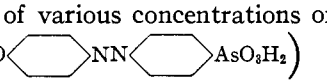
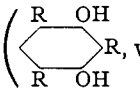
[CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, No. 907]

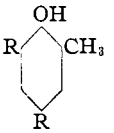
The Serological Properties of Simple Substances. IV. Hapten Inhibition of Precipitation of Antibodies and Polyhaptenic Simple Substances

BY DAVID PRESSMAN, DAVID H. BROWN, AND LINUS PAULING

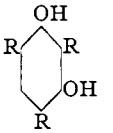
As part of a series of studies of the antigen-antibody reaction by use of polyhaptenic simple substances as antigens,[1,2,3] we have made a quantitative investigation of the ability of various substituted phenylarsonic acids to inhibit the precipitation of compounds containing two or more phenylarsonic acid groups with antiserum to sheep serum coupled with diazotized *p*-arsanilic acid.[4] The investigation included study of the effect of various concentrations of hapten XXIII  on the amount of precipitate obtained with antiserum and various concentrations of antigen VI  , where R is the *p*-azophenylarsonic acid group); the effect of changed conditions of precipitation for the same system; the effects of four haptens on the precipitation of each of five antigens and each of two pools of antiserum; and the effects of 24 different haptens on one precipitin reaction. The results are discussed in this paper.

Experimental Methods

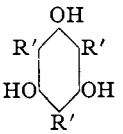
Simple Antigens and Haptens.—The substances used are the polyhaptenic compounds



III



VI



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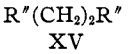
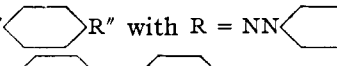
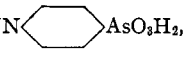
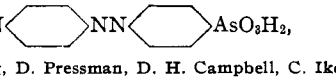
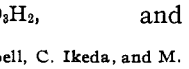
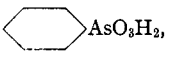
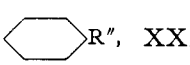
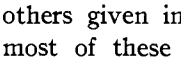
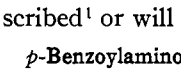


XV

and XX  with R =  , R′ =  , and R″ =  , and the haptens XXI  , XXIII  , XXVIII  , XXIX  , and twenty others given in Table VI. The preparation of most of these substances has already been described[1] or will be discussed elsewhere.[5]

***p*-Benzoylaminophenylarsonic acid** was prepared from *p*-arsanilic acid and benzoyl chloride by the Schotten–Baumann reaction. The product was precipitated with hydrochloric acid and purified by extraction with boiling ethanol.

***p*-Acetaminophenylarsonic acid** was prepared from *p*-arsanilic acid and acetic anhydride in basic solution. The compound was purified by precipitation with hydrochloric acid and recrystallization from boiling water.

Antisera to sheep serum coupled with diazotized arsanilic acid were prepared as previously described, with use of the same rabbits.

Method of Analysis.—Each precipitate was centrifuged and washed thoroughly with three 10-ml. portions of 0.9% saline solution at room temperature. The amount of protein in the precipitate was determined with the Folin–Ciocalteu reagent[6] by a modification to be discussed elsewhere.

Buffer.—The borate buffer of *p*H 8.0 was prepared by adding 0.16 *N* sodium hydroxide solution to 0.2 *M* boric acid in 0.9% sodium chloride solution. The antigen and hapten solutions were all diluted with this buffer.

TABLE I

EFFECT OF STANDING AT ROOM TEMPERATURE AND IN THE REFRIGERATOR ON AMOUNT OF PRECIPITATE OBTAINED IN THE PRESENCE OF HAPTEN

Analyses for 5-ml. aliquots of a mixture of equal volumes of serum S, antigen VI (25 μg./ml.), and hapten XXIII (60/μg.(ml.). *p*H of all supernates 8.2.

Time at room temp.	Nights in refrigerator	Amount of precipitated antibody, μg.
0	1	125 131 138
0	2	131 131 144
3/4 hour	1	125 125 131
3/4 hour	2	131 144 144
2 hours	1	106 106 144
2 hours	2	106 119 125 125 125 138
Overnight	0	63 63 63
Overnight	1	94 100 100 106
Hapten replaced by buffer		
2 hours	2	810

(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) The first experiments on the precipitation of polyhaptenic simple substances by antisera and its inhibition by haptens were carried out by K. Landsteiner and J. van der Scheer, *J. Exp. Med.*, **56**, 399 (1932).

(5) D. Pressman and D. H. Brown, to be published.

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

The Effect of Changed Conditions on Amount of Precipitate.—In order to observe the effect of standing on the amount of precipitate obtained in the presence of hapten, equal volumes of serum S, antigen VI (25 μg./ml.), and hapten XXIII (60 μg./ml.) were mixed and 5-ml. aliquots of the mixture were permitted to stand zero, three-quarters, or two hours or overnight at room temperature and were then placed in the refrigerator at 3° over zero, one, or two nights. The amount of precipitate obtained in each test was about 15% of that obtained in the absence of hapten. The results are given in Table I.

Only when the tubes stood overnight at room temperature without subsequent refrigeration was a significant decrease in the amount of precipitate observed. This effect was also observed with specific precipitates in the absence of hapten.[2] A slight decrease was observed when the tubes stood overnight at room temperature and then overnight in the refrigerator.

TABLE II

INHIBITION OF PRECIPITATION OF ANTISERUM S AND ANTIGEN VI BY HAPTEN XXIII

Antigen solution, hapten solution, and antiserum, 1 ml. each; 2 hours at room temperature, overnight in refrigerator. Blanks of antiserum and buffer: 0, 0, 0 μg. *p*H of all supernates 8.2.

Amount of hapten, μg.	Amount of antigen, μg. 6.3	12.5	25	50	100
	Amount of antibody precipitated, μg.[a]				
0	122	335	660	305	(144)
4.1	(131)	262	356	181	131
8.3	125	212	285	147	106
16.5	88	163	178	109	103
31	41	94	116	72	
63	9	41	31	35	(31)
125	6	6	19	12	12
250	0	0	3	0	3
500	0	0	6	0	3

[a] Values are averages of duplicate analyses, with mean deviation of ±5% from the averages, except for the values in parentheses, which represent single analyses.

The Effect of Various Amounts of Hapten XXIII on Amount of Precipitate Obtained with Various Amounts of Antigen VI.—The results of the study of inhibition by hapten XXIII of the precipitation of antigen VI are given in Table II. As previously observed with phenylarsonic acid as the hapten,[2] the optimum zone does not shift significantly with increasing amounts of hapten.

Experiments on the Relative Inhibiting Powers of Different Haptens.—Since the effect of hapten inhibition is most easily given theoretical interpretation in the antigen–antibody equivalence zone, experiments were carried out with antiserum pools S and T to determine the optimum zones (which may be taken as the equivalence zones) for antigens III, VI, XI, XV, and XX. The results are given in Table III. It is seen that antiserum S contained much more antibody than T. As previously observed,[1] antigen XI, with long haptenic groups, gave much larger amounts of precipitate than the others. It is interesting, as an example of the variability of antisera, that antigen XX gave more precipitate than antigens III, VI, and XV with antiserum T but less with antiserum S.

Hapten inhibition experiments were then carried out with haptens XXI, XXIII, XXVIII, and XXIX and these five antigens in the optimum zones for the individual antigens with each of the antisera S and T. The results are given in Tables IV and V.

Experiments were also carried out with antiserum S and antigen VI at the optimum concentration in the presence of each of twenty-four haptens at various concentrations, with the results shown in Table VI.

Discussion

The data on hapten inhibition given in the foregoing tables can be most effectively discussed by comparison with the simple theory developed in a preceding paper of this series.[2] It was shown that for the system postulated, with both antigen and antibody assumed to be bivalent, a plot of the amount of antibody precipitated, for the case

TABLE III

PRECIPITATION OF ANTISERA S AND T AND FIVE ANTIGENS

Antigen solution 0.5 ml.; antiserum, 0.5 ml.; 1 hour at room temperature and overnight in refrigerator.

	Amount of antigen used, μg. 4.7	6.2	8.0	10.4	13.5	17.5	22.8	29.6	38.5	50
	Amount of antibody precipitated, μg.[a]									
Antiserum S										
Antigen III	194	228	303	(275)	306	226	191	169	135	(106)
VI	101	157	278	426	372	263	(156)	85	69	32
XI	147	226	291	438	603	859	1070	1410	1340	1300
XV	(138)	160	(187)	178	125	115	(75)	47	32	28
XX	(69)	100	97	107	54	47	28	28	22	22
	Blanks of antiserum and buffer: 0, 0, 0. *p*H of supernates 8.2									
Antiserum T										
Antigen III	10	16	0							
VI	47	42	10							
XI	153	(187)	253	319	438	500	460	472	469	359
XV	0	19	22	13	13					
XX	88	63	63	56	81	41	44	28	50	31
	Blanks of antiserum and buffer: 0, 0, 0. *p*H of supernates 8.3									

[a] Averages of duplicate analyses, with mean deviation ±7%; single analyses in parentheses.

TABLE IV

HAPTEN INHIBITION OF PRECIPITATION OF ANTISERUM S AND ANTIGEN

Antigen solution, hapten solution, and antiserum, 1 ml. each; overnight at room temperature and overnight in refrigerator. Blanks of antiserum and buffer: 0, 0, 0 μg. *p*H of all supernates 8.2.

Antigen, μg.	Hapten	Amount of hapten, μg. 0	1.95	3.9	7.8	15.6	31.3	62.5	125	250
		Amount of antibody precipitated, μg.[a]								
III	XXI	540	522	488	(456)	413	394	365		241
25	XXIII		468	488	(438)	285	257	181	113	50
	XXVIII		563	541	479	410	(406)	276	219	166
	XXIX		528	538	525	507	463	432	403	378
VI	XXI	556	500	432	369	266	191	147	101	97
25	XXIII		485	363	254	127	91	47	6	0
	XXVIII		457	397	319	213	122	60	28	6
	XXIX		528	519	488	441	362	294	222	197
XI	XXI	2310	2290	2160	(2070)	1935	1640	1400	1360	1090
59	XXIII		2255	2130	1955	1585	1170	794	416	194
	XXVIII		2285	2190	1860	1800	1320	970	663	435
	XXIX		2410	2330	2280	2210	2115	(2060)	1740	1490
XV	XXI	386	416		347	307	332	225	188	119
25	XXIII		366	347	228	131	79	37	16	0
	XXVIII		360	363	310	225	178	88	41	13
	XXIX		388	(400)	(419)	(425)	402	347	366	385
XX	XXI	167	194	178	178	169	147	125	94	60
16.7	XXIII		135	125	88	50	37	6		0
	XXVIII		141	125	106	63	37	6	0	0
	XXIX		190	181	181	147	141	125	116	94

[a] Averages of duplicate analyses, with mean deviation ±3%; single analyses in parentheses.

TABLE V

HAPTEN INHIBITION OF PRECIPITATION OF ANTISERUM T AND ANTIGEN

Antigen solution, hapten solution, and antiserum, 3 ml. each for antigen VI, 2 ml. for XI and XX; 2 hours at room temperature and overnight in refrigerator. Blanks of antiserum and buffer: 0, 0, 0 μg. *p*H of all supernates 7.8–7.9.

Antigen, μg./ml.	Hapten	Concentration of hapten solution, μg./ml. 0	1.95	3.9	7.8	15.6	31.2
		Amount of antibody precipitated, μg.[a]					
VI	XXI	373	353	(325)	254	197	(119)
10	XXIII		353	347	294	185	63
	XXVIII		303	178	57	22	3
	XXIX		356	344	291	244	166
XI	XXI	1320	1220	1110	832	804	581
61	XXIII		1245	1150	950	715	532
	XXVIII		1210	844	675	478	313
	XXIX		1155	1050	885	819	675
XX	XXI	491	516	472	(488)	438	485
6.3	XXIII		481	495	460	378	400
	XXVIII		485	466	422	354	222
	XXIX		479	485	479		446

[a] Averages of duplicate analyses, with mean deviation ±3%; single analyses in parentheses.

of equivalent amounts of antigen and antibody, against the amount of hapten present should be a straight line. Some examples of plots of this sort are given in Fig. 1, taken from the data of Table VI, which correspond to the optimum zones. It is seen that the curves approach the theoretical linear form only at low hapten concentrations; the deviation from linearity at higher concentration we attribute to the heterogeneity of the antiserum, which may contain antibodies with greatly varying combining powers.

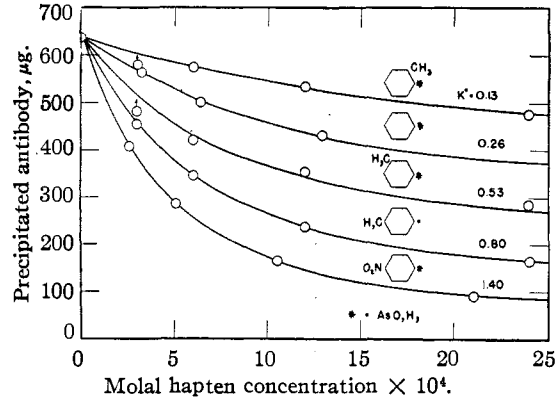


Fig. 1.—Effect of haptens in decreasing the amount of antibody precipitated by the trihaptenic antigen VI. Curves are shown for six of the twenty-four haptens for which data are given in Table VI.

Equation V of the earlier paper,[2] giving the value of the initial slope of the hapten-inhibition curve, may be rewritten in the form

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

with

$$C = \frac{(Ks + K^2s^2)^{1/2}}{s\{1/2 + Ks + K^2s^2 + (Ks + K^2s^2)^{1/2}\}}$$

and

$$C' = \frac{1 + Ks + (Ks + K^2s^2)^{1/2}}{1/2 + Ks + (Ks + K^2s^2)^{1/2}}$$

Here H is the amount of hapten added, $AB(pp)$ is the amount of antibody precipitated, s is the solubility of the antigen-antibody complex, and K and K' are the bond-strength constants for the antigen-antibody bond and the hapten-antibody bond, respectively. Both C and C' for any antiserum depend only on the antigen and are independent of the hapten. The constant K' is characteristic of the hapten, indicating the strength of the bond between the hapten and the antibody. Thus for any given antiserum and antigen the reciprocal of the rate of decrease of amount of precipitate with increasing amount of hapten is a linear function of the reciprocal of the hapten constant K'.

From the form of Equation 1 we should expect the order of inhibitory activity of various haptens for a given antiserum to be the same for various antigens. This is the case for our experiments. With serum S the order of inhibition was XXIII > XXVIII > XXI > XXIX for each of the five antigens used (Table IV). The order observed for serum T was different; XXVIII > XXIII > XXI > XXIX; but this order was again observed for each of the five antigens (data for antigens VI, XI, and XX are given in Table V; less reliable results obtained for III and XV, not included in the table, also placed the haptens in the same order).

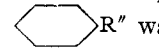
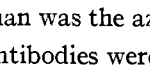
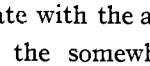
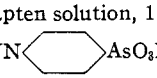
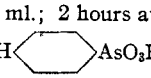
It is surprising that the amide compound  R″ was more effective in inhibition with serum T than was the azo compound HO  R, since the antibodies were produced by inoculation with an azoprotein and would accordingly be expected to combine preferentially with azo groups. Antiserum T was consistent in its greater reactivity with amide groups than with azo groups, insofar as it also gave a larger amount of precipitate with the amide antigen R″  R″ than with the somewhat similar azo antigens

TABLE VI

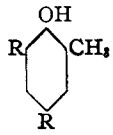
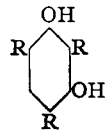
INHIBITION OF PRECIPITATION OF ANTISERUM S AND ANTIGEN VI BY HAPTENS

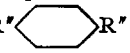
Antigen solution, 1 ml. (25 μg.); hapten solution, 1 ml.; antiserum, 1 ml.; 2 hours at room temperature and 2 nights in refrigerator. * = AsO_3H_2, R = NN  AsO_3H_2, R″ = —CONH  AsO_3H_2.

Hapten	K'	K_{A_2}[a] × 10^9	Amount of hapten, μg. 1.95	3.9	7.8	15.6	31.3	62.5	125	250
			Amount of precipitated antibody, μg.[b]							
o-$NH_2C_6H_4$*	0.13	2.2	662	550	500	(488)	(463)	375	269	200
o-$CH_3C_6H_4$*	.13	1.4	581	575	535	475	385	354	272	206
1-*-4-$NH_2C_{10}H_6$	.17	0.7	590	(588)	516	432	385	(275)	238	175
1-*-$C_{10}H_7$	.23	2.2	565	532	475	379	347	272	210	182
C_6H_5*	.26	3.3	566	501	432	325	235	169	131	91
o-$NO_2C_6H_4$*	.28	2.9	516	463	447	357	266	213	191	154
m-$NH_2C_6H_4$*	.29	2.4	566	538	387	306	(206)	181	131	119
p-$HOOCC_6H_4$*	.29	3.6	560	447	429	338	241	188	147	122
p-$NH_2C_6H_4$*	.44	1.2	538	(494)	376	276	216	172	128	116
p-$CH_3C_6H_4$*	.53	1.5	479	419	354	285	216	166	144	128
p-HOC_6H_4*	.60	3.9	475	422	372	213	173	134	103	85
2-*-$C_{10}H_7$	.66	3.4	473	388	344	250	182	(144)	116	79
m-$NO_2C_6H_4$*	.75	16	463	372	294	210	166	137	119	106
p-$CH_3C_6H_4$*	.80	2.1	457	344	238	163	144	91	63	41
p-ClC_6H_4*	.80	5.6	450	347	275	188	125	100	72	37
p-BrC_6H_4*	.80	6.5	494	400	285	216	103	106	78	66
p-IC_6H_4*	.80	5.7	(475)	404	297	194	169	131	94	50
C_6H_5R''	.80		541	454	332	225	175	101	50	6
p-$NH_2C_6H_4R''$	.89		559	457	291	194	135	78	34	10
p-$NO_2C_6H_4R''$	.89		513	419	335	216	153	88	41	13
p-HOC_6H_4R	.98		494	391	310	184	119	60	6	0
p-$NH_2C_6H_4R$	1.02		550	(438)	269	160	85	38	0	0
H_3CR''	1.02		451	341	203	141	85	50	19	0
p-$NO_2C_6H_4$*	1.40	16	407	285	163	91	44	16	0	0

Average value for no hapten 638 μg. Control, antiserum and buffer 0, 0, 0, 0 μg. *p*H of all supernates 8.1–8.2.

[a] Second acid dissociation constant.[5] [b] Averages of duplicate analyses, with mean deviation ±3%; single analyses in parentheses.


and

(Table III). This is the reverse of the behavior observed for serum S and the antisera tested previously.[1]

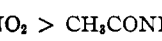
Another example of the variability in properties of the antisera is that the amide antigen  gave more precipitate than the other amide antigen $R''(CH_2)_2R''$ with serum T but less with serum S.

In order to obtain from the data of Table VI numbers representing the inhibiting power of the twenty-four haptens, the following treatment was used. Plots were made of the amount of precipitated antibody for each hapten as a function of the number of moles of hapten added, as illustrated in Fig. 1. Smoothed curves were drawn through the points, and the initial slopes were read from the curves. Some consideration was given to the course of the curve throughout the range of concentration covered by the experiment in determining the value of the initial slope, with use of a family of curves deduced from the whole set of data. Plots against the log of hapten concentration were also used. The reciprocals of the negative slopes $-\mathrm{d}\,AB(\mathrm{pp})/\mathrm{d}\,H$ are given in Table VI, under the heading K'. These reciprocals would be proportional to K' if the value of C' in Equation 1 were negligible compared to the other term. In any case these numbers would be expected to represent qualitatively the combining power of the haptens with antibody. A constant factor was introduced such that the average value of K' for the two azo haptens became unity.

Many interesting correlations of the values of K' with the molecular structure of the haptens can be observed. The effects of a substituent on the bond-strength constants of the substituted phenylarsonic acid molecules are seen to be dependent both on the position of the substituent in the benzene ring and on the nature of the substituent. It would also be expected that, aside from direct structural effects, the constituents would be of influence through their effect on the second dissociation constant of the acids. The antibodies, produced presumably in about neutral solution in the animal, probably have combining groups for both singly ionized and doubly ionized arsonic acid molecules. The combining power of the latter is probably greater than that of the former because of the stronger forces resulting from the doubled electric charge,[7] as has been pointed out by Haurowitz.[8] Accordingly it would be expected that increase in the second dissociation constant of the acid, leading to increase in the number of doubly charged ions present, would lead to increase in bond-strength constants with the antibody. There are given in Table VI values of the second ionization constant for eighteen of the haptens.[5] It is seen that the correlation with K' is not very striking.

The ortho-substituted phenylarsonic acids are seen to be, with one exception (the *o*-nitro acid), the least effective of all in combining with antibody. An α-naphthyl residue is about equivalent to an ortho-substituted benzene ring. Meta-substituted compounds are somewhat more effective in general than phenylarsonic acid, and para-substituted compounds are still more effective. β-Naphthylarsonic acid is intermediate between the meta- and para-substituted phenylarsonic acids.

All of the para-substituted phenylarsonic acids are more effective in inhibition than phenylarsonic acid itself. This increased effectiveness on replacement of the para hydrogen atom by a larger atom or group is presumably the result of increased van der Waals interaction between the substituent and the antibody. The order of effectiveness of the para substituents in increasing the value of the constant K' is

$$NO_2 > CH_3CONH > \text{  } > \text{  } >$$
$$Cl, Br, I, CH_3 > OH > NH_2 > COOH > H$$

The same order holds also in the ortho and meta positions for the substituents NO_2, CH_3, and NH_2, data not having been obtained for others.

Whereas replacement of a hydrogen atom in the para position by a substituent group always leads to an increase in the value of K', this is not so for the ortho position. Both *o*-methylphenylarsonic acid and *o*-aminophenylarsonic acid, as well as the α-naphthyl compounds, are less effective in inhibition than phenylarsonic acid itself. This difference in behavior from the para-substituted compounds is attributed to steric effects. The immunizing antigen, a protein with attached *p*-azophenylarsonic acid groups, gives rise to

(7) This may be responsible for the fact that the maximum amount of precipitate with variation of *p*H is obtained at *p*H 8, instead of at about 7, which presumably prevails at the site of antibody formation.

(8) F. Haurowitz, *Z. physiol. Chem.*, **245**, 24 (1937).

antibodies which are molded to the *p*-azophenylarsonic acid group, and which in general do not allow sufficient space for a larger group than hydrogen in the ortho position. On the other hand, a para substituent may fit into the space provided for the azo nitrogen atoms, and so by increased van der Waals attraction for the antibody makes the hapten more effective than phenylarsonic acid itself.

The most striking and unexpected observation is that the nitro group in any position in the benzene ring causes a large increase in the bond-strength constant over the value for any other substituent in the same position. For the ortho compound this increase is so great as to overcome the steric effect and make the hapten more effective than phenylarsonic acid. The reason for this large effect of the nitro group is not obvious. The second acid dissociation cannot be advanced as the cause, since the value of K_{A_2} for *o*-nitrophenylarsonic acid is less than that of phenylarsonic acid, whereas its value of K' is greater. We suggest that this action of the nitro group is the result of the well-known effect of the group in aromatic compounds in causing increased van der Waals attraction for other molecules, which shows itself in the great ability of aromatic nitro compounds to form molecular compounds with other substances.

It is surprising that haptens containing the amide group —CONH— are nearly as effective as the corresponding haptens containing the azo group —NN—. As mentioned above, for antiserum T the amide group was found to be even more effective than the azo group. It will be interesting to see, by experiments with other pools of antisera, to what extent the order of combining power with antibodies of the haptens listed in Table VI varies with the antiserum used.

In the larger haptens the effect of substituents far removed from the phenylarsonic acid group is small. Thus substitution of an amino or nitro group in the para position in phenylcarbamidophenylarsonic acid leads to an increase of only 10% in the value of K', with the nitro group showing no greater effect than the amino group. Similarly *p*-hydroxyphenylazophenylarsonic acid and *p*-aminophenylazophenylarsonic acid show nearly the same value of K'.

There is now under way an investigation of the inhibition by various haptens of precipitation with simple antigens of antisera made with use of a protein with attached azophenylazophenylarsonic acid groups as the immunizing antigen.

This investigation was carried out with the aid of a grant from the Rockefeller Foundation. Mrs. Elizabeth Swingle, Mr. Frank Lanni, Mr. Stanley Swingle, and Mr. Shelton Steinle assisted with the analyses.

Summary

A study has been made of the inhibiting action of haptens (substituted phenylarsonic acids) on the precipitation of polyhaptenic simple substances containing two or more phenylarsonic acid groups by antiserum obtained by inoculating rabbits with sheep serum coupled with diazotized *p*-arsanilic acid.

It was found that the order of inhibitory activity of four haptens was the same for each of five test antigens with a given antiserum. The order depended on the antiserum used. The antiserum which showed stronger inhibition by amide haptens than by azo haptens also gave larger amounts of precipitate with amide antigens than with azo antigens.

The effect of each of twenty-four haptens on one antigen-antibody reaction was studied, and the data were interpreted to give relative values of the bond-strength constant of the haptens with the antibody. These values are shown to be correlated with the structure of the haptens.

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