates were obtained with the polyhaptenic substances VI, XIV, XX, and XXXI. These all gave precipitates with anti-R serum,[1] although not so large as given by antigen XI by a factor of three or four.

With anti-R′ serum B, which was obtained several months after anti-R′ serum A, a slight amount of precipitate was obtained with antigen VI. Antigen IX gave an equal amount of precipitate while antigen XII gave over thirty



times as much. The results are in Table II.

TABLE II

PRECIPITATION OF ANTI-R′ SERUM B AND SIMPLE ANTIGENS

Antigen solution and antiserum, 1 ml. each. *p*H of supernates 8.1. Control, antiserum and buffer, 0 μg.

	Moles of antigen added × 10^9				
	8	16	32	63	126
Antigen	Amount of antibody precipitated (μg.)[a]				
XII	24	160	676	919	476
IX	0	19	29	12	
VI	0	26	27	2.2	

[a] Averages of triplicate analyses.

Precipitation of Anti-R′ Serum and Anti-R Serum by R′-ovalbumin and R-ovalbumin.—In order to see whether the failure of some R-antigens to form appreciable precipitates with anti-R′ serum extended also to azoproteins, precipitation tests were carried out with anti-R′ serum and anti-R serum and the two azo-ovalbumins, with the results given in Table III. It is seen that each of the antigens R′-ovalbumin and R-ovalbumin formed precipitates with each of the antisera. Although the number of haptenic groups per molecule was nearly the same for the two azo-ovalbumins (8.4 for R′, 8.8 for R), the antigen with the long groups R′ was found to precipitate much more antibody from each antiserum than that with the short groups R. The ratio of R′-precipitable antibody to R-precipitable antibody in each of the antisera was about 4, whereas the ratio in the case of the dyes is about 30 for anti-R′ serum and, as previously determined, 3 to 4 for anti-R serum.[1]

The Effect of Hydrogen Ion Concentration on the Precipitation of Anti-R′ Serum and Antigen XXX.—Precipitation experiments were carried out at five different hydrogen ion concentrations from *p*H 7.6 to 9.1 as controlled by borate buffer and also in normal saline solution at *p*H 7.9, with the results given in Table IV. Optimum precipitation was found to occur at about *p*H 7.9, and the replacement of buffer by saline solution was found to have small effect. The antigen concentration at which the maximum amount of antibody is precipitated becomes lower with increasing *p*H. These observations are similar to those made[2] with anti-R serum and antigen VI.

The Relative Inhibiting Powers of Different Haptens.—To extend our studies of the inhibiting powers of haptens, hapten inhibition experiments similar to those made with anti-R serum[4] were carried out with anti-R′ serum. In Table V there are given the results obtained with antigens XXX and XII and anti-R′ serum C and the haptens XXI, XXXII, XXXIII, XXXIV, and XXXV.

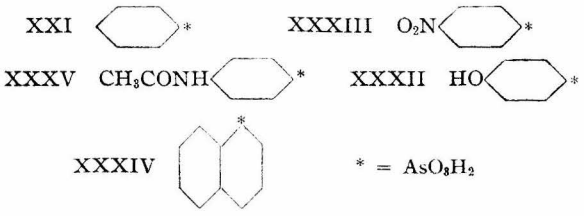


TABLE III

PRECIPITATION OF ANTI-R′ SERUM B AND ANTI-R SERUM U BY R′-OVALBUMIN (8.4 HAPTENIC GROUPS PER MOLECULE) AND R-OVALBUMIN (8.8 HAPTENIC GROUPS PER MOLECULE)

2 ml. each of R-ovalbumin solution and antiserum or 1 ml. each of R′-ovalbumin solution and antiserum. Data are given on basis of 1 ml. of antiserum. *p*H of supernates 8.1 to 8.2. Blank of serum and buffer, 0.0 μg. B is amount of antibody in precipitate (μg.). Average of triplicate analyses with mean deviation ±2.7%. A is amount of antigen in precipitate (μg.). Average of duplicate analyses with mean deviation ±3.0% for amount greater than 50 μg. and ±11% otherwise. The small values are unreliable.

Amount of antigen (μg.)	46		93		185		370		730		1460	
	B	A	B	A	B	A	B	A	B	A	B	A
R′-Ovalbumin, anti-R′ serum	330	22	813	54	1343	118	1248	145	840	140		
R′-Ovalbumin, anti-R serum	298	51	603	53	848	75	898	119	633	120		
R-Ovalbumin, anti-R′ serum			100	20	211	34	330	46	332	40	198	28
R-Ovalbumin, anti-R serum			77	14	171	16	230	30	181	31	67	23

TABLE IV

THE EFFECT OF HYDROGEN ION CONCENTRATION ON PRECIPITATION OF ANTI-R′ SERUM A AND ANTIGEN XXX

Antigen solution, 1 ml.; antiserum and buffer, 1/2 ml. each.

*p*H of supernates	Amount of antigen added (μg.) 8.3	12.5	18.7	29.6	44.4	66.7
	Amount of antibody precipitated (μg.)[a]					
7.6	89	139	218	266	(305)	259
7.9	161	200	288	356	331	306
7.9[b]	114	197	297	362	372	320
8.3	130	176	226	235	(217)	176
8.7	132	157	139	114	(89)	(70)
9.1	121	141	105	83	58	56

[a] Averages of triplicate analyses with mean deviation ±6%; duplicate analyses in parentheses. [b] Run made with saline solution replacing borate buffer.

Experiments were also made with antigen XXX and anti-R′ serum A and the twenty-six haptens listed in Table VI, in which the results are recorded.

TABLE V

INHIBITION BY HAPTENS OF PRECIPITATION OF ANTI-R′ SERUM C AND ANTIGEN XII AND XXX AT OPTIMUM ANTIGEN CONCENTRATION

Antigen solution, hapten solution, and antiserum, 1 ml each.

Antigen	Hapten	Moles of hapten × 10^9 0	25	50	100	200
		Amount of antibody precipitated (μg.)[a]				
XXX, 33 μg.	XXI	295	252	251	(209)	169
	XXXII		222	195	165	(120)
	XXXIII		224	188	145	109
	XXXIV		211	162	140	79
	XXXV		204	159	134	101
XII, 50 μg.	XXI	404	(310)	(295)	213	167
	XXXII		266	224	170	133
	XXXIII		235	(184)	119	70
	XXXIV		248	186	133	87
	XXXV		226	175	121	76

[a] Averages of analyses in triplicate, with mean deviation ±4%; duplicate analyses in parentheses.

Discussion

The data given in Tables IV, V, and VI are similar to those found for anti-R serum and reported in earlier papers of this series. In particular the data given in Table V provide some further support for the prediction of our simple quantitative theory of hapten inhibition[2,4] that the order of inhibiting power of a series of haptens is for a given antiserum the same for different antigens; for antigens XXX and XII and anti-R′ serum the same order was found for the five haptens listed in Table V except for interchange of haptens XXXIII and XXXIV.

With use of the procedure described in the preceding paper,[4] values of the constant K' representing the strength of the hapten-antibody bonds were derived from the initial slopes of the curves of amount of precipitate plotted against the number of moles of hapten. The average for the two azohaptens was placed equal to 1. These values are given in the second column of Table VI. In the third column are the values of K' (listed as K'^*) for anti-R serum as found previously.[4]

It is seen, as was found also for anti-R serum in the preceding investigation,[4] that there is no correlation between these values of K' and the values of the second ionization constant of the arsonic acids, which are in the fourth column.

The most striking feature of the K' values is their independence of the position of the substituent in the substituted phenylarsonic acids. It was found in the earlier study with anti-R serum that for a given substituent the *o*-substituted phenylarsonic acid had a small value of K', the *m*-substituted acid a larger one, and the *p*-substituted acid a still larger one, with the value of K' increasing by about 100% at each step. This phenomenon was attributed to steric hindrance of the group (methyl, amino, nitro) in the ortho or meta position; it was assumed that the pocket formed as the antibody folded about a *p*-azophenylarsonic acid group of the immunizing azoprotein would fit a *p*-substituted group closely, and a *m*-substituted or *o*-substituted group less closely. But the data of Table VI for anti-R′

TABLE VI

INHIBITION OF PRECIPITATION OF ANTI-R′ SERUM A AND ANTIGEN XXX BY HAPTENS

Antigen solution, 1.25 ml. (43.5 μg.); antiserum, 0.75 ml.; hapten solution, 1 ml.

	K′	K′*[a]	$K_{A_2} \times 10^9$[b]	Moles of hapten added × 10^9: 6.25	12.5	25	50	100	200
				Amount of antibody precipitated (μg.)[c]					
Methylarsonic acid[d]	0.00								
o-Aminophenylarsonic acid	.10	0.13	2.2			485	468	420	382
m-Aminophenylarsonic acid	.11	.29	2.4			471	447	385	(360)
p-Carboxyphenylarsonic acid	.14	.29	3.6			435	(411)	392	315
p-Aminophenylarsonic acid	.15	.44	1.2			438	(412)	347	302
Phenylarsonic acid	.15	.26	3.3			438	397	350	295
p-Hydroxyphenylarsonic acid	.16	.60	3.9			432	395	337	232
p-Methylphenylarsonic acid	.18	.80	2.1			444	393	293	211
m-Methylphenylarsonic acid	.19	.53	1.5			417	372	300	209
o-Methylphenylarsonic acid	.20	.13	1.4			425	310	263	189
β-Naphthylarsonic acid	.21	.66	3.4			423	387	261	171
p-Chlorophenylarsonic acid	.25	.80	5.6			421	373	255	184
p-Bromophenylarsonic acid	.27	.80	6.5			419	347	232	157
p-Iodophenylarsonic acid	.31	.80	5.7			382	310	199	110
p-Benzoylaminophenylarsonic acid	.35	.80		476	443	369	258		
p-Nitrophenylarsonic acid	.39	1.40	13.5	465	416	353	252		
o-Nitrophenylarsonic acid	.39	0.28	2.9			(345)	253	161	114
m-Nitrophenylarsonic acid	.40	.75	18.2			311	247	(144)	103
p-(*p*-Aminobenzoylamino)phenylarsonic acid	.46	.89		(428)	(410)	311	212		
p-Acetaminophenylarsonic acid	.48	1.02		407	365	319	239		
p-(*p*-Nitrobenzoylamino)phenylarsonic acid	.50	0.89		384	338	299	(202)		
α-Naphthylarsonic acid	.59	.23	2.2			253	191	128	79
1,4-Aminonaphthylarsonic acid	.65	.17	0.7			258	162	117	63
p-(*p*-Hydroxyphenylazo)phenylarsonic acid	.94	.98		(419)	311	213	124		
p-(*p*-Aminophenylazo)phenylarsonic acid	1.06	1.02		(290)	215	125	62		
VI	1.60			(155)	(65)	(32)	(13.2)		

Average value with buffer in place of hapten, 537 μg. Blank with 2 ml. buffer and 1 ml. antiserum, 3 μg. *p*H of supernates 8.05 to 8.10.

[a] Values for anti-R serum found previously.[4] [b] Second dissociation constant of the arsonic acid.[6] [c] Averages of triplicate analyses with mean deviation of ±4%; duplicate analyses in parentheses. [d] Values 518 and 532 μg. protein obtained at 400 and 800 × 10^{-9} moles hapten, respectively.

serum do not show this effect; the values of the *o*, *m*, and *p*-substituted acids lie very close together for the methyl, amino, and nitro compounds. This observation was verified by similar tests with another pool of anti-R′ serum.

An interpretation of these results which we suggest is the following. It was pointed out in a theoretical discussion[9] that in general all effective antigen–antibody bonds should have about the same strength, and that in consequence antibodies to antigens containing strong groups should show low specificity, and those to antigens containing weak groups should show high specificity. We may consider that in order to form a sufficiently strong bond with the short haptenic group —NN⟨⟩AsO_3H_2 the antibody would have to fit it closely, whereas an equally effective antibody to the longer haptenic groups —NN⟨⟩NN⟨⟩AsO_3H_2 might fit the group only very loosely—so loosely as to permit the easy attachment of ortho- and meta-substituted phenylarsonic acids. This looseness of fit is also indicated by the large values of K' shown by α-naphthylarsonic acid and 1,4-aminonaphthylarsonic acid.

Aside from this, the values of K' for the haptens with anti-R′ serum show essentially the same reasonable dependence on the structure of the hapten as found for anti-R serum. The values of K' for the *p*-substituted phenylarsonic acids depend on the substituent in the order HOC_6H_4NN > CH_3CONH > NO_2 > C_6H_5CONH > I > Br > Cl > CH_3 > OH > H, NH_2 > COOH. This order is nearly the same as that found for anti-R serum, which was NO_2 > CH_3CONH > HOC_6H_4NN > C_6H_5CONH > Cl, Br, I, CH_3 > OH > NH_2 > COOH > H, the only differences being the interchange of the azo and nitro groups, the change in position of phenylarsonic acid itself, and the small differences found for methyl, chloro, bromo, and iodo groups with anti-R′ serum. These

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

changes in order may not be characteristic of anti-R and anti-R′ serum, since different pools of the same antiserum are observed to give somewhat different results.

The importance of the phenyl group is shown by the fact that methylarsonic acid has practically no inhibitory power.[10] On the other hand, the haptens $HOC_6H_4NNC_6H_4AsO_3H_2$ and $H_2NC_6H_4NNC_6H_4AsO_3H_2$, which are very like the immunizing haptenic group $-NNC_6H_4NNC_6H_4AsO_3H_2$, show very strong inhibition.

The very strong inhibition shown by the trihaptenic substance VI is probably due to the entropy effect of the three haptenic groups.

The fact that this substance and the other polyhaptenic substances containing R groups give at most only slight precipitates with anti-R′ serum we attribute to steric repulsion of antibodies; their strong inhibitory action shows that a strong bond can be formed with one antibody molecule. Presumably a group R with part of the nucleus to which it is attached occupies the cavity of an anti-R′ antibody; the remaining groups R then protrude from the antibody by such a small distance that another antibody cannot approach closely enough to form a bond. This steric effect is similar to that invoked earlier[3] in explanation of the observed effective bivalence of trihaptenic and tetrahaptenic antigens. The formation of an appreciable amount of precipitate by the exceptional substance IX we attribute to the large size of its molecules, in which arsonic acid groups are separated by six benzene rings. The same explanation—large distances between haptenic groups—applies to the precipitation of anti-R′ serum by R-ovalbumin (Table II).

(10) See also F. Haurowitz and F. Breinl, *Z. physiol. Chem.*, **214**, 111 (1933).

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Summary

Quantitative data are reported for the precipitation reactions of polyhaptenic simple substances and antisera prepared by inoculating rabbits with azoproteins made from *p*-(*p*-azophenylazo)phenylarsonic acid. Measurements were made of the inhibitory effect of each of twenty-six haptens on one antigen-antibody reaction, and interpreted to give values of the bond-strength constant of the haptens with the antibody. These values are discussed in relation to the structure of the hapten molecules and in comparison with the values previously found for an antiserum homologous to the *p*-azophenylarsonic acid haptenic group. An explanation in terms of steric hindrance is given of the failure of some polyhaptenic substances to give precipitates with the antiserum.

PASADENA, CALIFORNIA RECEIVED DECEMBER 23, 1942

Discussion.--As an addition to the published discussion given above, it might be well to mention something concerning the methods used in obtaining interpretations regarding structure and its effect on the reactions involved. As is mentioned in the publication, the values of K' are obtained by plotting the amount of antibody precipitated against the total amount of hapten present, drawing a smooth curve and finding the relative initial slopes of the curves for each hapten. This procedure involves a good deal of arbitrariness in weighting the points far removed from the initial portion of the curve, since there is often "crossing over" of curves in the region of higher hapten concentration. The use of the extended theory of hapten inhibition developed by Professor Pauling, which takes into account heterogeneous antisera, does away with this arbitrariness in determining K'. This theory will be discussed below.

An observation worthy of emphasis and one which will be referred to again below is that R'-containing antigens precipitate more antibody from both anti-R and anti-R' sera than do R-containing antigens. This fact is brought out especially by the data in Table III above.

II.

Comparison of Inhibition of Anti-R and Anti-R' Precipitation

As an aid in obtaining more thorough knowledge of the relations between the structure and the serological reactions of simple substances, hapten inhibition studies using 24 haptens were carried out involving precipitation from the same pools of anti-R and anti-R' sera by both antigen XXX[6] and

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[6] Ref. 4 V, p. 729.

R'-ovalbumin.

Experimental

Preparation of Antisera, Antigens and Haptens.--The preparation of these materials is described or referred to in the appended publication (ref. 4 V). The R'-ovalbumin contained 2% arsenic.

The Hapten Inhibition Reaction.--The following description of the procedure used in carrying out the hapten inhibition reaction applies to all of the hapten inhibition data presented in this thesis, except for specific alterations in the method which are mentioned in the tables of data:

The antiserum is added to tubes containing the hapten solution, made up in borate buffer, 0.16 M. in salts, of the required pH. The antigen solution, in the same buffer, is added and the tubes are well shaken and allowed to stand one hour at room temperature and then over two nights in the refrigerator at 5°. The tubes are then centrifuged for five minutes, the supernates decanted and the precipitates transferred to other tubes and washed three times with 10 ml.

portions of 0.9% saline solution, centrifuging for five minutes after each washing. The washed precipitates are then analyzed by the standard method reported in reference 5.

In the above manner the inhibition of the 24 haptens was studied in four different antigen-antibody systems. Tables II-A and II-B give the results involving anti-R serum and antigens XXX and R'-ovalbumin, respectively, and Tables II-C and II-D give the results involving anti-R' serum and the same antigens, respectively. (These tables are made up from the data as given in Tables I and II of reference 4 VII.)

Discussion

The following discussion of the quantitative theory of hapten inhibition involving heterogeneous antisera is taken directly from reference 4 VII, as written by Professor Pauling:

"Let us assume that the heterogeneity of the antiserum is such that it can be described by a probability distribution function which is an error function in the effective free energy of combination of hapten and antibody (in competition with antigen); that is, in the quantity $\ln(\underline{K}'/\underline{K}'_0)$, where $\underline{K}'$ is the effective hapten inhibition constant of the particular antibody molecule under consideration and $\underline{K}'_0$ is an average effective hapten inhibition constant. The normalized distribution function itself is

$$\frac{1}{\sqrt{\pi}\,\sigma}\, e^{-\left\{\ln\left(\underline{K}'/\underline{K}'_0\right)\right\}^2/\sigma^2} \qquad (3)$$

and the fractional number of antibody molecules with given value of $\underline{K}'$ in a differential region is

Table II-A

Hapten Inhibition of Precipitation of Anti-R Serum with Antigen XXX

For antigen XXX: antigen solution, 1.75 ml. (22 μg.); anti-R serum, 0.25 ml.; hapten solution, 1 ml. pH of all supernates 8.05 - 8.10

	Antigen XXX		Moles of hapten added, x 10^9						
	K'_o	σ	2.4	4.7	9.4	18.8	37.5	75	150
			Amount of precipitate with antigen XXX[a]						
p-(p-Aminophenylazo)-phenylarsonic acid	1.00	2.0	906[b]	872[b]	813[b]				
p-(p-Hydroxyphenylazo)-phenylarsonic acid	1.00	1.5	945[b]	920[b]	843[b]				
p-Acetaminophenylarsonic acid	1.00	1.2	815	663	440				
p-(p-Nitrobenzoylamino)-phenylarsonic acid	0.43	2.1	849	765	627				
p-(p-Aminobenzoylamino)-phenylarsonic acid	.54	2.0	815	758	578				
p-Benzoylaminophenylarsonic acid	.63	1.5	859	760	585				
p-Nitrophenylarsonic acid	1.52	1.2	719	540	291				
m-Nitrophenylarsonic acid	0.47	1.0			696	[452]	218		
o-Nitrophenylarsonic acid	.095	1.2					725	527	288
p-Iodophenylarsonic acid	.89	1.0			494	226	80		
p-Bromophenylarsonic acid	.81	1.0			521	252	99		
p-Chlorophenylarsonic acid	.71	1.0			562	301	122		
p-Methylphenylarsonic acid	.50	1.1			660	448	192		
m-Methylphenylarsonic acid	.22	1.0			835	732	500		
o-Methylphenylarsonic acid	.022	1.8					913	846	725
β-Naphthylarsonic acid	.41	1.1			723	518	262		
α-Naphthylarsonic acid	.061	1.5					802	645	453
1,4-Aminonaphthylarsonic acid	.088	1.0					764	558	321
p-Hydroxyphenylarsonic acid	.22	1.8			795	655	480		
p-Carboxyphenylarsonic acid	.19	1.0					543	282	122
Phenylarsonic acid	.137	1.2					630	410	179
p-Aminophenylarsonic acid	.19	0.9			895	765	540		
m-Aminophenylarsonic acid	.127	1.1					657	428	192
o-Aminophenylarsonic acid	.027	1.5					915	807	679

a. The amounts are tabulated as fractions per mille of the amount precipitated in absence of hapten: this was 715 μg. Values are averages for triplicate analyses, with mean deviation ± 2%; single analyses are given in brackets.

b. These values are for hapten concentrations one-fifth of those indicated.

Table II-B

Hapten Inhibition of Precipitation of Anti-R Serum with R'-ovalbumin

For R'-ovalbumin: antigen solution, 1.80 ml. (140 μg.); anti-R serum, 0.20 ml.; hapten solution, 1 ml. pH of all supernates 8.05 - 8.10

	R'-ovalbumin		Moles of hapten added, x 10^9					
			9.4	18.8	37.5	75	150	300
	K'_o	σ	Amount of precipitate with R'-ovalbumin[a]					
p-(p-Aminophenylazo)-phenylarsonic acid	1.34	1.0	954[b]	895[b]	830[b]			
p-(p-Hydroxyphenylazo)-phenylarsonic acid	1.34	1.0	955[b]	914[b]	828[b]			
p-Acetaminophenylarsonic acid	1.07	1.5	760	634	421			
p-(p-Nitrobenzoylamino)-phenylarsonic acid	0.98	1.2	821	666	440			
p-(p-Aminobenzoylamino)-phenylarsonic acid	.77	1.5	844	710	530			
p-Benzoylaminophenylarsonic acid	.71	1.5	[865]	[745]	[555]			
p-Nitrophenylarsonic acid	2.06	1.2	648	437	193			
m-Nitrophenylarsonic acid	0.46	1.5		813	673	474		
o-Nitrophenylarsonic acid	.089	1.2				880	775	600
p-Iodophenylarsonic acid	.98	1.2		661	469	216		
p-Bromophenylarsonic acid	.81	1.5		687	514	291		
p-Chlorophenylarsonic acid	.69	1.2		759	562	322		
p-Methylphenylarsonic acid	.50	1.0		839	669	453		
m-Methylphenylarsonic acid	.18	1.5		915	844	743		
o-Methylphenylarsonic acid	.031	1.0				980	918	832
β-Naphthylarsonic acid	.49	1.2		820	668	445		
α-Naphthylarsonic acid	.049	1.5				919	832	707
1,4-Aminonaphthylarsonic acid	.079	1.0				884	798	602
p-Hydroxyphenylarsonic acid	.20	1.5		919	824	700		
p-Carboxyphenylarsonic acid	.17	1.5				751	576	358
Phenylarsonic acid	.125	1.5				804	660	434
p-Aminophenylarsonic acid	.18	1.5		909	851	733		
m-Aminophenylarsonic acid	.125	1.5				781	661	437
o-Aminophenylarsonic acid	.035	1.0				964	906	804

a. The amounts are tabulated as fractions per mille of the amount precipitated in absence of hapten: this was 769 μg. Values for R'-ovalbumin include the precipitated antigen protein. Values are average for triplicate analyses, with mean deviation ± 2%; single analyses are given in brackets.

b. These values are for hapten concentrations one-fifth of those indicated.

Table II-C

Hapten Inhibition of Precipitation of Anti-R' Serum with Antigen XXX

For antigen XXX: antigen solution, 1.25 ml. (67 μg.); anti-R' serum, 0.75 ml.; hapten solution, 1.0 ml. pH of all supernates 8.1 - 8.2

	Antigen XXX		Moles of hapten added, x 10^9					
	K'_o	σ	12.5	25	50	100	200	400
			Amount of precipitate with antigen XXX[a]					
p-(p-Aminophenylazo)-phenylarsonic acid	1.20	1.9	629	452	270			
p-(p-Hydroxyphenylazo)-phenylarsonic acid	1.05	1.8	651	501	305			
p-Acetaminophenylarsonic acid	0.24	1.7		812	670	510		
p-(p-Nitrobenzoylamino)-phenylarsonic acid	.32	2.0		736	619	436		
p-(p-Aminobenzoylamino)-phenylarsonic acid	.47	2.0		675	501	343		
p-Benzoylaminophenylarsonic acid	.37	1.8		754	568	424		
p-Nitrophenylarsonic acid	.22	1.8		825	701	539		
m-Nitrophenylarsonic acid	.25	2.0				502	318	190
o-Nitrophenylarsonic acid	.16	2.0				602	426	288
p-Iodophenylarsonic acid	.32	1.5				420	234	134
p-Bromophenylarsonic acid	.22	2.0				531	357	198
p-Chlorophenylarsonic acid	.17	1.5				596	417	250
p-Methylphenylarsonic acid	.17	2.0				602	411	268
m-Methylphenylarsonic acid	.089	1.5				750	607	404
o-Methylphenylarsonic acid	.084	2.0				725	575	430
β-Naphthylarsonic acid	.19	1.5				603	404	196
α-Naphthylarsonic acid	.25	1.5				516	313	182
1,4-Aminonaphthylarsonic acid	.32	1.7				431	252	137
p-Hydroxyphenylarsonic acid	.095	1.5				800	600	381
p-Carboxyphenylarsonic acid	.074	1.5				827	620	482
Phenylarsonic acid	.067	1.5				821	680	495
p-Aminophenylarsonic acid	.074	1.5				801	669	460
m-Aminophenylarsonic acid	.054	1.0				915	778	578
o-Aminophenylarsonic acid	.029	2.0				867	809	671

a. The amounts are tabulated as fractions per mille of the amount precipitated in absence of hapten: this was 748 μg. Values are averages of triplicate analyses, with mean deviation ± 4%.

Table II-D

Hapten Inhibition of Precipitation of Anti-R' Serum with R'-ovalbumin

For R'-ovalbumin: antigen solution, 1.50 ml. (175 μg.); anti-R' serum, 0.50 ml.; hapten solution, 1.0 ml. pH of all supernates 8.1 - 8.2

	R'-ovalbumin		Moles of hapten added, x 10^9					
	K'_o	σ	12.5	25	50	100	200	400
			Amount of precipitate with R'-ovalbumin					
p-(p-Aminophenylazo)-phenylarsonic acid	1.20	2.0	680	481	340			
p-(p-Hydroxyphenylazo)-phenylarsonic acid	1.02	2.0	690	541	386			
p-Acetaminophenylarsonic acid	0.20	2.0		813	772	605		
p-(p-Nitrobenzoylamino)-phenylarsonic acid	.30	2.5		755	648	497		
p-(p-Aminobenzoylamino)-phenylarsonic acid	.38	2.0		775	571	446		
p-Benzoylaminophenylarsonic acid	.29	2.5		780	648	521		
p-Nitrophenylarsonic acid	.30	1.0		903	773	541		
m-Nitrophenylarsonic acid	.22	2.0				588	413	260
o-Nitrophenylarsonic acid	.11	2.5				706	566	445
p-Iodophenylarsonic acid	.34	2.0				483	282	187
p-Bromophenylarsonic acid	.19	1.5				656	454	278
p-Chlorophenylarsonic acid	.17	1.5				694	457	295
p-Methylphenylarsonic acid	.16	2.0				660	465	324
m-Methylphenylarsonic acid	.063	3.0				744	655	530
o-Methylphenylarsonic acid	.060	2.5				795	684	550
β-Naphthylarsonic acid	.15	2.0				664	468	349
α-Naphthylarsonic acid	.15	2.0				678	494	347
1,4-Aminonaphthylarsonic acid	.19	1.7				641	434	276
p-Hydroxyphenylarsonic acid	.069	1.5				884	687	557
p-Carboxyphenylarsonic acid	.054	1.5				890	720	655
Phenylarsonic acid	.060	1.5				875	703	606
p-Aminophenylarsonic acid	.062	1.5				880	709	598
m-Aminophenylarsonic acid	.041	1.5				957	834	686
o-Aminophenylarsonic acid	.038	2.0				880	765	673

a. The amounts are tabulated as fractions per mille of the amount precipitated in absence of hapten: this was 562 μg. Values for R'-ovalbumin include the precipitated antigen protein. Values are averages of triplicate analyses, with mean deviation ± 4%.

$$\frac{dN}{N} = \frac{1}{\sqrt{\pi}\sigma} e^{-\left\{\ln (K'/K'_0)\right\}^2/\sigma^2} \; d \ln (K'/K'_0) \qquad (4)$$

Curves showing the distribution function for several values of the heterogeneity index σ, ...(indicate that)....for $\sigma = 1$ most of the antibody molecules (84%) have K' values in the range from 0.368 K'_0 to 2.718 K'_0, for $\sigma = 2$ this range is from 0.135 K'_0 to 7.48 K'_0, for $\sigma = 3$ from 0.050 K'_0 to 20.1 K'_0, and for $\sigma = 4$ from 0.018 K'_0 to 54.5 K'_0. The corresponding ranges of values of free energy of interaction of antibody and hapten have widths of 1200, 2400, ... cal. per mole for $\sigma = 1, 2, \ldots$.

This distribution of antibodies would occur in an antiserum if the heterogeneity were the result of a very large number of independent influences, each of which could increase or descrease the free energy of bond formation by an additive contribution. Since this is not unreasonable, in the light of current concepts of the structure of antibodies, we may well expect this distribution to be rather closely approximated by the antiserum from a single animal or by the combined antisera from a large number of animals.[6] On the other hand, the combined antisera from only a few animals or a combination of unlike fractions obtained in the course of fractionation of antibodies might not have an error-function distribution and might not correspond in their properties to the present theory; the theory could, however, easily be extended to cover such

[6] It would not be surprising if the antiserum from a single rabbit inoculated with an azoprotein were to have a different type of distribution. The antibodies produced by the rabbit would be influenced by the nature of the place of attachment of the hapten to the protein (histidine or tyrosine side-chains). The distribution function for such an antiserum might be closely approximated by the sum of two or more error functions.

special cases.

If we assume that for each kind of antibody the amount of precipitate is linear in the amount of hapten, with slope proportional to $\underline{K}'$, the amount of precipitate formed by the heterogeneous antiserum is given by the equation

$$\underline{P} = \frac{\underline{AB}\,(pp)}{\underline{AB}\,(pp)_{\underline{H}_{total}=0}} = \frac{1}{\sqrt{\pi}\,\sigma}\int_{-\infty}^{\ln\,(1/\underline{H}_{total}\,\underline{K}'_o)} (1 - \underline{K}'\underline{H}_{total}) \cdot \underline{e}^{-\{\ln\,(\underline{K}'/\underline{K}'_o)\}^2/\sigma^2}\, d\ln\,(\underline{K}'/\underline{K}'_o) \qquad (5)$$

The upper limit of the integral represents the value of $\underline{K}'$ at which the hapten just inhibits completely the precipitation of the corresponding antibody: no precipitate is formed by antibody with larger vlaues of $\underline{K}'$. The integral is easily evaluated in terms of the Gaussian probability integral $H(\underline{x})$, for which numerical values are given in tables[7]; the equation

7 Tables of Probability Functions. Vol. I. Federal Works Agency, Works Projects Administration, New York, 1941.

then assumes the form

$$\underline{P} = \tfrac{1}{2}\{1 + H(\underline{x}_1)\} - \tfrac{1}{2}\underline{H}_{total}\underline{K}'_o\,\underline{e}^{\sigma^2/4}\{1 + H(\underline{x}_2)\} \qquad (6)$$

with $\underline{x}_1 = \frac{1}{\sigma}\ln\,(1/\underline{H}_{total}\underline{K}'_o)$ and $\underline{x}_2 = \underline{x}_1 - \sigma/2$. The function $H(\underline{x})$ is to be taken with the same sign as $\underline{x}$.

Curves of the calculated amounts of precipitate for $\sigma = 0, 1, 2, \ldots$ plotted against $\underline{H}_{total}$ ·····(show)····· that with increase in σ there is increasing deviation from the linear relation, which holds for $\sigma = 0$. .....

A very satisfactory way of comparing experiment and theory is to use a logarithmic scale for the amount of hapten. Since $\underline{K}'_o$ and $\underline{H}_{total}$ occur in Equation 6 only as their product, the curves showing the amount of precipitate plotted against the

logarithm of $\underline{H}_{total}$ have the same shape when they correspond to the same value of σ ; the effect of changing $\underline{K}'_o$ is simply to shift the curve along the log $\underline{H}_{total}$ axis. Hence only a single family of such curves, covering a range of values of σ, is needed for the evaluation of σ and $\underline{K}'_o$ from a set of experimental points. Such a set of curves[8], for σ = 0, 1, 2, 3, 4, 5, is shown in Figure 1.

8 In interpreting the experimental data it was found convenient to prepare and use a set of transparent templates, covering the range σ = 0 to 5 at intervals of 0.5.

Only two experimental points are needed to evaluate $\underline{K}'_o$ and σ ; the agreement of three or more points with a theoretical curve provides a test of the theory." (End of quotation)

The fit of all experimental points to the theoretical curves is quite close, well within the expected error of $\pm$4% due to the uncertainty in analysis, so that they constitute a demonstration of the essential correctness of the assumptions involved in the theory.

Relative values of $\underline{K}'_o$ are calculated from the values of the hapten concentration at 50% inhibition, $\underline{K}'_o$ being inversely proportional to the hapten concentration for this amount of inhibition on each curve. An arbitrary value of 1.00 for the $\underline{K}'_o$ of p-(p-hydroxyphenylazo)-phenylarsonic acid in inhibition involving antigen XXX is used.

The values for $\underline{K}'_o$ extend over a larger range than those of $\underline{K}'$ and are to be considered more meaningful, for the reasons mentioned in part I of this thesis. The generalization, first deduced from the simple quantitative theory[5], that the relative order of values of $\underline{K}'$ should be independent of the antigen used, for a given antiserum, is seen to be upheld also for

To follow page 22

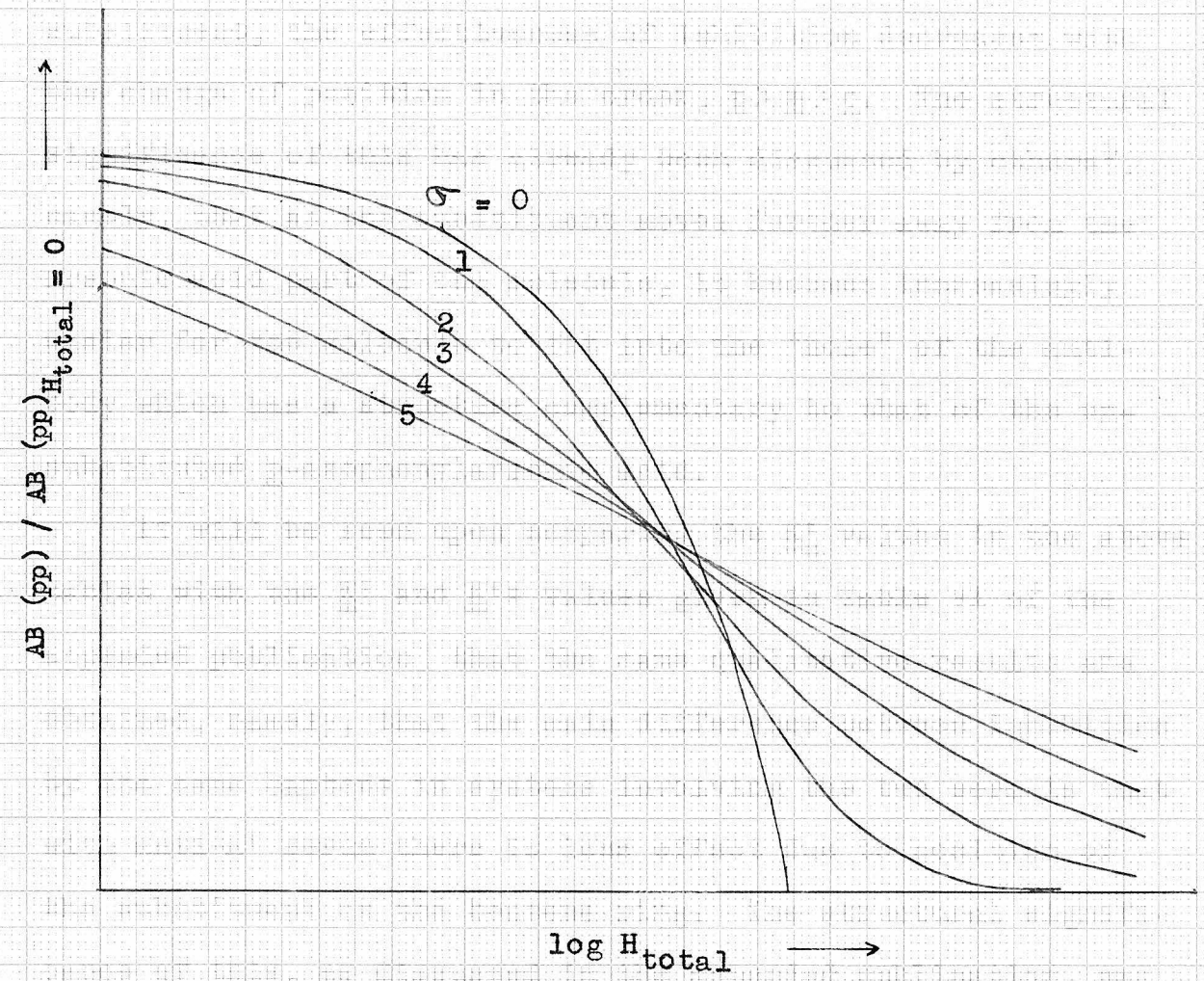


Figure 1.

Family of Curves for Determining σ and $\underline{K}'_o$ from Hapten Inhibition Data

K'_o values by the data in the above tables.

The effect of position of substituents on the benzene ring on the value of K'_o for anti-R inhibition is the same as was observed for K' (reference 4 IV). That is, for any given substituent, the effectiveness of inhibition decreases with the change of position in the order, p > m > o. The structural significance of this has already been discussed by others[5], namely, that as the substituent moves further away from the arsonic acid part of the molecule, it becomes increasingly easier for the molecule to fit into the "hole" of the antibody which has a structure complementary to that of the unsubstituted p-azophenylarsonic acid.

It will be seen upon comparing the K'_o values in the above tables with the K' and K'^* values given in Table VI of the appended publication that the same qualitative results are obtained, namely, that the main difference between inhibition by the same haptens in systems involving the two sera is that with anti-R' serum there is less effect due to position of the substituent on the benzene ring. The structural significance of this, as discussed in the appended publication, was taken to be that the specific complementary portion of the anti-R' molecule gives a much looser fit with the homologous haptenic group than does the corresponding structure of the anti-R molecule, so that enlarging the hapten molecule by putting substituents on the ortho or meta positions does not bring about very significant steric hindrance with the anti-R' molecule. The magnitude of this difference of effect is brought out by the figures given in Table II-E, which are calculated from the data in the above tables, the method of comparison

having been suggested by Professor Pauling. The table gives the ratio of the K'_o values for ortho and meta substituents to the K'_o values for the para substituent in each case.

Table II-E

Substituent	Anti-R serum		Anti-R' serum	
	A	B	C	D
		Meta/para		
Nitro	0.31	0.22	1.15	0.81
Methyl	.44	.36	0.54	.39
Amino	.67	.69	.73	.66
		Ortho/para		
Nitro	.063	.043	.73	.40
Methyl	.044	.062	.50	.37
Amino	.14	.19	.40	.60

Letters refer to the tables in this section (part II) of the thesis.

Table II-E also brings out the fact that the amino group shows much less of a relative "position effect" than do either the nitro or the methyl groups. The reason for this is not clear. The same effect has also been observed by the author and Dr. Stanley Swingle[9] in inhibition involving anti-serum specific for the p-azobenzoic acid group, coupled to bovine serum protein, so that it evidently has some general significance. As has been mentioned[5], it might be thought that part of the position effect would be due to the influence of substituents on the second acid constant of phenylarsonic acid, but such effect was not at all apparent[5], and it is

9 Work to be published in the J.Amer.Chem. Soc.

ruled out altogether in the case of the benzoic acid derivatives, since at the pH at which the inhibition is carried out (8.5) the carboxyl group is completely ionized.

The slight possibility of some chelation of the ortho amino group with the acid group, giving a more compact structure and thus less steric hindrance, does not appear as a very plausable explanation, since such chelation would reduce the available negative charge on the acid group, which would render the hapten less attracted to the specific structure of the antibody. Also, chelation would occur even more readily in the ortho hydroxy compound and such an effect, of decreased ortho effect was not noticed with salicylic acid[9]. The hydrogen bonding ability of the amino group, which would be expected to enable a molecule to be more strongly bound to the protein complimentary structure, cannot be invoked for the same reason, since the hydroxyl group is a better hydrogen bond former than the amino group.

It is interesting to speculate that the effect may be due to the presence of an amino group occurring close to the haptenic structure in the immunizing azoprotein. Such a free amino group, for example from lysine, while not attached to the haptenic group, might be attracted to its vicinity by the possibility of hydrogen bonding or even zwitter ion formation with the haptenic group, and thus influence the complimentary structure being built up around it during the process of antibody formation. This would probably necessitate that lysine occur one amino acid removed from tyrosine or histidine in the azoprotein peptide chain, so that the suggested interaction could take place.

It is clear, however, that such considerations are all complicated by the fact that the effect depends on both steric

effects and ion attraction and other forces, so that no clean-cut explanation can be given for inhibition effects in terms of structures involved until experiments are performed which enable the relative contributions of these effects to be studied. More will be said in this connection in part IV of this thesis.

Summary for Part II.

Hapten inhibition data in systems involving precipitation of antisera homologous to the p-azophenylarsonic acid group and to the p-(p-azophenylazo)-phenylarsonic acid group are treated in the light of an extended quantitative theory of hapten inhibition, taking into account the heterogeneity of antisera, developed by Professor Linus Pauling. The theory is found to be in good agreement with the experimental data.

Various possible explanations for some of the observed hapten inhibition effects are discussed.

III.

The Effect of Haptens with an Active Group Different from that of the Antigen Involved-- Evidence of Enhancement of Precipitation

An investigation of the effect of substances whose active (ionized) group is different from that of the antigen involved on the precipitation of anti-R and anti-R' sera by antigens containing the R and R' groups was undertaken. In some of the antigen-antibody systems it was observed that the substances caused enhancement of precipitation in a specific manner. An observation of this type has not previously been reported.

Experimental

Antisera and Antigens.--The antisera and antigens used have all been previously described in references 4 I and 4 V.

Haptenic Substances.--The substances investigated for their effect on the various antigen-antibody systems were:

(a) benzoic acid; (b) p-nitrobenzoic acid; (c) benzene sulfonic acid; (d) p-nitrobenzene sulfonic acid; (e) p-nitrophenol; (f) picric acid; (g) p-(p-hydroxyphenylazo)-benzoic acid; (h) p-(p-hydroxyphenylazo)-benzene sulfonic acid.

The first six substances listed were obtained as reagent grade industrial preparations. (g) and (h) were made by diazotizing p-aminobenzoic acid and p-sulfanilic acid, respectively, and coupling to an excess of phenol by the usual procedure. Recrystallization of the resulting products, from dilute aqueous hydrochloric acid in the case of (g) and from 40% ethanol containing aqueous HCl in the case of (h), gave well-formed yellow-orange crystals in each case. Melting point of the benzoic acid derivative was 267-268° (dec.). The sulfonic acid derivative crystallized as the sodium salt, even from

strongly acid solutions. Carbon-hydrogen analyses gave:

	for (g) $C_{13}H_{10}O_3N_2$			for (h) $C_{12}H_9O_4N_2SNa$	
	calc.	found		calc.	found
%C	64.5	64.1	%C	46.0	46.1
%H	4.2	4.1	%H	3.4	3.6
			%Na	7.7	7.8 (from ash)

Hapten Inhibition.--The substances were tested for their effect by using the same hapten inhibition technique as outlined above (part II of this thesis). Analyses involving precipitates containing anti-R protein and both dye and azoprotein antigens are given in Table III-A. Analyses involving anti-R' protein and dye and azoprotein antigens are given in Table III-B. Note that the inhibition due to phenylarsonic acid was tested with most of the systems to serve as a check.

The results represented by the data given in Tables III-A and III-B may be summarized as follows:

1. In only some of the systems involving anti-R serum is there appreciable inhibition of precipitation by the substances tested.

2. In all systems involving anti-R' serum the substances tested inhibited precipitation to a greater or lesser degree. The best inhibitors were the two substances (g) and (h).

3. Some of the substances, in certain of the systems involving anti-R serum, showed a marked tendency to cause more precipitate to form in their presence than did in their absence.

The very interesting observation of enhancement of precipitation was investigated further by means of the following experiments:

Table III-A

Effect of Certain Haptenic Substances on Precipitation of Anti-R Serum by Both R- and R'-Antigens

1.0 ml. antiserum solution; 1.0 ml. antigen solution; 1.0 ml. hapten solution; tubes stood one hour at room temperature and over one night at 5°. pH of all supernates: 8.0-8.1.

Antigen[c]	Amount of Hapten, moles x 10^7	Hapten[a] (a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)[b]
		μgm. of protein in precipitate								
XXXI	0	609	609		609		609	609	609	609
(29.4)[d]	2	570	598		564		556	594	566	387
	10	561	547		591		539	570	568	283
0.50 ml.[e]	50	583	540		576		475	493	492	185
VI	0	527	527		527		527	527	527	527
(16.7)	2	492	491		501		484	483	498	216
	10	490	491		479		494	473	489	122
0.25 ml.	50	476	466		455		513	484	416	67
oval.-R[f]	0							703	703	703
(57)	2							741	709	668
	10							739	726	632
0.50 ml.	50							738	698	595
XXX	0	715	715	715	715	715	715	715	715	
(33.3)	40	915	902	850	896	948	919	918	825	
	80	926	896	867	898	954	945	900	728	
0.25 ml.	160	927	928	890	894	935	1010			
XII	0	310	310		310		310	310	310	310
(22.2)	2	337	338		319		341	343	334	165
	10	355	351		355		328	347	323	102
0.125 ml.	50	336	340		362		348	354	295	42
oval.-R'[g]	0							509	509	509
(80)	2							507	505	458
	10							499	497	391
0.125 ml.	50							485	461	328

Analyses are averages of triplicates with mean deviation of ±4%.

[a] Letters refer to the substances listed on p. 27.

[b] This column gives inhibition results involving phenylarsonic acid; the hapten amounts in this case are, reading down for each set, 0, 0.25, 0.5 and 1.0 moles of phenylarsonic acid x 10^7.

[c] The numbered antigens are those given in references 4 I and 4 V.

[d] The figure under each antigen number gives the number of μgm. of antigen used in each 1.0 ml. of antigen solution.

[e] This figure is the amount of anti-R serum, pool A, used to make up the 1.0 ml. of serum solution.

[f] This azoprotein contained 8.8 haptenic groups per molecule

[g] This azoprotein contained 11 haptenic groups per molecule.

Table III-B

Effect of Certain Haptenic Substances on Precipitation of Anti-R' Serum by Both R- and R'-Antigens

1.0 ml. antiserum solution; 1.0 ml. antigen solution; 1.0 ml. hapten solution; tubes stood one hour at room temperature and over one night at 5°. pH of all supernates: 8.0-8.1.

Antigen[c]	Amount[i] of Hapten, moles x 10^7	Hapten[a] (a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)[b]
		μgm. of protein in precipitate								
IX[h]	0	163						163		
(33.3)[d]	3.1	152						143		
	12.5	159						161		
2.50 ml.	50	143						238		
XII	0	232	232		232		232	232	232	232
(40)	2	279	251		230		229	222	211	165
	10	255	228		217		227	211	204	121
0.40 ml.	50	258	243		222		273	179	139	89
XXX[i]	0	233	233	233	233	233		233	233	
(44.4)	40	166	166	209	169	158		138	112	
	80	135	121	200	145	135		118	106	
2.00 ml.	160	109	105	165	133	112				
XXX	0	239	239		239		239	239	239	239
(33.3)	2	225	219		231		225	236	219	215
	10	195	161		210		145	175	161	186
0.40 ml.	50	136	113		146		91	117	92	164
oval-R'[g]	0							650	650	650
(160)	2							623	659	617
	10							599	623	612
0.50 ml.	50							563	571	550

Analyses are averages of triplicates with mean deviation of ±4%.

Superscripts a, b, c, d, and g refer to Table III-A.

The anti-R' serum pools used with the above antigens all consisted of sera from the same individual rabbits, but the pools were made up from bleedings at different times.

h 2.50 ml. of all reactants were used in this case.

i 2.00 ml. of all reactants were used in this case.

i This column gives the amount of hapten present per 1.00 ml. of hapten solution.

A. The effect of the substances on precipitation of ovalbumin by anti-ovalbumin rabbit serum was tested. Only a very slight effect was observed in the course of over 100 separate analyses. Typical data are given in Table III-C. It was also determined that when hapten and dye antigen together were added to anti-ovalbumin serum, its precipitation by ovalbumin was not changed from the precipitation occurring in their absence.

B. The effect of the two azo substances, which give the most marked enhancement, on the antiserum alone was tested and it was found that no precipitate was brought down.

C. The dependence of the effect on the order of mixing reactants was investigated in the following manner:

10 ml. of anti-R serum, pool A, were mixed with 10 ml. of a solution of antigen XXX containing 22 μgm. of the dye per ml. and the mixture was allowed to stand one hour at room temperature and overnight at 5°. The precipitate formed was centrifuged down and 1 ml. portions of the supernate were added to 1 ml. portions of solutions of haptens (g) and (h) and, as a control, buffer at pH 8. After allowing the tubes to stand overnight at 5° it was found that the tubes which contained the haptens (g) and (h) had present 33 μgm. and 16 μgm. of specific protein precipitate, respectively, while the control with buffer contained 0 μgm. These figures are averages of triplicate analyses.

The above experiment was repeated, using the supernate from a precipitate of anti-ovalbumin and ovalbumin, from a mixture of 1.2 ml. of anti-ovalbumin serum diluted to 10 ml. with pH 8 borate buffer and 10 ml. of an ovalbumin solution in the same buffer, containing 1 mg. of ovalbumin.

Table III-C

The Effect of Certain Haptenic Substances

on Anti-Ovalbumin Precipitation

0.00625 ml. of anti-ovalbumin serum diluted to 0.5 ml. with pH 8 borate buffer; 0.5 ml. of ovalbumin solution containing 100 μgm. of ovalbumin; 0.5 ml. of hapten solution. The tubes stood one hour at room temperature and over one night at 5°. pH of supernates: 8.0-8.2.

Hapten	Moles of hapten added x 10^7		
	40	80	160
	μgm. protein in precipitate[a]		
Benzoic acid	408	412	380
p-Nitrobenzoic acid	424	420	408
p-Nitrobenzene sulfonic acid	440	427	440
p-Nitrophenol	420	405	411
Picric acid	398	390	413
p-(p-Hydroxyphenylazo)-benzoic acid	395[b]	404[b]	
p-(p-Hydroxyphenylazo)-benzene sulfonic acid	378[b]	373[b]	

[a] The amount of protein in the precipitate with zero hapten present amounted to 404 μgm.

[b] In this case the amount of protein in the precipitate with zero hapten present amounted to 379 μgm.

All analyses are in triplicate, with mean deviation of ±4%.

No precipitate was formed in any of the tubes after 48 hours standing at 5°.

D. The effect of the azo substances (haptens (g) and (h)) over a wide range of concentration was tested. The data are given in Table III-D.

E. The effect of p-phthalic acid, compared with that of benzoic acid, was tested. The data are given in Table III-E.

F. As an aid in interpreting the results, over 200 separate analyses were made of anti-R precipitates brought down by antigen XXX in the presence of concentrations of homologous haptens, such as phenylarsonic acid, p-(p-hydroxyphenylazo)-phenylarsonic acid and o-methylphenylarsonic acid, which were in the range just below the concentration necessary to give noticeable inhibition. In no case was there any effect of enhancement of precipitation. The concentration range covered was from that just necessary to give noticeable inhibition to a concentration one-thousandth as great, through a series of 17 1.5-fold dilutions.

G. The effect of different concentrations of antigen on this enhancement effect was tested. The data are given in Table III-F.

H. The effect of variation of the time the mixtures stand at 5° was investigated. It was found that the enhancement effect had appeared before 10 hours standing and did not change appreciably after 40 hours standing, except for an apparent slight increase in the effect after this longer time. In this experiment analyses were made after each five to ten hour interval in the standing time.

I. The effect of the presence of the simple substances in a mixture to be analyzed by the standard method (ref. 5)

Table III-D

The Effect on Anti-R Precipitation of Azo-

Substances Over a Wide Range of Concentration

0.25 ml. of anti-R serum, pool A, diluted to 1.0 ml.; 1.0 ml. of antigen solution containing 22.2 μgm. of antigen XXX; 1.0 ml. of hapten solution. The tubes stood over one night at 5° except where noted differently. pH of supernates: 8.0-8.1.

Hapten[a]	Moles of Hapten Added x 10^7									
	0	0.16	0.32	0.63	1.25	2.5	5.1	10.2	20.5	41.0	82.0
	μgm. of protein in precipitate										
(h)	(686)	(682)	(689)	(739)	(741)	(785)	(831)	(818)	(818)	(709)	(700)
(g)	716		716		749		849		906		862
(g)[b]	701		693		755		838		887		865

Analyses in triplicate with mean deviation of ±4%; single analyses in parentheses.

[a] The letters refer to the substances listed on p. 27.
[b] This series stood over two nights at 5°.

Table III-E

The Effect of a Divalent Hapten

on the Enhancement Effect

0.25 ml. of anti-R serum, pool A, diluted to 1.0 ml.; 1.0 ml. of a solution containing 22.2 μgm. of antigen XXX; 1.0 ml. of hapten solution. The tubes stood one hour at room temperature and over night at 5°. pH of supernates: 8.0.

Hapten	Moles of Hapten Added x 10^7			
	1.56	6.25	25	100
	μgm. of protein in precipitate[a]			
Benzoic acid	959	1013	1051	1100
p-phthalic acid	784	785	801	860

[a] Amount of protein in precipitate when zero hapten present, 795 μgm.
Analyses in triplicate with mean deviation of ±4%.

Table III-F

The Effect of Different Concentrations of Antigen on the Enhancement Effect

0.25 ml. of anti-R serum, pool A, diluted to 1.0 ml.; 1.0 ml. of antigen solution; 1.0 ml. of a solution of hapten (g) (p. 27). The tubes stood one hour at room temperature and overnight at 5°. pH of supernates: 8.0.

Amount of Antigen XXX (μgm.)	Moles of Hapten Added x 10^7					
	0	1.25	5	20	80	%[b]
	μgm. of protein in precipitate					
7.4[a]	168	166	184	181	160	9.6
11	389	417	469	494	447	27.0
22	782	837	877	909	856	16.3
44	727	759	807	838	783	15.1

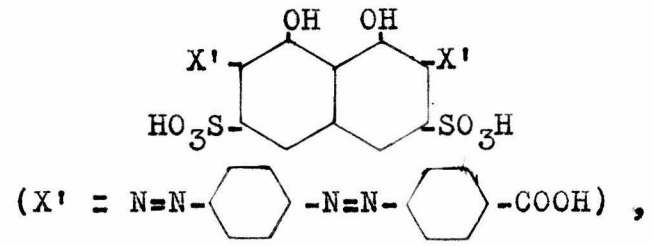
Analyses in triplicate with mean deviation of ±4%

[a] Double amounts of all reacts were used in this case. Results are reported on the basis of 1.0 ml. of each reactant solution.

[b] The figures in this column give the maximum percentage enhancement of precipitation for each antigen concentration.

was investigated. It was found that most of the substances did not affect the analyses at all, and that those that did, namely the azo substances, (g) and (h), would have to be present to the amount of several μgm. in order to give the observed effect. The presence of this amount of hapten in the specific precipitate is extremely unlikely, since the experiments with anti-ovalbumin precipitation indicate that these haptens are not brought down non-specifically with the precipitate.

In the course of further investigations concerned with the precipitation of antibodies against p-azobenzoic acid coupled to bovine serum proteins[9] by the simple dye antigen, Chrom X_2^1 :



and the inhibition of this precipitation by simple haptens, it was found by the author and Dr. Stanley Swingle[9] that certain haptens of a structure different from that of the benzoic acid group also gave the effect of enhancement of precipitation. The substances most active in this case were the members of the straight chain aliphatic acid series. Data illustrating this effect are given in Table III-G.

Discussion

The experiments outlined above lead to a consistent picture of the effect of the haptenic substances studied on the precipitation of antibody by its hapten-homologous antigen.

Comparing the res lts given in Tables III-A and III-B brings out the fact that the substances act like poorly

Table III-G

The Enhancement Effect in

Anti-p-azobenzoic Acid Precipitation

0.75 ml. of anti-p-azobenzoic acid serum, pool A, diluted to 1.0 ml.; 1.0 ml. of antigen solution containing 55 μgm. of Chrom X_2^1; 1.0 ml. of hapten solution. Diluent used was pH 8.5 borate buffer, 0.16 M in salts. The tubes stood one hour at room temperature and over two nights at 5°. pH of supernates: 8.3-8.4.

Hapten	Moles of Hapten Added x 10^7		
	4	20	100
	μgm. of protein in precipitate[a]		
Acetic acid	695	697	704
Propionic acid	702	713	717
n-Butyric acid	730	700	728
i-Butyric acid	737	741	752
n-Valeric acid	726	788	836
n-Caproic acid	745	799	848
n-Enanthic acid	788	884	1007
n-Caprylic acid	836	971	1088
Cyclohexane carboxylic acid	730	750	816
p-aminobenzene-sulfonamide	709	717	767

[a] The amount of protein in the precipitate formed with zero hapten present was 682 μgm.

Analyses in triplicate with mean deviation of ±4%.

inhibiting homologous haptens in anti-R' precipitation in all cases, while with R-antigens and the azoprotein antigens in anti-R precipitation they act this way, but with the R'-antigens and anti-R serum they give the enhancement effect. This observation is in accord with the structural differences between anti-R and anti-R' molecules, as suggested in reference 4 V. That is, the anti-R' molecule gives a "looser fit" with the haptenic group and so allows more widely varying haptens to interact than does the anti-R molecule. The haptenic substances studied thus act toward anti-R' molecules as phenylarsonic acids with the acid group substituted by other similar acid groups. They also act in this manner toward anti-R molecules when precipitated by R-antigens. Since R'-antigens give much stronger interaction with anti-R molecules (cf. p.13 of this manuscript) than do R-antigens, presumably they prevent any effective interaction of these "substituted arsonic acids" with the anti-R molecule.

The reason for the appearance of an enhancement effect in this latter case is harder to understand. The experiments outlined under A above indicate that the effect of enhancement depends on the presence of an antibody structure which is sufficiently similar to the hapten to allow for the possibility of some interaction between the two. They indicate that it it this type of specific structure that is involved and that the effect is not due to some entirely non-specific reaction. The fact that, as shown in Table III-C, there is a slight amount of enhancement of precipitation of the anti-protein molecule can be explained either as that part of the effect which is due to a non-specific salt effect, or more probably, as due to the fact that some of the anti-ovalbumin molecules

are structurally complementary in some degree to the haptenic substances, since ovalbumin contains tyrosine and histidine groups. On the basis of this explanation, it would be expected that the simple straight chain aliphatic acids should show an even greater enhancement of anti-ovalbumin precipitation than do the substances tested, since more of the protein antigen molecule is of an aliphatic nature.

The experiments outlined under B, C, H and I above give further evidence that the enhancement effect involves the specific complementary regions of the antibody molecule and is not non-specific in nature.

The experiments outlined under D above point out the fact that for some of the substances there is an optimum in the enhancement effect and that at higher concentrations the substances give less effect, thus acting in a manner analogous to inhibiting haptens of structure homologous to the antigen.

The experiments outlined under G above bring out the fact that the enhancement effect is greatest in the optimum zone of precipitation and decreases on either side of this zone.

The experiments with anti-p-azobenzoic acid precipitation show that the enhancement effect is not limited to the arsonic acid system, but has a general significance.

One explanation here suggested for the enhancement effect is that it is due to the rendering insoluble by the haptenic substances of the soluble complexes of antigen and antibody postulated as existing in any mixture of antigen and antibody (ref. 3; ref. 4 a, p. 3001). The argument is that these complexes, consisting of a chain of several antibody molecules bound together by antigen molecules, may be rendered insoluble

by the specific attachment of several of these haptenic molecules to the specific antibody structures at the ends of the chain and any third valence positions (ref. 4 III) available in the antibodies, since these molecules present essentially non-polar surfaces once they are specifically bound. A piece of evidence in support of this explanation is given in Table III-E, in which it is shown that a hapten with a polar group at the other end of the molecule does not give such a large enhancement effect as the corresponding hapten with only one polar group. However, according to this explanation, phenylarsonic acid haptens at sufficiently low concentrations should also act in the same manner, as was brought to the author's attention by Dr. David Pressman. No such effect was observed, as mentioned as the result of experiments outlined under F above. However, theoretical calculations indicate that the concentration range within which such an effect might be found might be extremely narrow, so that it is barely possible that the effect remained unnoticed due to this cause.

Another possible explanation for the enhancement effect, which was first brought to the author's attention by Dr. Stanley Swingle, is that it is due to interaction of the haptenic substances with weak, non-precipitating antibody molecules--univalent antibodies, which in the free state act to inhibit precipitation by combining with antigen present and thus reducing the effective antigen concentration. The experiment outlined under G above was devised to test this possibility, since it would be expected that the greatest effect of enhancement should then occur in the region of antibody excess. This is not the case, as the results given in Table III-F indicate. The result given in Table III-E is also hardly

explainable on this basis, since it would be expected that a "divalent" hapten would be twice as effective as a monovalent one in interacting with the weak antibodies, due to the entropy factor involved. Also, as pointed out to the author by Dr. Pressman, it is reasonable to expect that the action of small concentrations of homologous haptens, mentioned above, would occur on the basis of this explanation also.

As in the case of other serological effects involving a consideration of molecular structure, this effect is complicated by many factors. It is suggested that much progress toward a better understanding of the effect could be made by using purified fractions of antisera, prepared by precipitation of the antisera with known antigens and dissociation of the resulting precipitates.

Summary of Part III.

A study is presented of the effects of simple substances which are different from the homologous haptens on the precipitation of anti-R, anti-R' and anti-p-azobenzoic acid sera by antigens containing the specific groups involved. An effect of enhancement of precipitation in some cases is reported.

The actions of the simple substances on the precipitation reactions involving the various antigen-antibody systems are discussed in terms of the structures of the entities involved, and the plausability of some explanations of the enhancement effect is discussed.

IV.

Inhibition by Polyhaptenic Substances

On the basis of the simple theory of hapten inhibition as developed in reference 4 IV, it would be expected that polyvalent haptens would give a greater inhibition than the corresponding univalent hapten of similar structure, due to the entropy factor involved. If the entropy factor alone were involved, the relative K'_o values should be in the ratio of the number of groups present in the various polyhaptenic substances having the same active group. To examine the experimental effect, hapten inhibition studies were carried out in systems involving anti-R' and anti-p-azobenzoic acid sera (the latter will hereafter be referred to as anti-X serum), since in these two systems R-containing and X-containing polyhaptenic simple substances, respectively, do not give precipitates.

Experimental

Preparation of antisera.--The preparation of anti-R serum has been described in reference 4 I. Anti-X serum, as mentioned above, was prepared in a similar manner by injecting rabbits with bovine serum proteins to which were coupled p-azobenzoic acid. This serum was prepared by Dr. Stanley Swingle.

Preparation of antigens.--The preparation of antigen XXX has been described above in the appended reference 4 V. Chrom X'_2 (cf. p.30), the analogous benzoic acid compound, was prepared by Dr. Stanley Swingle in a similar manner and purified by repeated washings with acetone, which remove the monovalent impurity. Carbon-hydrogen analysis indicated

that the sodium salt was present.

Preparation of Haptens.--The arsonic acid haptens used are of two kinds--haptens containing the azo link and haptens containing the amide link. They were prepared by Mr. Carol Ikeda and Mr. Miyoshi Ikawa by methods described in reference 4 I, except for the compound, HOOC-  -R, (R = N=N-  -AsO_3H_2), which was prepared by Mr. Shelton Steinle from p-nitrosophenylarsonic acid and p-aminobenzoic acid by a method similar to that of Karrer.[11]

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[11] S. Karrer, Ber., 45, 2066, 2376 (1912)

The benzoic acid haptens were also of the azo and amide types and were prepared by Dr. Stanley Swingle by methods similar to those used for the corresponding arsonic acid haptens.

Hapten Inhibition.--The usual hapten inhibition technique, as described above in part II, was used. The data for inhibition studies with anti-R' serum and the azo haptens are given in Table IV-A and for the amide haptens in Table IV-B. The data for inhibition studies with anti-X serum are given in Table IV-C.

Discussion

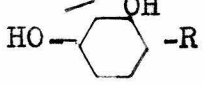
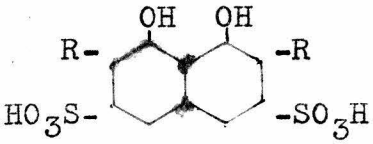
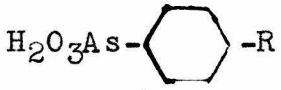
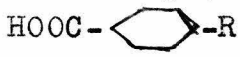
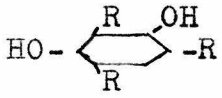
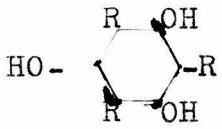
In the second column of each of the tables, following the formulae of the haptens, is given the value of K'_o, the relative hapten inhibition constant for each substance, calculated from the data by making use of the extended theory of hapten inhibition which takes into account heterogeneity of the antiserum, as discussed above in part II. The values are based on unity for the hapten for which this value is given.

Table IV-A

Hapten Inhibition of Anti-R' Precipitation--

Comparison of Effects of Uni- and Polyvalent Azo Haptens

0.5 ml. of anti-R' serum, pool A, diluted to 1.0 ml.; 1.0 ml. of antigen solution containing 40 μ gm. of antigen XXX; 1.0 ml. of hapten solution. The tubes stood one hour at room temperature and overnight at 5°. pH of supernates: 8.1.

Hapten[a]	K'_o	σ	Moles of Hapten Added x 10^9		
			2.5	5	10
			Amount of precipitate[b]		
	1.00	1.5	1008	918	763
	1.81	1.0	920	862	619
	2.13	1.5	891	773	635
	2.44	1.5	874	759	581
	2.81	1.0	878	725	552
	5.00	2.0	719	572	385
	7.60	2.0	648	461	295

[a] R = N=N-  -AsO_3H_2.

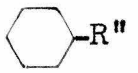
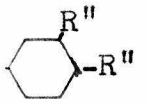
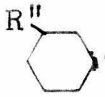
[b] The amounts are tabulated as fractions per mille of the amount precipitated in absence of hapten, which was 372 μ gm. Values are averages of triplicate analyses with mean deviation of ± 3 %.

Table IV-B

Hapten Inhibition of Anti-R' Precipitation--

Comparison of Effects of Uni- and Polyvalent Amide Haptens

0.5 ml. of anti-R' serum, pool A, diluted to 1.0 ml.; 1.0 ml. of antigen solution containing 40 μgm. of antigen XXX; 1.0 ml. of hapten solution. The tubes stood one hour at room temperature and overnight at 5°. pH of supernates: 8.1.

Haptens[a]	K'_o	σ	Moles of Hapten Added x 10^9		
			6.25	12.5	25
				[b]	
CH_3-R"	1.00	2.0	918	861	765
 -R"	1.95	2.0	859	773	635
 -R"	3.17	2.5	751	655	522
R"-$(CH_2)_8$-R"	5.15	2.0	707	531	412
R"-R"	6.15	2.5	635	515	381
R"-  -R"	6.65	2.0	665	505	355
R"-$(CH_2)_4$-R"	8.00	3.0	583	460	365
R"-$(CH_2)_2$-R"	8.00	3.0	570	485	335
 -R"	8.84	2.5	603	418	312

[a] R" = CONH-  -AsO_3H_2.

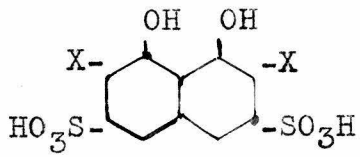
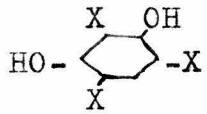
[b] The figures in the last three columns give the amounts of precipitate, tabulated as fractions per mille of the amount precipitated in absence of hapten, which was 416 μgm. Values are averages of triplicate analyses with mean deviation of ±3%.

Table IV-C

Hapten Inhibition of Anti-X Precipitation--

Comparison of Effects of Uni- and Polyvalent Haptens

0.28 ml. of anti-X serum, pool B, diluted to 1.0 ml. with pH 8.5 borate buffer; 1.0 ml. of antigen solution, containing 40 μgm. of Chrom X_2^1; 1.0 ml. of hapten solution. The tubes stood one hour at room temperature and overnight at 5°. pH of supernates: 8.4.

Haptens[a]	K_o^1	σ	Moles of Hapten Added x 10^9		
			1.1	3.3	10
			Amount of Precipitate[b]		
CH_3-X"	1.00	0.5	882	668	260
X"-X"	1.26	0.8	870	529	100
X"-$(CH_2)_2$-X"	2.87	1.5	568	345	55
X"-  -X"	2.74	0.9	673	215	50
HO-  -X	1.00	0.1	1010	943	605
		0.1[c]	943[c]	941[c]	842[c]
	5.75	0.8	785	345	55
	4.05[c]	0.5[c]	768[c]	505[c]	12[c]
	10.2	0.8	605	152	17
	7.75[c]	0.5[c]	710[c]	235[c]	3[c]

[a] X" = CONH-  -COOH; X = N=N-  -COOH.

[b] The amounts are tabulated as fractions per mille of the amount precipitated in absence of hapten, which was 180 μgm. Values are averages of triplicate analyses with mean deviation of ±3%.

[c] These values are for an antigen-antibody mixture containing 0.17 ml. of anti-X serum, pool B, and 1.2 mg. of azoprotein, X-ovalbumin (number of haptenic groups per molecule not known) per 2.0 ml. of mixture. 1.0 ml. of hapten solution was added to this mixture. The amount of precipitate, including the antigen protein, in the absence of hapten was 643 μgm.

The third column gives the value of σ, the heterogeneity index for the antiserum interacting with the antigen and the hapten involved.

These data bring out some interesting correlations. First of all, due to the combination of structural effects with the entropy effect, there is no clean-cut demonstration of an entropy effect *per se*. This is particularly noticeable upon comparing the K_o' values for HO-  -R and HO-  -R in Table IV-A, the latter substance having a higher value for this constant. This is probably due to the fact that the latter hapten, being larger and containing the extra hydroxyl group, more effectively fills the rather loose "hole" of the anti-R' molecule and allows more molecular attraction forces to come into play.

As further demonstration of this combination of effects, in Table IV-B the succinanilic and adipanilic haptens have higher values of K_o' than the oxanilic hapten because of the added separation of the active groups, while the sebacanilic hapten may have a reduced value of K_o' because of the greater tendency for this longer chain to bend back on itself and so bring the acid groups closer together.

Another interesting observation, brought out in both Tables IV-B and IV-C, is that the haptens of more aliphatic character have a higher heterogeneity index than the corresponding aromatic compounds. This is to be expected, since the antibodies are specific for the aromatic type of coupled compound and thus would tend to behave as more heterogeneous serum toward a compound of less aromatic character, since antibodies of less specific complementariness would come into play.

The amide haptens, on the average, have a higher heterogeneity index than the corresponding azo compounds, as would be expected from the fact that the antibodies are directed against the azo link.

All the various possible effects just discussed illustrate again how difficult it is to find serological systems in which the effect of only one variable can be studied.

Summary for Part IV.

The effect of polyhaptenic substances in inhibition involving antisera homologous for the p-(p-azophenylazo)-phenylarsonic acid and the p-azobenzoic acid groups is reported. It is found that the magnitude of the inhibition by polyhaptenic substances is not wholly accounted for by the entropy factors involved, but that various other complicating factors enter into consideration. Some of these possible factors are discussed.

V.

Precipitation of Azoprotein and Antiserum Homologous to the p-Azophenyltrimethylammonium Ion and its Inhibition by Haptens

Although most workers investigating serological reactions with conjugated proteins have used anionic or neutral groups to modify the protein, it has been demonstrated[2c] that it is equally easy to obtain antiserum to a positively charged group. It was felt that investigation of the reactions of such an antiserum would prove useful in eliciting further information as to the relative effects of structure and charged group on the specificity of antigens and antibodies.

Experimental

Preparation of the Immunizing Antigen.--The immunizing antigen was prepared by coupling diazotized p-aminophenyltrimethylammonium chloride, kindly prepared by Dr. Leland Pence of Reed College, to crude sheep serum. Three equal portions of sheep serum, having respectively 2%, 4% and 8% by weight of hapten coupled to the serum protein, were allowed to stand overnight at 5° and pH 8.5 in order to make sure that coupling was as complete as possible. The solutions were then neutralized, combined and dialyzed at pH 7 against three changes of saline over an 84 hour period in order to remove as much uncoupled material as possible. The antigen was injected as a 1.0% protein solution, and was preserved with phenol.

Preparation of Antiserum.--Four rabbits were injected on alternate days with one to two ml. of the antigen over a period of several months. Serum was taken after about one

month of injections and every 20 - 25 days thereafter, with a rest period of a week to 10 days after the last injection before bleeding. The serum was pooled according to titer. The serum so obtained will be designated hereafter as anti-Z serum.

Preparation of the Test Antigen.--The test antigen was prepared by coupling the homologous azo hapten to ovalbumin at pH 8-8.5. It was found that preparations containing less than about 10 or more than about 25 groups per molecule of protein (assuming molecular weight of ovalbumin = 40,000) were unsatisfactory, the former giving no precipitate with fairly high titer antiserum, while the latter were too insoluble at pH 7. The preparations which proved suitable were purified by precipitating the azoprotein several times at pH 4.7-5, redissolving, and finally dialyzing against saline. The yields were very poor due to irreversible denaturation of much of the product during the purification procedure. The two preparations used, designated as $(Z\text{-oval.})_1$ and $(Z\text{-oval.})_2$ below, were made using enough hapten to give 25 and 15 groups per molecule, respectively.

Preparation of Haptens.--Most of the haptens were prepared by Dr. Pence, using standard procedures, and were characterized, in the case of known compounds, by melting points. Table V-A gives data for some of the haptens. The following two compounds were analyzed for halogen, using the method of Reber and McNabb[12].

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12 Reber, L.A. and McNabb, W.M., Ind. Eng. Chem., Anal. Ed., 9, 529 (1937)

Table V-A

Melting Points of Some Quaternary Ammonium Salts

Substance		Obs. m.p. (corr.) °C	Reported m.p. °C	Ref.
Cation	Anion			
H_3C-C$_6$H$_4$-*[a]	I^-	200-0.5	216-220	13
H_3C-C$_6$H$_4$-*	I^-	187-8	177	14
CH_3-C$_6$H$_4$-*	I^-	208-10(subl)	209	15
C$_{10}$H$_7$-*	I^-	161.5-3.5(dec)	164	16
H_3CCONH-C$_6$H$_4$-*	Cl^-	220		
C$_6$H$_4$-$N(C_2H_5)_3^+$	I^-	125-27		
ClH_3N-C$_6$H$_4$-*	Cl^-	217-18(dec)	219	17
H_3CCONH-C$_6$H$_4$-CH_2-*	I^-	226-7		
C$_6$H$_5$-*	I^-	212.5-13.5	210-12	18

[a] * = $N(CH_3)_3^+$

13 Wedekind, Ber., 35, 773 (1902)
14 v. Braun, J. and Kruber, O., Ber., 46, 3473 (1913)
15 v. Braun, J., Ber., 49, 1107 (1916)
16 Landshoff, Ber., 11, 645 (1878)
17 Reilly, J.,and Drumm, P.J., J. Chem. Soc., 1935 II, 871
18 Willstätter, R. et. al., Ann., 346, 217 (1905)

p-(p-Hydroxyphenylazo)-phenyltrimethylammonium chloride.

(m.p. 212-213°): for $C_{15}H_{18}ON_3Cl$,

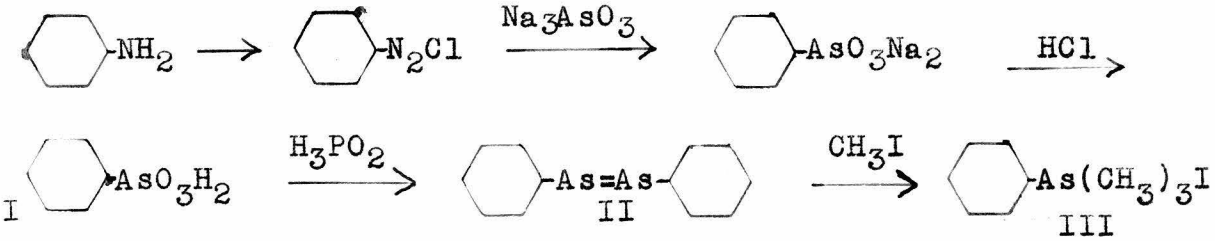
	calc.	found
%Cl	12.2	12.4, 12.3

p-Acetylaminobenzyltriethylammonium iodide.

(m.p. 191-197.5°): for $C_{15}H_{25}ON_2I$,

	calc.	found
%I	33.7	33.8, 33.9

The substance, phenyltrimethylarsonium iodide, was prepared by the method outlined by the following scheme:



The preparation of phenylarsonic acid was carried out according to the method given in Organic Syntheses, Vol. 15, p. 59, giving I. The preparation of arsenobenzene (II) was performed by the method of Bing[19]. The product, obtained in 31% yield,

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19 Bing, A., et.al., Ber., 53, 427 (1920)

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was heated with 5% excess of methyl iodide in a sealed tube at 100° for one hour[20] to give the desired compound, III, in

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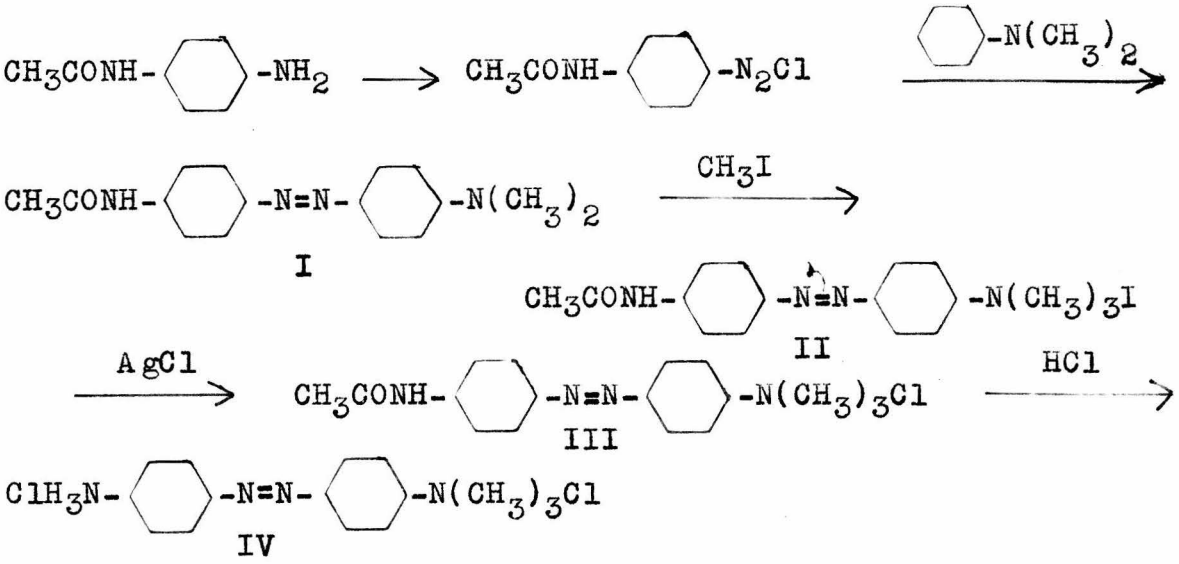
20 Bertheim, A., Ber., 47, 273 (1914)

20% yield after recrystallization from alcohol.

The aliphatic quaternary ammonium salts and the amine salts used in the hapten inhibition studies reported below were obtained as commercial preparations.

Attempted Preparation of Polyhaptenic Substances Containing the p-(p-Azophenylazo)-phenyltrimethylammonium Chloride Group.--In an attempt to prepare a polyhaptenic precipitating dye antigen of known structure for anti-Z serum, the following synthesis to obtain the required amine was carried out as outlined:



I was obtained by slow addition of sodium hydroxide solution to the cold acidic mixture of the diazo salt and the tertiary amine hydrochloride in aqueous solution. Precipitation by addition of the base required brought down a brownish-red solid which gave well-formed orange-red crystals of I from 90% ethanol, with the reported m.p. of 226-227°. Yield: 55%

II was obtained by heating I with 300% excess of methyl iodide in a sealed tube at 100° for 2.5 hours and then recrystallizing the product from water. II appeared as orange-red plates, m.p. 193-194°. Yield: 70%

III was obtained by digestion of an aqueous solution of II with freshly precipitated AgCl until no test for I^- could be obtained with Fe^{+++} and starch paper. The solid AgI was filtered off and the acetyl group was removed from III by adding an equal volume of 12 N HCl to the solution and

refluxing for one hour. The solution was then evaporated at 200 mm. pressure, the original volume of 400 ml., containing about 9 gm. of product, being evaporated to about one-tenth that volume. The solid (IV), present upon cooling to 0°, was filtered off and the remaining solution was evaporated almost to dryness, the solid so obtained being washed with acetone. IV appeared as orange-red crystals, m.p. 193-8°. Yield: 70%

An attempt was made to diazotize IV with nitrous acid in the cold, and couple it at pH 8.5-9 to chromotropic acid or to resorcinol, in order to obtain the compounds chromotropic acid bis-p-(p-azophenylazo)-phenyltrimethylammonium chloride and 2,4,6-tri-p-(p-azophenylazo)-phenyltrimethylammonium chloride resorcinol. Due to the fact that the diazo compound decomposes and couples with itself rather rapidly, while at the same time coupling with the desired nuclei proceeds rather slowly, such a complex mixture was obtained in both cases that no product could be isolated which would give a specific precipitate with anti-Z serum. Further attempts to prepare the desired compounds were discontinued at the time since an insufficient amount of the amine was available.

The Effect of pH on Anti-Z Precipitation.--The effect of pH on the system involved was roughly tested by bringing the antiserum to pH 5.7 with HCl and then adding the azoprotein antigen solutions, made up in 0.16 M boric acid (pH 6) and in borate buffers, 0.16 M in salts, of pH's 7.5, 8.5, and 9.5. The data for this experiment are given in Table V-B.

Hapten Inhibition of Anti-Z Precipitation with Azoprotein.--Hapten inhibition studies were carried out at pH 8 with the

Table V-B

The Effect of pH on Anti-Z Precipitation

1.0 ml. anti-Z serum, pool I-F, brought to pH 5.7 with HCl; 1.0 ml. of appropriate borate buffer; 1.0 ml. of a saline solution of $(Z\text{-oval.})_2$. The tubes stood one hour at room temperature and overnight at 5°.

pH		μgm. of $(Z\text{-oval.})_2$ Added		
buffer	supernates	667	1000	1500
		μgm. protein in precipitate[a]		
6.0[b]	6.1	424	544	665
7.5	7.0	338	431	505
8.5	7.9	284	290	(288)
9.5	8.7	(219)	[219]	(273)

[a] These figures include the antigen protein and are averages of triplicate analyses, except where noted, with mean deviation of ±4%. Duplicate analyses in parentheses and single analysis in brackets.

[b] 0.16 M boric acid solution was used here.

following substances:

(1) p-(p-hydroxyphenylazo)-phenyltrimethylammonium chloride

(2) phenyltriethylammonium iodide

(3) ∝-naphthyltrimethylammonium iodide

(4) chromotropic acid bis-p-azophenyltrimethylammonium chloride

(5) p-tolyltrimethylammonium iodide

(6) m-tolyltrimethylammonium iodide

(7) phenyltrimethylammonium iodide

(8) p-aminophenyltrimethylammonium chloride

(9) o-tolyltrimethylammonium iodide

(10) p-aminobenzyltrimethylammonium chloride

(11) phenyltrimethylarsonium iodide

(12) p-acetylaminobenzyltriethylammonium iodide

(13) tetraethylammonium bromide

(14) tetramethylammonium iodide

(15) chromotropic acid mono-p-azotertiarybutylbenzene

The results of this study are given in Table V-C, in which the haptens are designated by the numbers given in the above list, with an abbreviated name following to make the reading of the table easier. σ^- and K'_o are calculated as previously described.

The observation that the aliphatic quaternary ammonium salts gave appreciable inhibition of anti-Z precipitation (Table V-C) made it seem worthwhile to test some of the related primary, secondary and tertiary amine salts for inhibition in this antigen-antibody system at pH 6 (K_B for these amines is close to 5×10^{-4}). The results of this study, in which the effect of the amines is compared with that of some of the quaternary ammonium compounds, are given in Table V-D.

Table V-C

Hapten Inhibition of Anti-Z Precipitation

1.0 ml. anti-Z serum, pool I-F; 1.0 ml. antigen solution, containing 585±30 μgm. of $(Z\text{-oval.})_1$; 1.0 ml. of hapten solution. The hapten and antigen solutions were made up in pH 8.0 borate buffer, 0.16 M in salts. The tubes stood 1.5 hours at room temperature and overnight at 5°. pH of supernates: 7.7-7.8.

Hapten[a]	K'_o	σ	Moles of Hapten Added x 10^8						
			0.4	2	10	40	50	200	1000
			Amount of precipitate[b]						
(1) p-hydroxy-phenylazo	1.00	1.5		711	234		23		
(2) phenyltri-ethyl	0.85	2.0		708	296		127		
(3) α-naphthyl	0.51	2.0		792	435		127		
(4) chrom.Z_2	0.45	0.5	1065	893	390				
(5) p-tolyl	0.22	1.0		974	669		156		
(6) m-tolyl	0.20	1.5		1075	685		234		
(7) unsubstituted	0.19	1.0		987	704		214		
(8) p-amino	0.16	1.3		1078	753		256		
(9) o-tolyl	0.155	1.5		1000	692		338		
(10) p-aminobenzyl	0.096	1.5		1110[c]	841		471		
(11) arsonium	0.096	0.8		1124[c]	942		435		
(12) benzyl-ethyl	0.072	2.5				552		221	114
(13) tetraethyl	0.020	2.5				809		416	247
(14) tetramethyl	0.0050	2.5				1087		744	477
(15) tertiarybutyl	0.0038	0.1				1182[c]		1133[c]	562

a The numbers refer to the list on p. 56.

b The amounts are tabulated as fractions per mille of the amount precipitated in absence of hapten, which was 308 μgm. The values are uncorrected for antigen protein in the precipitate. Analyses in triplicate with mean deviation of ±4%.

c These values were checked in a separate experiment and are significantly above 1000. Other values above 1000 in this table probably represent experimental errors.

Table V-D

Hapten Inhibition of Anti-Z Precipitation--

The Action of Some Amine Salts

1.0 ml. of anti-Z serum pool I-F, brought to pH 5.7 with HCl; 1.0 ml. of antigen solution containing 750 μgm. of $(Z\text{-oval.})_2$ in saline at pH 6; 1.0 ml. of hapten solution in saline at pH 6. The tubes stood four hours at room temperature and overnight at 5°. pH of supernates: 6.3-6.5.

Hapten[a]	K'_o	σ	Moles of Hapten Added x 10^8					
			2	10	40	50	200	1000
			Amount of Precipitate[b]					
(3) α-naphthyl	0.75	3.5	894	734		535		
(7) unsubstituted	0.45	4.0	877	779		584		
(13) tetraethyl	0.042	5.0			787		645	500
(14) tetramethyl	0.020	5.0			844		734	584
$(C_2H_5)_3NHCl$	<0.001	>5.0			832		791	734
$(CH_3)_3NHCl$	<0.001	>5.0			939		930	791
$(C_2H_5)_2NH_2Cl$	$\ll 0.001$	$\gg 5.0$			967		971	900
$(CH_3)_2NH_2Cl$					967		1008	949
$C_2H_5NH_3Cl$					967		959	975
CH_3NH_3Cl					975		967	1008

[a] Numbers refer to the list on p. 56.

[b] The amounts are tabulated as fractions per mille of the amount precipitated in absence of hapten, which was 488 μ gm. The values are uncorrected for the amount of antigen protein present. Analyses are in triplicate with mean deviation of $\pm 4\%$.

Discussion

Due to the instability of the azoprotein antigen at low pH's, the investigation of the effect of pH on the precipitin reaction was only incompletely carried out. Due also to the scarcity of suitable azoprotein, the region of antigen excess was not investigated. However, the data in Table V-B perhaps can be considered to bear out a result which might have been predicted, namely, that since the ionic group involved in the specific interaction is not affected by pH, the strength of combination should not be affected by pH as it is in the case of the arsonic acid antigens. The whole effect of pH is then concerned only with the rendering of the proteins involved more insoluble as their isoelectric point is approached, so that it would be expected that more precipitate would be obtained at the lower pH values, which is what is observed. However, another reservation which must be held in mind in considering these results is that the effect of salts on this reaction has not been investigated, so that salt effects due to the difference in character between the precipitation at pH 6 in the presence of boric acid, and the precipitation at higher pH's in the presence of borates may explain part of the effect. Also, the very fact that the antigen becomes more unstable at lower pH's makes it entirely possible that the whole effect is merely due to nonspecific precipitation of antigen, which carries down antibody also. It should be mentioned, however, that the azoprotein antigen does precipitate the antibody <u>specifically</u> up to a certain amount, at least, since the antigen does not cause precipitation in heterologous sera at pH 7.

The significance of the inhibition studies is complicated by the fact that the azoprotein antigen contains much non-precipitating material, as is evident from the amount of antigen necessary to give a small amount of specific precipitate. This nonprecipitating antigen material may combine so strongly with most of the antibody molecules present which have structures closely complementary to it that the haptens whose effects were studied are competing with the small amount of precipitating antigen for a fraction of the serum which is only a small part of the total "good" antibody and which may have a complementariness more heterogeneous than that of the actual serum as a whole. This would explain many of the rather anomalous effects appearing in Tables V-C and V-D, namely, that haptens of a structure quite different from that of the immunizing haptenic group, such as phenyltriethylammonium and α-naphthyltrimethylammonium, have larger values of $\underline{K}'_o$ than does the more homologous hapten phenyltrimethylammonium. As more of the specific complementary structure of the antibody is brought into play, this effect of higher $\underline{K}'_o$ values for structures deviating from the homologous structure would tend to disappear, and this is indeed the case, since the p-(p-hydroxyphenylazo)-phenyltrimethylammonium chloride hapten is the best inhibitor of the substances studied. Incidentally, it should be stated that the values of $\underline{K}'_o$ in Table V-C are made relative to the value of 1.00 for this last-named hapten. In Table V-D, the $\underline{K}'_o$ values are make relative to the value 0.75 for the α-naphthyltrimethylammonium iodide.

It is interesting that there are antibodies present which react strongly enough through the mere presence of a structure which is presumably shaped complementary to the "three pronged"

trimethylammonium part of the homologous antigen so that the hapten containing the tertiarybutyl group can show some inhibition. The very small value of σ found for this hapten indicates that the antibody involved is all of the same kind with respect to the combining capacity involved, which would be expected in the case of such a special kind of interaction.

An interesting example of the dependence of σ on the antigen involved in a given hapten-antibody system is provided by the data in Tables V-C and V-D for the two aliphatic quaternary ammonium salts. The antigen involved in the data of Table V-D has a stronger combining power for the antibody than does the other antigen, as is evidenced by the smaller amount of inhibition at the same hapten concentration in the former case. This stronger interaction is reflected in the value of σ, which is larger, since antibodies of a wider heterogeneity presumably would be involved in the interactions of the former case in order for the antigen to be able to compete more effectively with the haptens of relatively low $\underline{K}'_o$ values, which presumably interact with antibodies of a more or less different structure from that complementary to the homologous haptenic group of the antigen.

It is interesting to note that the effect of enhancement of precipitation, discussed in part III, occurs to some extent in this antibody-antigen system.

Summary of Part V.

Antiserum homologous to the p-azophenyltrimethylammonium ion was prepared in rabbits and its precipitation by azoprotein and the inhibition of this precipitation were studied. The results of these studies are discussed in terms of the peculiarities of the azoprotein antigen and the observed heterogeneity of the antiserum.

The preparation of azoprotein and of some substances used in investigating the serological reactions is described.

References

1. Landsteiner, Karl, The Specificity of Serological Reactions, Charles C. Thomas, Springfield, Ill., 1936.
2. Marrack, J. R., The Chemistry of Antigens and Antibodies, His Majesty's Stationery Office, London, 1939.
3. Pauling, Linus, A Theory of the Structure and Method of Formation of Antibodies, J.A.C.S., 62, 2643 (1940)
4. Pauling, Linus, and co-workers, The Serological Properties of Simple Substances
   - I. Linus Pauling, David Pressman, Dan H. Campbell, Carol Ikeda and Miyoshi Ikawa, J.A.C.S., 64, 2994 (1942)
   - II. Linus Pauling, David Pressman, Dan H. Campbell and Carol Ikeda, ibid., 64, 3003 (1942)
   - III. Linus Pauling, David Pressman and Carol Ikeda, ibid., 64, 3010 (1942)
   - IV. David Pressman, David H. Brown and Linus Pauling, ibid., 64, 3015 (1942)
   - V. David Pressman, John T. Maynard, Allan L. Grossberg and Linus Pauling, ibid., 65, 728 (1943)
   - VII. Linus Pauling, David Pressman and Allan L. Grossberg, ibid., to be published
5. Pressman, David, Determination of Microquantities of Certain Proteins, Ind. Eng. Chem., Anal.Ed., 15, 357 (1943)