each hydrogen shifts among three nitrogen atoms. Case one is unlikely because it appears highly probable that hydrogen bonds are present.[3,4,5] If the actual situation is that of case four or five, which does not appear at all unlikely, there would be no isomerism due to the position of the hydrogen atoms.

It appears from a study of the Fisher models that a flat giant ring of this type can be constructed without undue strain. However, there is much interference between the pyrrole rings and the benzene rings if an attempt is made to place the latter in the same plane as the rest of the molecule. This suggests that the benzene rings do not have free rotation and are probably oscillating about a position perpendicular to the plane of the main ring. The general shape of the molecule would then be that of a flat disc with the four benzene rings cutting it at about right angles. If the benzene rings do not have free rotation then derivatives of *ms*-tetraphenylporphine having ortho or meta substituents should exist in a number of stereoisometric forms depending on whether the substituents are above or below the plane of the giant ring.

Experimental

The dielectric constant measurements were carried out with the same apparatus and in the same manner as described previously.[6] The refractive indices were measured with a Pulfrich refractometer using a neon Geissler tube as a light source.

The *ms*-tetraphenylporphine was recrystallized twice from benzene.

Acknowledgment.—We are indebted to Dr. O. L. Inman and Dr. Paul Rothemund of the C. F. Kettering Foundation at Antioch College for the sample of *ms*-tetraphenylporphine.

Summary

The more abundant isomer of *ms*-tetraphenylporphine was found to have a polarization of 310 and an electronic polarization of 297. Measurement of polarizations at different temperatures gave no evidence of a decrease of polarizations with increasing temperatures. Both methods indicate the compound has zero moment although the data do not enable one to distinguish between a moment of a few tenths and a zero moment.

The electronic polarization of the compound was obtained by measuring the refractive index of the solutions although they were highly colored. This was accomplished by using the orange line of a neon tube. This light had a wave length that was not absorbed by the solution.

The zero moment indicates the compound is symmetrical. The resonance demands that the giant ring and the pyrrole rings be in one plane. A study of the Fisher models suggests the benzene rings do not have free rotation and are perpendicular to this plane. With ortho or meta substituents on the benzene rings this hindered rotation will give rise to a number of stereoisomers.

SAN FRANCISCO, CALIF. RECEIVED SEPTEMBER 24, 1942

(3) Corwin and Quattlebaum, THIS JOURNAL, **58**, 1081 (1936).
(4) Vestling and Downing, *ibid.*, **61**, 3511 (1939).
(5) Aronoff and Weast, *J. Org. Chem.*, **6**, 550 (1941).
(6) Kumler, THIS JOURNAL, **62**, 3292 (1940).

[CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, No. 885]

The Serological Properties of Simple Substances. I. Precipitation Reactions between Antibodies and Substances Containing Two or More Haptenic Groups

BY LINUS PAULING, DAVID PRESSMAN, DAN H. CAMPBELL, CAROL IKEDA, AND MIYOSHI IKAWA

The study of serological precipitation reactions is complicated by the fact that ordinarily these reactions involve two proteins, the antigen and the antibody. The understanding of these reactions was greatly advanced by the introduction into their study of precise microanalytical methods and a further simplification involving the use of a nitrogen-free multivalent hapten of pneumococcus polysaccharide.[1] A few years ago it was reported by Landsteiner and van der Scheer[2a] that the precipitin reaction and anaphylaxis could be produced by simple substances formed by coupling two haptenic groups with resorcinol or tyrosine, in place of the azoprotein (containing the same haptenic group) which has been used as the antibody-producing antigen. Landsteiner[2b] suggested that "the ready precipitability of these

(1) M. Heidelberger and F. E. Kendall, *J. Expil. Med.*, **50**, 809 (1929); **61**, 559, 563 (1935).

(2) (a) K. Landsteiner and J. van der Scheer, *Proc. Soc. Expil. Biol. Med.*, **29**, 747 (1932); *J. Expil. Med.*, **56**, 399 (1932); **57**, 633 (1933); **67**, 79 (1938). (b) K. Landsteiner, "The Specificity of Serological Reactions," Charles C Thomas, Baltimore, Md., 1936, p. 120.

dyes is dependent upon peculiarities in constitution which, like those of fatty groups, diminish solubility in water and favor the formation of colloidal solutions." However, it seemed probable to us, on the basis of a theory of the structure of antibodies and the nature of the precipitin reaction,[3] that simple substances containing two or more haptenic groups would react with antibodies in essentially the same way as the homologous protein antigens containing the same haptenic groups; we accordingly prepared a half-dozen simple substances of this sort, each with two or more phenylarsonic acid groups per molecule, and observed each to precipitate antisera homologous to phenylarsonic acid azoprotein,[4] thus obtaining evidence for the generality of the phenomenon discovered by Landsteiner and van der Scheer.

The problem of obtaining from precipitation experiments evidence about the structure of antibodies and the nature of serological reactions is obviously greatly simplified by the replacement of protein antigens by simple substances of known structure. For this reason we began and are carrying on an extensive program of investigation of the reactions of simple substances with antisera. In this paper we report the quantitative study of the precipitin reaction for twenty simple substances containing two or more haptenic groups, and the results of tests of seven substances containing one group. It is found that the observations support the framework theory of serological precipitates.[5]

Discussion of Experimental Methods

Simple Antigens.—The simple antigens and haptens used in the investigation are listed in Table I; methods of preparations of these substances and the intermediates used are described in the following section.

Protein Antigens.—The immunizing antigens used for inoculations were made from diazotized arsanilic acid and sheep serum by the method described by Landsteiner and van der Scheer.[6] The ratio of arsenic to protein in these antigens ranged from 2 to 3%.

Test antigens were similarly made from purified ovalbumin by treatment with diazotized arsanilic acid, diazotized *p*-(*p*-aminophenylazo)-phenylarsonic acid, or diazotized *p*-aminobenzanilide-*p'*-arsonic acid. The azo-ovalbumins contained, respectively, 0.16, 4.0, and 2.0% arsenic.

Arsanilic acid was diazotized in hydrochloric acid solution by the addition of sodium nitrite solution at 0° to the starch–iodide end-point. The diazotizations of *p*-(*p*-aminophenylazo)-phenylarsonic acid and of *p*-aminobenzanilide-*p'*-arsonic acid were similarly carried out at 10° with end-point the disappearance of the slightly soluble amine hydrochlorides.

Preparation of Antisera.—Twenty-five rabbits were injected intraperitoneally or intravenously with 1- or 2-ml. portions of the atoxylazo-sheep-serum antigen described above, containing 0.5% protein. Several weekly courses of 3 to 5 injections were given, with intervening rest periods of a week or more. The rabbits were bled from the ear on the eighth, ninth, and tenth days after the last injection, 40 ml. of blood being taken from each rabbit each day. The blood was permitted to clot, and the antisera were pooled according to titer. The courses of injections and subsequent bleedings were repeated to obtain more pools of serum.

A measure of the total amount of antibody homologous to the atoxyl hapten (the phenylarsonic acid group) was made by determining the maximum amounts of antibody precipitated by the azo-ovalbumin test antigens. The most effective test antigen, that made from *p*-(*p*-aminophenylazo)-phenylarsonic acid, precipitated 2 mg. of antibody per ml. of antiserum A, 4 mg. per ml. of B, 1.5 mg. per ml. of C, and 4 mg. per ml. of D.

The Reaction of Antigen and Antiserum.—The precipitation tests were carried out by mixing portions of undiluted antiserum, usually 2 ml., with equal volumes of saline solution containing dye; usually four to six dye concentrations were tested, differing by powers of 2. The tubes were allowed to stand for one hour at room temperature and then overnight in the refrigerator. The precipitates were then centrifuged down, washed with three or four 10-ml. portions of normal saline, and analyzed for nitrogen. In some experiments colorimetric determinations were made of the amount of dye in the redissolved precipitates. In the tests with serum A the customary visual estimates of cloudiness were made one-half hour after the solutions were mixed.

Methods of Analysis.—Analyses for nitrogen were made by the semimicro Kjeldahl method, using the apparatus described by Redemann.[7] Sulfuric acid, copper sulfate, and potassium sulfate were used in the digestion mixture; hydrogen peroxide was found not to be needed.

The arsenic determinations were carried out by the method of Haurowitz and Breinl.[8] Carbon and hydrogen analyses were made with the usual semimicro technique. Colorimetric determinations of dye and azoprotein were made with a Klett photoelectric colorimeter after dissolving the precipitates in a few drops of 2 *N* sodium carbonate solution.

The reported values of antibody in precipitates are the values of antibody nitrogen multiplied by the factor 6.25, the antibody nitrogen being the difference between total nitrogen in the precipitate and antigen nitrogen calculated

(3) L. Pauling, THIS JOURNAL, **62**, 2643 (1940). J. R. Marrack and F. C. Smith, *Brit. J. Expil. Path.*, **13**, 394 (1932), had made the tentative suggestion that precipitation by azohaptens depends upon the presence in the molecule of two or more haptenic groups.

(4) L. Pauling, Dan H. Campbell and D. Pressman, *Proc. Nat. Acad. Sci.*, **27**, 125 (1941).

(5) R. J. Marrack, "The Chemistry of Antigens and Antibodies," His Majesty's Stationery Office, London, 1938; M. Heidelberger, *Chem. Rev.*, **24**, 323 (1939); *Bact. Rev.*, **3**, 49 (1939); L. Pauling, ref. 3.

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

(7) C. E. Redemann, *Ind. Eng. Chem., Anal. Ed.*, **11**, 635 (1939).

(8) F. Haurowitz and F. Breinl, *Z. physiol. Chem.*, **205**, 259 (1932).

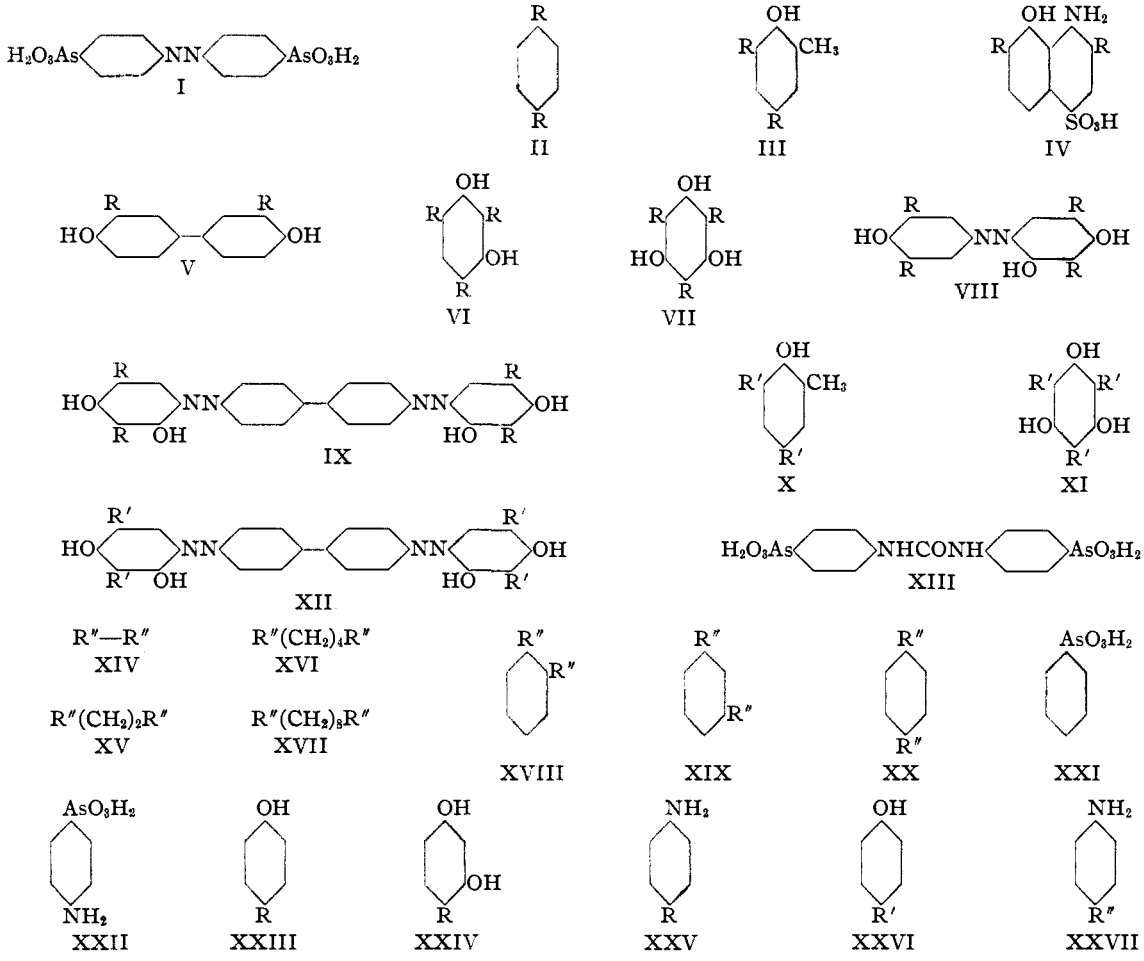
TABLE I

SUBSTANCES USED IN PRECIPITATION TESTS

R = *p*-azophenylarsonic acid, —NN⟨⟩AsO_3H_2

R′ = *p*-(*p*-azophenylazo)-phenylarsonic acid, —NN⟨⟩NN⟨⟩AsO_3H_2

R″ = phenylcarbamyl-*p*-arsonic acid, —CONH⟨⟩AsO_3H_2



from the amount of antigen as determined colorimetrically (for simple antigens this correction is very small).

The Preparation of Compounds

XXI, Phenylarsonic acid was prepared by Mr. David Brown by the Bart reaction.[9]

XXII, Arsanilic acid was prepared by the method of Bechamp.[10]

XXV, *p*-(*p*-Aminophenylazo)-phenylarsonic acid was made (a) by the hydrolysis in 2 *N* sodium hydroxide of the acetyl derivative made by condensing *p*-nitrosophenylarsonic acid with *p*-aminoacetanilide (20% excess) in glacial acetic acid by refluxing for three hours, and (b) by the hydrolysis for twenty minutes in boiling 1 *N* sodium hydroxide of the ω-methylsulfonate formed by reaction in 0.3 *N* sodium carbonate of diazotized arsanilic acid and aniline-ω-methylsulfonate (20% excess).[11] The two products, purified as the sodium salts, appeared to be identical.

Anal. Calcd. for $C_{12}H_{11}O_3N_3AsNa$: C, 42.04; H, 3.12. Found: (a) C, 41.98; H, 3.23; (b) C, 42.12; H, 3.40.

Nitrosophenylarsonic acid was made by the method of Karrer[12] from arsanilic acid and Caro's acid.

I, **Azobenzene-*p*,*p*′-diarsonic acid** was prepared by the method of Karrer[12] from *p*-nitrosophenylarsonic acid and arsanilic acid and was purified by repeated precipitation with acid from alkaline solution.

Anal. Calcd. for $C_{12}H_{12}O_6N_2As_2$: C, 33.50; H, 2.79 Found: C, 33.50, 33.56; H, 2.83, 2.97.

(9) H. Gilman, "Organic Syntheses," John Wiley and Sons, New York, N. Y., 1935, Vol. XV, p. 59.

(10) *Ibid.*, 1932, Coll. Vol. I, p. 63.

(11) F. G. Pope and W. I. Willett, *J. Chem. Soc.*, 1259 (1913); H. Bucherer and A. Schwalbe, *Ber.*, **39**, 2798 (1906).

(12) S. Karrer, *ibid.*, **45**, 2066, 2376 (1912).

TABLE II

Compound	Reactant	Excess of diazo compound	Formula	Analyses, % Calcd. C	Calcd. H	Found C	Found H	Color in alkali	Color in H_2SO_4
III	*o*-Cresol	50%	$C_{19}H_{16}O_7N_4As_2$	40.07	3.09	40.17	3.20	Orange	Purple
IV	1-Amino-8-naphthol-4-sulfonic acid	100%	$C_{32}H_{19}O_{11}N_6As_2S$					Purple	Green
V	*p,p*-Dihydroxybiphenyl	150	$C_{24}H_{20}O_8N_4As_2$	44.85	2.74	45.43	2.93	Light	Light
						45.47	2.83	yellow	yellow
VI	Resorcinol	33	$C_{24}H_{21}O_{11}N_6As_3$	36.27	2.67	36.52	2.62	Orange	Pink
						36.67	2.62		
VII	Phloroglucinol	33	$C_{24}H_{21}O_{12}N_6As_3$	35.55	2.61	35.58	2.91	Yellow	Pink
						35.65	2.91		
VIII	2,4,4′-Trihydroxyazobenzene	30	$C_{36}H_{30}O_{15}N_{10}As_4$	37.85	2.64	38.62	2.98	Brown	Pink
						38.80	3.18		
IX	4,4′-Bis-(azo-2,4-dihydroxy)-biphenyl	25	$C_{48}H_{28}O_{16}N_{12}As_4$	43.07	2.86	43.17	3.40	Brown	Purple
X	*o*-Cresol	50	$C_{31}H_{26}O_7N_8As_2$	48.21	3.37	48.42	3.64	Violet-	Purple
						48.27	3.64	red	
XI	Phloroglucinol	33	$C_{42}H_{33}O_{12}N_{12}As_3$	44.92	2.96	44.71	2.96	Violet	Blue
XII	4,4′-Bis-(azo-2,4-dihydroxy)-biphenyl	10	$C_{72}H_{54}O_{16}N_{20}As_4$	49.27	3.10	49.85	3.66	Brown	Violet
XXIII	Phenol	20[a]	$C_{12}H_{11}O_4N_2As$	44.60	3.42	44.68	3.42	Yellow	Yellow
XXIV	Resorcinol	100[a]	$C_{12}H_{11}O_5N_2As$	42.59	3.28	42.65	3.26	Orange	Yellow
XXVI[b]	Phenol	20[a]	$C_{18}H_{14}O_4N_4As$-Na	48.20	3.16	48.20	3.42	Red-orange	Blue-violet

[a] Excess of phenol (per cent.). [b] Sodium salt.

II, *p*-Di-(*p*-azophenylarsonic acid)-benzene was similarly made from *p*-nitrosophenylarsonic acid and *p*-phenylenediamine and similarly purified.

Anal. Calcd. for $C_{18}H_{16}O_6N_4As_2$: C, 40.46; H, 2.94. Found: C, 38.85, 38.81; H, 3.12, 3.19.

III, 2-Methyl-4,6-di-(*p*-azophenylarsonic acid)-phenol; IV, 1-Amino-2,7-di-(*p*-azophenylarsonic acid)-4-sulfo-8-naphthol; V, 3,3′-Di-(*p*-azophenylarsonic acid)-4,4′-dihydroxybiphenyl; VI, 1,3-Dihydroxy-2,4,6-tri-(*p*-azophenylarsonic acid)-benzene; VII, 1,3,5-Trihydroxy-2,4,6-tri-(*p*-azophenylarsonic acid)-benzene; VIII, 2,4,4′-Trihydroxy-3,5,3′,5′-tetra-(*p*-azophenylarsonic acid)-azobenzene; IX, 4,4′-Bis-(azo-2,4-dihydroxy)-3,5-di-(*p*-azophenylarsonic acid)-biphenyl; X, 2-Methyl-4,6-di(*p*-(*p*-azophenylazo)-phenylarsonic acid)-phenol; XI, 1,3,5-Trihydroxy-2,4,6-tri-(*p*-(*p*-azophenylazo)-phenylarsonic acid)-benzene; XII, 4,4′-Bis-(azo-2,4-dihydroxy)-3,5-di-(*p*-(*p*-azophenylazo)-phenylarsonic acid)-biphenyl; XXIII, *p*-(*p*-Azophenylarsonic acid)-phenol; XXIV, 1,3-Dihydroxy-4-(*p*-azophenylarsonic acid)-benzene; XXVI, *p*-(*p*-(*p*-azophenylazo)-phenylarsonic acid)-phenol.—Compounds III to XII, XXIII, XXIV, and XXVI were made by coupling diazotized arsanilic acid or diazotized *p*-(*p*-aminophenylazo)-phenylarsonic acid with the appropriate phenolic nucleus in dilute sodium carbonate solution or (for XXVI) in sodium acetate–acetic acid solution. For III, X, and XI pyridine was added[13] in amount about 10% of the volume of the reaction mixture. The reaction mixtures were allowed to stand for a few days (1 to 4) and the products were then precipitated with hydrochloric acid and purified by repeated solution in dilute sodium hydroxide and reprecipitation with acid. Compounds X, XI, and XII were in addition purified by dialysis through Visking sausage casing against dilute borax solution with *p*H 10; this membrane permits passage of molecules containing only one haptenic group R′ but not those containing two or more of these groups. The compounds were washed free of sodium chloride and dried *in vacuo*. Experimental details, analytical results, and colors of solutions in alkali and in concentrated sulfuric acid are given in Table II.

A chromatographic method of analysis of the purity of these compounds was developed and applied by Mr. A. Pardee. The column packing used was a mixture of 30% Celite and 70% Neutrol Filtrol. A dilute solution of the dye was poured into the packed column and the chromatogram was developed with phosphate buffer of *p*H varying from 8 to 12 in different cases. The rate of passage of the dyes was greater the higher the *p*H. Compounds VI, VII, X, XI, XXIII, XXIV, and XXVI appeared to be quite pure, having at most only a slight trace of a second band. On the other hand, compounds I, II, III, V, VIII, IX, and XII contain appreciable colored impurity, as shown by the appearance of more than one band on the column. Compounds V, VIII, IX, and XII might be expected to be impure from the large number of steps in their preparation. The impurity in compound II was identified as XXV from a mixed chromatogram.

2,4,4′-Trihydroxyazobenzene was prepared by coupling diazotized *p*-aminophenol with resorcinol (100% excess) in the presence of sodium hydroxide. The product was purified by dissolving it in sodium hydroxide and reprecipitating with acid and finally by two crystallizations from 70% alcohol.

Anal. Calcd. for $C_{12}H_{10}O_3N_2$; C, 62.60; H, 4.38. Found: C, 62.39; H, 4.46.

4,4′-Bis-(azo-2,4-dihydroxy)-biphenyl was prepared by adding bisdiazotized benzidine to resorcinol (100% excess) in a sodium acetate buffered solution. The mixture finally was made alkaline with sodium hydroxide. The product was purified by washing.

Anal. Calcd. for $C_{24}H_{18}O_4N_4$; C, 67.60; H, 4.25. Found: C, 66.37; H, 4.62.

XXVII, *p*-Aminobenzanilide-*p*′-arsonic acid was prepared by the method of King and Murch[14] by the hydrolysis of the carbethoxyamino compound obtained by coupling *p*-arsanilic acid with *p*-carbethoxyaminobenzoyl chloride.

(13) K. H. Saunders, "The Aromatic Diazo-Compounds," Edward Arnold and Company, London, 1938, p. 115.

(14) H. King and W. O. Murch, *J. Chem. Soc.*, 2595 (1924).

TABLE III

Compound	Reactant	Formula	Analyses, % Calcd. C	Calcd. H	Found C	Found H
XIII	Phosgene	$C_{13}H_{14}O_7N_2As_2$	33.93	3.07	33.83	3.09
XIV	Oxalyl chloride	$C_{14}H_{14}O_6N_2As_2$	34.45	2.89	34.30	3.05
XV	Succinic anhydride	$C_{16}H_{18}O_8N_2As_2$	37.23	3.52	37.15	3.70
XVI	Adipyl chloride	$C_{18}H_{22}O_8N_2As_2$	39.72	4.08	39.69	4.24
XVII	Sebacyl chloride	$C_{22}H_{30}O_8N_2As_2$	44.01	5.04	43.97	5.03
XVIII	*o*-Phthalyl chloride	$C_{20}H_{18}O_8N_2As_2$	42.57	3.22	42.55	3.53
XIX	Isophthalyl chloride	$C_{20}H_{18}O_8N_2As_2$	42.57	3.22	42.42	3.28
XX	Terephthalyl chloride	$C_{20}H_{18}O_8N_2As_2$	42.57	3.22	42.76	3.43

Anal. Calcd. for $C_{13}H_{13}O_4N_2As$; C, 46.44; H, 3.90. Found: C, 46.91, 46.74; H, 4.08, 3.97.

XIII, Carbanilide-*p,p'*-diarsonic Acid; XIV, Oxanilide-*p,p'*-diarsonic Acid; XV, Succinanilide-*p,p'*-diarsonic Acid; XVI, Adipanilide-*p,p'*-diarsonic Acid; XVII, Sebacanilide-*p,p'*-diarsonic Acid; XVIII, Phthalanilide-*p,p'*-diarsonic Acid; XIX, Isophthalanilide-*p,p'*-diarsonic Acid; XX, Terephthalanilide-*p,p'*-diarsonic Acid.—These dianilide compounds were prepared by essentially the methods of King and Murch[14] and Morgan and Walton,[15] involving the reaction of arsanilic acid with the required acid chloride or anhydride in a basic or buffered aqueous solution. The compounds were purified by repeated solution in sodium hydroxide and precipitation with hydrochloric acid followed by a thorough washing with hot water. The reactants used and the results of analyses are given in Table III.

Results of the Precipitation Experiments

The Precipitation Reactions between Multivalent Compounds and Antisera.—Precipitation tests were carried out between the antisera A, B, C, and D homologous to atoxylazoprotein and the twenty compounds I to XX, each of which contains in its molecule two or more haptenic groups R, R′, or R″. *In every case precipitation occurred.* The amount of precipitate was found to vary with concentration of the antigen in the same way as for ordinary antigens. There is an optimum antigen concentration (or amount while the volume of antigen solution is held constant) at which the amount of precipitate reaches a maximum.

The amounts of precipitated antibody found by analysis are given in Tables IV, V, VI, VII, and VIII and are represented graphically in Figs. 1, 2, 3, 4, and 5.

Errors in the determination of the amounts of precipitated antibody may arise in the separation and washing of the precipitate or in the nitrogen analysis. Examination of the reported results of duplicate determinations indicates a probable error of about 5% for individual determinations.

(15) G. T. Morgan and E. Walton, *J. Chem. Soc.*, 615 (1931); 91 (1933), 902 (1936).

TABLE IV

AMOUNTS OF ANTIBODY PRECIPITATED FROM ANTISERUM A BY ANTIGENS I TO XII

Amounts of solutions used: 2 ml. antiserum, 3 ml. saline-antigen. The *p*H of the supernatant solutions was between 8.3 and 8.5. In Tables IV to VIII, inclusive, the amount of antigen used, in micrograms per ml. of antiserum, is given at the top of each column. The numbers in the columns are the amounts of precipitated antibody, in micrograms per ml. of antiserum. The meaning of the other symbols is given in the text.

Amount of antigen	3.13	6.25	12.5	25	50
I	−+	−+	−++	−+	−−
II	−++	−84	++134	+119	−53
III	−47	±103	++150	+100	−22
IV	−69	+138	++225	++213	±75
V	±106	+222	++322	+213	−63
VI	±100	+188	++222	+135	− ↓
VII	−48	+116	++128	+94	− ↓
VIII	+141	++185	+++338	++250	±110
IX	±106	+141	++291	+++347	−69
X	±66	+144	++188	+++397	↓ 660
XI	++232	↓ 425	↓ 920	↓ 1560	↓ 1690
XII	+128	+197	+++456	↓ 890	↓ 1210

TABLE V

AMOUNTS OF ANTIBODY PRECIPITATED FROM ANTISERUM B BY ANTIGENS II TO IX

Amounts of solutions used: 2 ml. antiserum, 3 ml. saline-antigen. The *p*H of the supernate was in each test 8.3.

Amount of antigen	6.25	12.5	25	50	100
II	270	520	660	470	300
	250	490	670	460	280
III	120	290	470	120	0
	110	350	490	140	9
IV	100	440	950	330	80
	200	490	850	330	100
V	330	760	1330	520	80
	270	840	1120	460	
VI	330	920	(1600)[a]	230	60
	310		(1490)	180	20
VII	180	350	770	300	100
	180	370	710	190	50
VIII	330	840	1590	340	20
	310	860	1400	280	10
IX	110	160	650	920	260
	110	130	690	670	260

[a] There is strong evidence that these values are in error.

TABLE VI

AMOUNTS OF ANTIBODY PRECIPITATED FROM ANTISERUM C BY ANTIGENS VI, IX, X, XI, AND XII

Amounts of solutions used: 2 ml. antiserum, 2 ml. saline-antigen for VI and IX; 1 ml. antiserum, 1 ml. saline-antigen for X, XI, and XII. The *p*H of the supernate was 8.4 for VI and IX, 8.5 for X, XI, and XII.

Amount of antigen	6.25	12.5	25	50	100	200
VI	290	460	270	160		
	290	380	270	150		
IX	220	440	400	230		
	180	430	390	220		
X	110	220	380	540	640	600
	110	220	350	530	650	710
XI	310	710	1360	1330	1000	660
	320	610	1350	1360	1190	750
XII	310	440	900	910	640	350
	320	440	840	990	680	360

TABLE VII

AMOUNTS OF ANTIBODY PRECIPITATED FROM ANTISERUM B BY ANTIGENS XIII TO XX

Amounts of solutions used: 3 ml. antiserum, 3 ml. saline-antigen. The *p*H of the supernate was 8.2 for each antigen.

Amount of antigen	6.25	12.5	25	50	100
XIII	50	75			10
	25	75			50
XIV	180	385	440	340	265
	165	355	475	340	270
XV	130	345	315	200	150
	150	255	325	225	140
XVI	15	35	55	35	15
	35	50		35	
XVII	35	35	50	45	30
	35	50	50	40	40
XVIII	10	10	60	85	50
	0	0	55	65	55
XIX	65	305	465	200	75
	65	300		215	
XX	120	505	695	440	195
	120	435	735	500	175

Test of the Customary Visual Estimation Method.—For the series reported in Table IV we took advantage of the opportunity of testing the customary visual-estimation method of studying antigen-antibody precipitation reactions. The tubes were inspected one-half hour after the solutions were mixed, and a record made of their appearance; this is given by the symbols in the table, which have the following meanings: −, no apparent cloudiness; ±, slight cloudiness; +, ++, +++, increasingly pronounced cloudiness; ↓, formation of clumps of precipitate. Comparison of these with the amounts of antibody precipitated in twenty-four hours shows that the correlation is reasonably good, and suggests that visual estimates of the amount of precipitate may be trusted to within about ± 30%. In this case each symbol represented about 60% more precipitate than the preceding one, as follows: −, 75; ±, 100; +, 150; ++, 250; +++, 400; ↓, 500 μg/ml.

TABLE VIII

AMOUNTS OF ANTIBODY PRECIPITATED FROM ANTISERUM D BY ANTIGENS II AND XIII TO XX

Amounts of solutions used: 2 ml. antiserum, 3 ml. saline-antigen.

Amount of antigen	3.13	6.25	12.5	25	50	100
II	140	280	780	1160	1150	930
XIII			20	20		
XIV			570	690	700	570
			460	640	640	510
				740	760	
XV	60	190	440	600	480	
				700	660	
XVI	10	10	50	110	90	
XVII	10	60	80	100	120	100
			80	90	110	90
			80	90	110	
XVIII	20	30		50	40	
XIX	10	180		1060	420	
XX	120	250	610	1170	800	

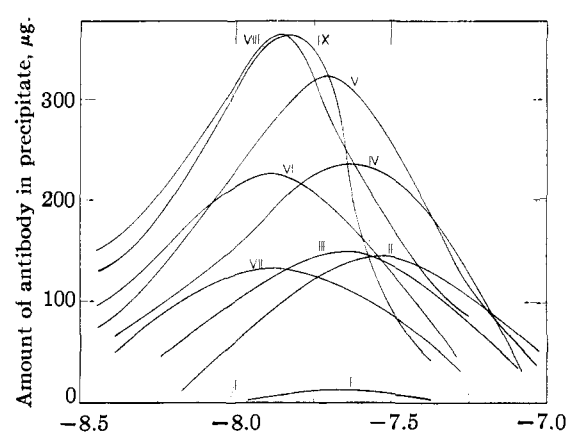


Fig. 1.—Amounts of antibody precipitated from antiserum A (per ml.) by antigens I to IX as functions of log of molal antigen concentration (Table IV). The curve for I represents an estimate. Smooth curves have been drawn through the experimental points, which are not shown.

Comparison Tests with Normal Serum.—Similar tubes were set up for each of the twenty antigens with normal rabbit serum in place of antiserum. In none did precipitation occur.

The Failure of Monohaptenic Compounds to Produce Precipitates with Antisera.—The seven

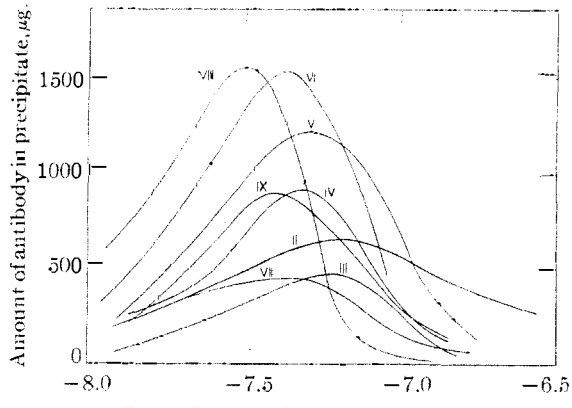


Fig. 2.—Amounts of antibody precipitated from antiserum B (per ml.) by antigens II to IX (Table V).

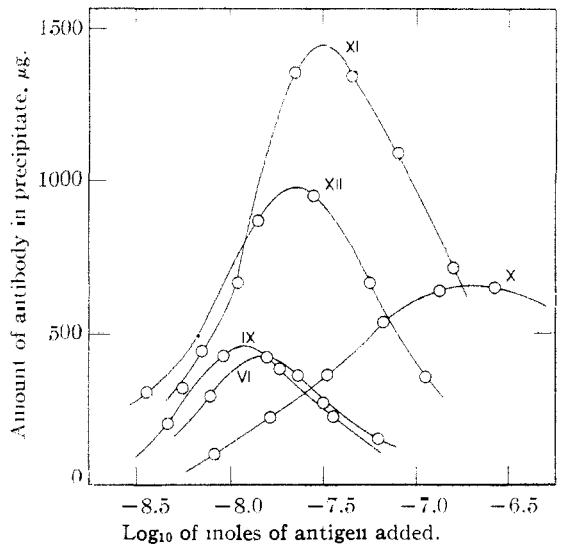


Fig. 3.—Amounts of antibody precipitated from antiserum C (per ml.) by antigens VI, IX, X, XI, and XII (Table VI).

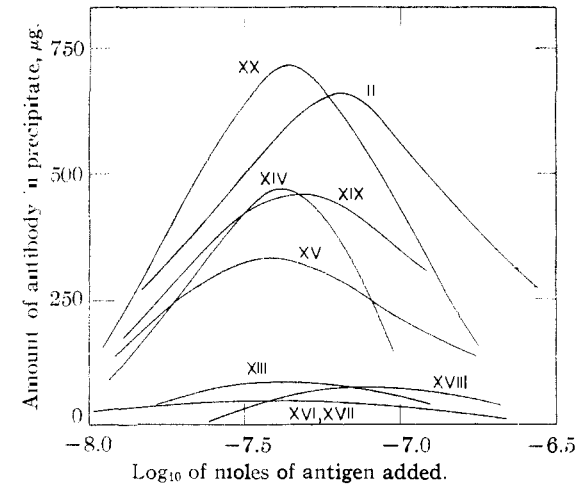


Fig. 4.—Amounts of antibody precipitated from antiserum B (per ml.) by antigens XIII to XX (Table VII) with antigen II for comparison (Table V).

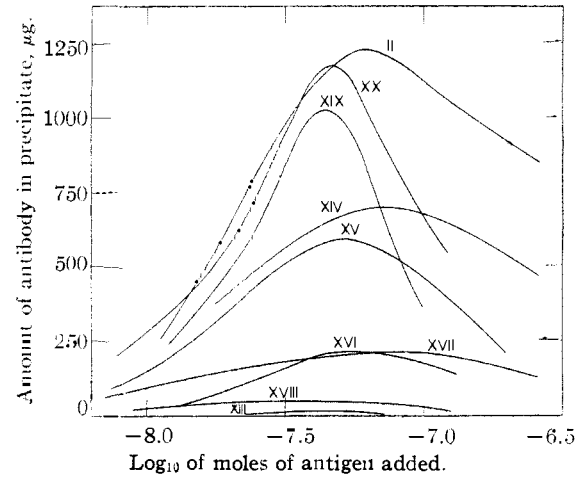


Fig. 5.—Amounts of antibody precipitated from antiserum D (per ml.) by antigens II and XIII to XX (Table VIII).

compounds XXI to XXVII, each of which contains one haptenic group per molecule, were tested with the antisera in the same way as the twenty compounds I to XX; no precipitate was formed by any of these "univalent" substances. This result is, of course, to be expected, in view of the failure of Landsteiner[16] to obtain precipitates between haptens and homologous antisera during his extensive experiments on the inhibition by homologous haptens of the azoprotein-antibody precipitation reaction.

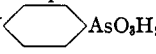
Discussion

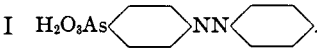
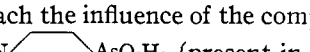
The fact that each of the twenty polyhaptenic substances precipitates hapten-homologous antibody, whereas substances containing only one haptenic group do not have this effect, provides strong support for the Marrack–Heidelberger framework theory (lattice theory) of the structure of serological precipitates, inasmuch as the framework theory gives a simple explanation of the phenomenon, and no other reasonable explanation has been proposed. Multivalent antibody molecules, whose existence is postulated in the framework theory, would be expected to combine with polyhaptenic molecules to form infinite aggregates, which would grow into visible precipitates, whereas with monohaptenic molecules they would form soluble small complexes containing one antibody molecule.

The general shape of all the curves of Figs. 1 to

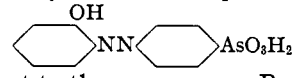
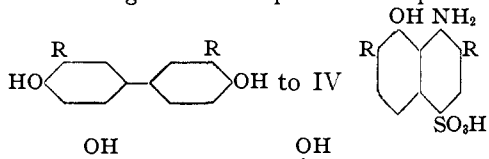
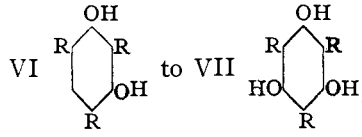
(16) K. Landsteiner, "The Specificity of Serological Reactions," Charles C. Thomas, Baltimore, 1936, p. 118.

5, showing the dependence of amount of antibody precipitated on the amount of added antigen, is the same; the amount of precipitated antibody first increases, then reaches a maximum, and finally decreases, reaching zero for large amounts of antigen. This inhibition of precipitation by excess antigen, which occurs also for protein antigens, is explained as resulting from the formation of soluble complexes, such as A–B–A (A_2B) for a bivalent antibody (A = antigen, B = antibody). There may also be formed soluble complexes AB_2, A_2B_3, etc., which contain an excess of antibody, saturating the effective valences of the antigen molecules. The interpretation of the precipitation data in terms of the strength of the antigen-antibody bond is complicated not only by the necessity of considering these soluble complexes but also by the heterogeneity of antibody molecules.

The data for nine compounds I to IX which contain the group  used in the immunizing azoprotein are reproduced in Figs. 1 and 2. It is seen that there is agreement between the results obtained with the two antisera in most respects. From Fig. 1 we obtain the following sequence of precipitating ability of the haptens: VIII = IX > V > VI = IV > VII = III = II > I. Nearly the same sequence is given by Fig. 2. The principal difference is the interchange of VI and IX, and other data (as in Fig. 3) indicate that the order given by Fig. 1 is to be preferred. It is possible that some gross error was made in the work; on the other hand, the difference between Figs. 1 and 2 may well be real, resulting from difference between the two antisera.

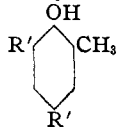
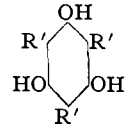
The smallness of the amount of precipitate given by I  we attribute to two causes. First, the molecule contains only one azo group, so that in holding two antibody molecules the molecule cannot exert toward each the influence of the complete haptenic group  (present in the immunizing azoprotein); and second, two large antibody molecules clasping the two end halves of this very small antigen would be expected to interfere sterically with each other, and be prevented by this steric interference from forming as strong an antigen-antibody bond as would otherwise be possible.

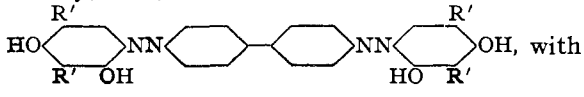
Of the five dihaptenic compounds I to V the two most effective, IV and V, are those with the greatest hapten-hapten distance. We attribute their relative effectiveness to the resultant diminution in steric interaction of the attached antibodies.

Since in the immunizing azoprotein the azophenylarsonic acid groups are attached in part to tyrosine residues, a very effective haptenic group is expected to be  with one hydroxyl adjacent to the azo group. By considering this we explain the superiority of V  to IV and of  to VII.

It is interesting that the trihaptenic (VI and VII) and tetrahaptenic (VIII and IX) compounds are superior but not greatly superior to the dihaptenic compounds of similar size and structure. It was expected[3] that dihaptenic compounds would be inferior in precipitating ability because of the formation with bivalent antibody of soluble strings A–B–A–B–A–B– rather than insoluble frameworks. The observation that good precipitates are obtained with dihaptenic compounds indicates either that the long strings themselves precipitate easily or that enough trivalent antibody molecules are present to link the strings together.

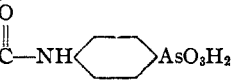
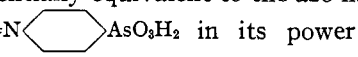
The compounds X, XI, and XII, which contain the long haptenic group

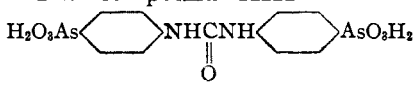
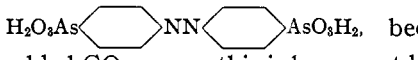
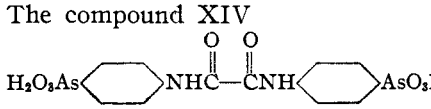
are much more effective than the corresponding compounds III, VII, and IX; this increased effectiveness we attribute to decreased steric interference between the attached antibodies. The comparison of X  and XI  is especially interesting. The superiority of the latter is clearly to be attributed to its having three haptenic groups instead of two.

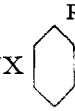
Why, then, does not XII  with

four, precipitate still more antibody? The answer may be that little gain is to be expected from increasing the number of haptenic groups from three to four, since three are enough to tie the strings together into a framework. In fact, XII is as effective as XI at low concentrations, and its later inferiority may be due to greater ability to form soluble complexes.

There is a striking relation between the number of haptenic groups per antigen molecule and the optimum antigen concentration for precipitation; a great change occurs from dihaptenic to trihaptenic antigens, and a much smaller change on addition of a fourth haptenic group. This is seen clearly in Fig. 3; the logarithm of the optimum molal concentration of X is -6.8, of XI -7.5, and of XII -7.65. Similarly the five dihaptenic antigens of Fig. 1 are grouped together at -7.6, and the others at -7.9.

The data represented in Figs. 4 and 5 show that the amide haptenic group  is essentially equivalent to the azo haptenic group  in its power to combine with antibodies homologous to the azo haptenic group. A straightforward comparison can be made between the amide compound XX  and the azo compound II  ; it is seen from the corresponding curves in Figs. 4 and 5 that these compounds are nearly equal in precipitating power.

The compound XIII  would be expected from steric considerations to be somewhat more effective than I  , because of the added CO group; this is borne out by experiment. The compound XIV  , containing two complete haptenic groups, should be still more effective. It is in fact surprisingly effective, with nearly the precipitating power of XX.

The compounds XVIII  , XIX  and XX  are increasingly effective in this order, with XVIII by far the weakest of the three. This is reasonably interpreted as due to steric interference of the attached antibody molecules.

This explanation cannot be extended, however, to the sequence XIV R″—R″, XV R″$(CH_2)_2$R″, XVI R″$(CH_2)_4$R″, XVII R″$(CH_2)_8$R″, since these compounds decrease in precipitating power in this order. A structural interpretation of this result in terms of the lack of rigidity of the polymethylene chain might be developed, but it is not very convincing.

The curves for compounds XIV and XV differ in shape from those for XIX and XX; no reasonable explanation of this has occurred to us.

It is important to note that the polyhaptenic simple antigens are not greatly inferior in precipitating power to azoproteins. Indeed, the best of the simple antigens, XI, was found to precipitate as much antibody as the test azoprotein.

Boyd[17] has recently reported failure to obtain precipitates between antisera and a number of simple substances containing two or more haptenic groups (including several substances also studied by us), and on the basis of this negative evidence he has drawn conclusions contrary to those which we have reached. In view of our observations, we consider it likely that his experiments were carried out under conditions unfavorable to precipitation—his antisera may have been weak, or his antigens may have contained monohaptenic impurities.

We are grateful to the Rockefeller Foundation for financial support of this work. We wish to thank Mr. Paul Faust and Mr. Shelton Steinle for their assistance in carrying out analyses, and Mr. David Brown for the preparation of phenylarsonic acid.

Summary

Twenty-seven simple substances containing the phenylarsonic acid group as haptenic group were prepared and used in precipitin tests with antisera made by injecting rabbits with azophenylarsonic acid sheep serum.

The twenty simple antigens containing two or more haptenic groups per molecule were found to give precipitates with the antisera, whereas

(17) W. C. Boyd, *J. Expll. Med.*, **75**, 407 (1942); S. B. Hooker and W. C. Boyd, *J. Immunol.*, **42**, 419 (1941).

the seven monohaptenic substances failed to precipitate. It is pointed out that these results provide strong support of the framework theory of the precipitin reaction.

Data on the amounts of precipitate formed are discussed in relation to the structure of the simple antigens.

PASADENA, CALIFORNIA RECEIVED JULY 6, 1942

[CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, No. 886]

The Serological Properties of Simple Substances. II. The Effects of Changed Conditions and of Added Haptens on Precipitation Reactions of Polyhaptenic Simple Substances

BY LINUS PAULING, DAVID PRESSMAN, DAN H. CAMPBELL, AND CAROL IKEDA

During the course of the investigation of precipitation reactions of polyhaptenic simple substances reported in the preceding paper of this series[1] we found it desirable to carry out a study of the effects of changed conditions of precipitation and washing on the amount of residual precipitate. We also made some experiments on the inhibition of precipitation by added haptens, in order to see how great would be the effects of monohaptenic impurities possibly present in the substances studied. The results obtained are presented and discussed in this paper.

Experimental Methods.—The experiments were carried out in the way described in the preceding paper (I). In addition to antisera C and D mentioned in paper I, three antisera, E, F, and G, were used. E and G contained amounts 0.6 and 3.2 mg. per ml., respectively, of antibody precipitable by azo-ovalbumin test antigen; the strength of F was not determined.

The borate buffer solutions were made by adding suitable amounts of 0.16 N sodium hydroxide solution to 0.2 M boric acid solution containing 0.9% sodium chloride.

The Effect of Changed Conditions of Precipitation and Washing on the Amount of Precipitate.—It is seen from the data reported in Tables I, II, and III, obtained with two antigens (VI and X) and three antisera (C, E, and F), that the antigen-antibody precipitate is either dissolved slightly or carried away mechanically by the saline or borate buffer solutions with which it is washed. The loss in this way is, however, small, amounting to about 5 to 15% for eight or ten extra washings with 10-ml. portions of solution.

A few experiments were made (Tables II and III) to test the effects of changing the time and temperature of precipitation. It was found that increasing the precipitation time from one day to two days increases the amount of precipitate by about 10%. The amount of precipitate formed seems to increase with increase in temperature of

TABLE I

EFFECT OF NUMBER OF WASHINGS ON AMOUNT OF RESIDUAL PRECIPITATE

	3 ml. antigen VI, 25 μg./ml. in saline solution, plus 3 ml. antiserum C					2 ml. antigen VI plus 2 ml. antiserum E		
	Composition of precipitate			Composition of precipitate			Composition of precipitate	
Washings	Antibody[a]	Antigen[a]	Washings	Antibody[a]	Antigen[a]	Washings	Antibody[a]	Antigen[a]
2	1665	8.9	6	1600	8.7	3	1110	5.7
	1660	8.4		1540	8.7		1125	6.2
	1660	8.9		1590	8.7	10	970	5.7
3	1600	8.7	7	1590	8.7		930	6.0
	1600	8.9		1530	8.9	15	960	6.0
	1545	8.7		1510	8.9			
4	1545	8.7	8	1510	9.2			
	1570	8.9		1530	8.7			
	1660	8.7		1480	8.1			
5	1570	8.9	10	1520	9.5			
	1570	8.9		1510	7.6			
	1570	9.5		1320	9.5			

[a] Amounts of precipitated antibody and antigen in micrograms. pH of all supernates 8.1.

(1) Linus Pauling, David Pressman, Dan H. Campbell, Carol Ikeda, and M. Ikawa, THIS JOURNAL, **64**, 2994 (1942). We shall refer to this as paper I.