Hapten Inhibition of Precipitation of Antisera Specific to the p-Azophenylarsonic Acid Group, the p-(p-Azophenylazo)phenylarsonic Acid Group, and the p-Azophenylmethylarsinic Acid Group.

Thesis

by

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This work was carried out as a part of the Immunochemistry program at the California Institute of Technology. A series of compounds similar to phenylarsonic acid were prepared for the purpose of examining the effect of changes in the structure of the acidic group on the haptenic action. Previous work has been done by Erlenmeyer and Berger[1] who found that either ovalbumin or horse serum globulin coupled with diazotized p-aminophenyl-phosphonic acid gave precipitates with antiserum homologous to the p-azo-phenylarsonic acid group. However proteins coupled with diazotized p-aminophenylstibonic acid did not react with this serum. Haurowitz and Berinl[2] tested the inhibition of sixteen simple, easily available substances, including phenylphosphonic acid, on the precipitation of an anti-azophenylarsonic acid serum. The amount of inhibition was estimated visually. Phenylphosphonic acid showed good inhibition, but the other substances, most of which were not very similar in structure, did not. Grossberg (unpublished results) found that benzoic and benzenesulfonic acids inhibit poorly.

Experimental Methods

Preparation of Compounds

Phenylarsonic acid.- Prepared by D. Brown.

Arsanilic acid.- Prepared by G. Cleland.

p-(p-Hydroxyphenylazo)phenylarsonic Acid.- Prepared by C. Ikeda.

Phenylphosphonic acid.- Prepared by the method of Michaelis

$$\phi H + PCl_3 \xrightarrow{AlCl_3} \phi PCl_2 \xrightarrow{Cl_2} \phi PCl_4 \xrightarrow{H_2O} PO_3H_2$$

A mixture of 60 ml. of phosphorus trichloride, 180 ml. of benzene and 16 g. of anhydrous aluminum chloride was refluxed in an all glass apparatus on an oil bath for 40 hours. The mixture turned dark and two phases appeared. The layers were separated, and the top layer was distilled up to 85° to eliminate unchanged phosphorus trichloride and benzene. The residue (30 ml.) was extracted with 50 ml. pet. ether. The ether layer was cooled to 0°, and was saturated with chlorine. A white precipitate appeared. Water was carefully added, drop by drop, at 0°. A very vigorous reaction ensued. Aqueous sodium hydroxide was added till the solution was just blue to congo red, the solution was evaporated to 75 cc. and cooled. There appeared to be two compounds present. The less soluble was phenylphosphonic acid. The product was recrystallized twice from water. M.P. 158° C Yield 0.5 g.

Phenylstibonic acid.- Prepared as described by Christiansen[4]. Half amounts were used. The final precipitate was extracted with alcohol, then with acetone, washed with water and dried. Yield 36%

Methylarsonic acid.- Prepared by W. Renfrow.

Benzylarsonic Acid.- Prepared by the method of Quick and Adams[5].

$$\phi CH_2Cl + As_2O_3 \longrightarrow \phi CH_2AsO_3H$$

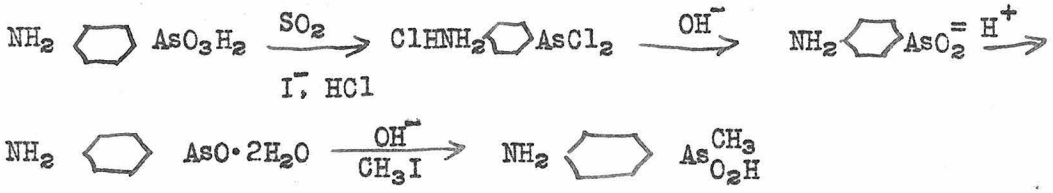
A mixture of 12.6 g. benzyl chloride, 5 g. arsenious oxide, and 15 cc. of 10N aqueous sodium hydroxide was refluxed with stirring for two hours. The upper layer was separated and discarded. The lower layer was made just neutral, and the slight precipitate which came down was discarded. Acid was added till the solution became just blue to congo red. The precipitate was immediately filtered off, washed with cold water and dried. M.P. 166-168°. Yield 1.7 g.

Phenylmethylarsonic Acid.- Prepared by the method of Bertheim[6].

$$\phi AsO_3H_2 \xrightarrow[I,\ HCl]{SO_2} \phi AsCl_2 \xrightarrow{H_2O} \phi AsO \xrightarrow[OH]{CH_3I} \phi CH_3AsO_2Na \xrightarrow{H^+} \phi CH_3AsO_2H$$

A solution of 10 g. of phenylarsonic acid and 0.1 g. potassium iodide in 50 ml. of 12 N hydrochloric acid was saturated with sulfur dioxide at 0° for 1 hour. The dichlorophenyl arsine separated as a heavy oil and was added to 100 ml. of water containing 15 g. of sodium carbonate. The phenylarsine oxide thus formed was dissolved in 8 ml. of 10 N sodium hydroxide solution to which was added 32 ml. ethanol and 4 ml. methyl iodide. The progress of the reaction was followed by withdrawing a sample and titrating with I_3^- in neutral solution. The reaction is complete in two hours. 100 ml. water was added and this was extracted with 20 ml. ether. The ether was discarded. With stirring, 8 g. of silver nitrate in 20 ml. of water was added to remove the iodide ion. As soon as a drop of acid gave no iodine color, 20 ml. of concentrated nitric acid was added. Silver iodide was filtered off. Then 10 g. more of silver nitrate in 20 ml. water was added and the solution was just neutralized with ammonium hydroxide. The silver phenylmethylarsinate was filtered off. The salt was added to 8 ml. 6 N hydrochloric acid in 150 ml. water. When all the precipitate seemed to have become silver chloride, the filtrate was evaporated to dryness on the water bath after neutralization to congo red with ammonium hydroxide. The residue was extracted with 10 ml. hot alcohol, filtered, and evaporated to dryness. This residue was dissolved in 1 ml. water and reprecipitated with warm acetone. The precipitation was repeated twice. Yield 0.5 g. M.P. 179.5° C.

p-Aminophenylmethylarsinic Acid.- Prepared by the method of Bertheim[6].

(a) 

10.8 g. (.05 mole) arsanilic acid was dissolved in 80 ml. 12 N hydrochloric acid. The solution was saturated with sulfur dioxide at 25° C. 0.1 g. of potassium iodide in 3 ml. water was added with stirring. After a few minutes the entire contents became almost solid and was filtered. The residue was added to 6 g. sodium hydroxide in 50 ml. water. Ammonium hydroxide was added in excess to the hot solution. On cooling, p-aminophenylarsine oxide crystallized out. Yield 90%.

0.06 mole of the p-aminophenylarsine oxide was dissolved in 12 ml. 10 N sodium hydroxide. 4 ml. of methyl iodide was added. The mixture was shaken until the titer with triiodide ion in neutral solution became small. This required about two hours. Freshly prepared silver chloride from 8 g. silver nitrate was added with stirring until a drop of acid gave no iodine color. Then 20 ml. of 6 N hydrochloric acid was poured in, making the solution blue to congo red. The silver chloride and silver iodide were filtered off. On evaporation to about 40 ml. an orange color appeared. The solution was dried in a desiccator over calcium chloride and the resulting solid mass was extracted with 50 ml. alcohol and treated with animal charcoal. After filtering off the charcoal, 100 ml. isopropyl ether was added to the alcoholic solution. A light colored solid separated. It was redissolved in alcohol and reprecipitated. Crystals were obtained by dissolveing in a minimum of alcohol, adding a large excess of nitrobenzene, and allowing the solution to evaporate slowly. Yield 31%. M.P. 201°

Phenyldimethylarsinedihydroxide.-

$$\phi AsCl_2 \xrightarrow{CH_3MgI} \phi As(CH_3)_2 \xrightarrow[H_2O]{Br_2} \phi As(CH_3)_2OHBr \xrightarrow{Ag_2O} \phi As(CH_3)_2(OH)_2$$

Methyl Grignard was made from 30 g., methyl iodide by the usual method. 0.1 mole dichlorophenylarsine in 30 ml. ether was dropped in with stirring, at 0° C. After refluxing 1/2 hour, ice and dilute HCl were added. The ether phase was separated, and the ether distilled off. The phenyldimethylarsine was distilled at 60 mm. B.p. 135°[7].

To this point, starting with phenylarsonic acid, the yield was 40%.

The phenyldimethylarsine was dissolved in 150 ml. petroleum ether (80-100°) and 10 ml. water was added. A 25% bromine solution in petroleum ether was added drop-wise with shaking, until there was a very faint yellow color. The water layer was separated and evaporated to 3 ml. The brownish solid which came out was dissolved in 30 ml. hot acetone. The insoluble residue was filtered off and discarded. On cooling the solution, thick, pale brown crystals came out. On recrystallization from 5 ml. water and 30 ml. acetone, the hydroxybromide melted at 161-162°. Yield, 0.6 g.

An aqueous solution of the hydroxybromide was shaken with excess silver oxide[8]. The solid was filtered off, and the solution of the dehydroxide was used directly.

Dissociation Constant of Phenylmethylarsinic Acid at 21.5° C.

A portion of 0.0103 g. of the acid was titrated at 25.5° C., in 20 ml. H_2O, with 0.025 N NaOH. A Beckman Model G. pH meter was used. 2.00 ml. were required for complete neutralization.

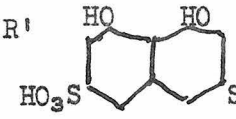
Data:

ml NaOH	pH	$H^+ \times 10^6$	Ratio $\frac{A^-}{HA} = \frac{(\text{ml. NaOH})}{(2-\text{ml. NaOH})}$	$10^6 \times K = (H^+)\frac{(A^-)}{(HA)} \times 10^6$
0.6	5.32	4.8	0.43	2.11
0.7	5.40	4.0	0.54	2.16
0.85	5.54	2.9	0.74	2.15
1.00	5.68	2.1	1.00	2.10
1.15	5.82	1.52	1.35	2.05
1.30	5.96	1.10	1.86	2.05
				2.1

From the inflection point pK = 5.65. This gives K = 2.2×10^{-6}. pK of

As O_3H_2 was similarly found to be = 3.7. This result was less accurate than the previous one because too dilute a solution was used. Pressman and Brown[9] found pK = 3.5 for this acid.

Experimental Procedure for Inhibition Tests.

Two separate sets of hapten inhibition tests were made with two different sera. One cc. each of hapten, and of R'  R' (antigen XXX)

(R' = -N=N  N=N AsO_3H_2) in a buffer of 0.16 M boric acid containing 0.9% sodium chloride at pH 8.0 were mixed and one cc. serum was added. This mixture was allowed to stand at room temperature for one hour, and then for approximately 24 hours in the refrigerator. The centrifuged precipitate was then analyzed by the method described by Pressman[10].

Analyses were run in triplicate. Average deviation from mean was less than 2.5%. pH of the supernate, unless otherwise stated, lay between 7.9 and 8.1. Thanks are due to Mr. Grossberg for help with these analyses.

Data: Set No.1. Serum: Anti-azophenlarsonic acid sheep serum. (anti-R serum) undiluted. Antigen diluted 1/26,000 for maximum precipitate. Values are tabulated as fractions ~~per mille~~ of the amount precipitated in absence of hapten, 230 μg.

Data: Set No. 2. Serum: Anti-azophenyl-azophenylarsonic acid sheep serum, (anti-R' serum) diluted to 1/2 concentration. Antigen diluted 1/40,000 to obtain maximum precipitate. Values are of the amount precipitated in the absence of hapten, 162μg.

Data: Set No. 3. Inhibition by some inorganic salts.

Conditions were the same as in set No. 1 pH 8.0 - 8.1. In triplicate.

Data: Set No. 4. Four rabbits were injected with p-azophenylmethylarsinic acid sheep serum. Three produced anitbodies. These three sera were pooled and used for inhibition tests. 2 cc. of serum and testing antigen p-azophenylmethylarsinic acid ovalbumin were mixed and varying amounts of hapten in 2 cc. of buffer at pH 8.0 added. Stood 1 hour at room temperature then 24 hours at 0° C. Values are tabulated as fractions ~~per mille~~ of the amount precipitated in the absence of hapten, 89 μg.

Data: Set No. 1

Inhibition by Haptens of the Precipitation of Anti -R Serum

Antigen solution, 1 ml. (38 μg); hapten solution, 1 ml.; antiserum, 1 ml. pH of supernates, 8.1

	Moles hapten added X10^8						
	1.4	4.1	123	37	111	333	1000
	Fract. amount of precipitate[a]						
p-Aminophenylarsonic acid	0.97	0.81	0.58	0.34			
Phenylarsonic acid	1.00	0.90	0.67	0.39			
Phenylphosphonic acid	0.99	0.90	0.77	0.50	0.17		
Benzylarsonic acid			0.95	0.89	0.77	0.65	0.47
Phenylstibonic acid			0.99	0.95	0.93[b]		
p-Aminophenylmethylarsinic acid			1.02	0.98	0.95	0.80	0.61
Phenylmethylarsinic acid			1.00	1.01	1.02	0.92	0.66
Methylarsonic acid			1.00	1.03	1.00	0.91	0.72
Phenyldimethylarsine hydroxybromide			0.97	0.96	0.98	0.98	0.97
p-Aminophenylarsinoxide						0.99	

a Amount in absence of hapten, 230 μg.

b pH, 8.2

Data: Set No. 2

Inhibition by Haptens of the Precipitation of Anti -R' Serum

Antigen solution, 1 ml. (25 μg); hapten solution, 1 ml.; antiserum, diluted with 1 vol. buffer, 1 ml. pH of supernates, 8.1

	Moles hapten added X10^8						
	1.4	4.1	123	37	111	333	1000
	Fract. amount of precipitate[a]						
p-Aminophenylarsonic acid		0.74	0.58	0.43			
Phenylarsonic acid		0.90	0.72	0.51			
Phenylphosphonic acid	0.90	0.86	0.61	0.42			
Benzylarsonic acid		1.09	0.88	0.88	0.27	0.16	
Phenylstibonic acid		1.12	1.05	1.11	0.99		
p-Aminophenylmethylarsinic acid			1.03	0.95	1.07	0.90	0.78
Phenylmethylarsinic acid			1.00	0.96	0.97	0.93	0.78
Methylarsonic acid			1.03	1.08	0.95	0.93	0.77
Phenyldimethylarsine hydroxybromide				1.03	1.03	1.06	1.05
p-Aminophenylarsinoxide						1.03	

a Amount in absence of hapten 162 μg.

Data: Set No. 3

Inhibition by Inorganic Salts of the Precipitation of Anti -R Serum

Antigen solution, 1 ml. (38 μg); salt solution, 1 ml.; antiserum 1 ml. pH of supernates 8.0 - 8.1

	Moles added $X10^5$	
Salt	1	10
	Fract. amount of precipitate[a]	
Na_2HAsO_4	0.92	0.44
$NaAsO_2$	1.16	0.40[b]
Na_2HPO_4	0.94	.40
Na_2SO_4	.95	.79
NaCl		1.00

a Amount in absence of added salt 230 μg.

b pH of supernatant, 8.4.

Data: Set No. 4

Inhibition by Haptens of the Precipitation of Anti-azophenylmethylarsinic Acid Serum

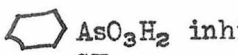
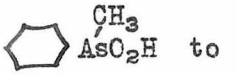
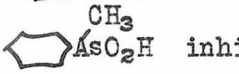
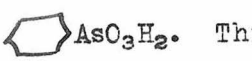
Antigen solution, 2 ml.; hapten solution, 2 ml.; antiserum 2 ml. pH of supernates, 8.1.

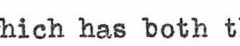
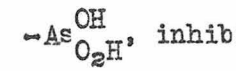
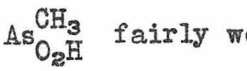
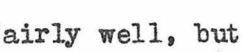
	Moles of hapten added $\times 10^8$				
	1	3	10	100	1000
	Fract. amount of precipitate[a]				
Phenylmethylarsinic acid	0.93	0.81	0.73	0.00	0.00
Methylarsonic acid			.79	.64	.53
p-(p-Hydroxyphenylazo)phenylarsonic acid			.98	.71	
Phenylarsonic acid			.93	.90	.57
Benzylarsonic acid			.95	1.03	.68
Phenylphosphonic acid			1.03	1.05	.69

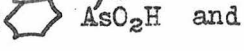
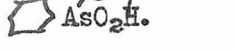
a Amount in absence of hapten, 89 μg.

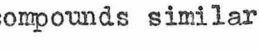
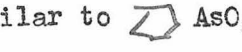
Conclusions

1. Substitution of $-CH_3$ for $-OH$ on the end group has a far greater effect than has substitution of groups onto the ring. Two substitutions have a still greater effect. It is a question as to whether this is a steric or a charge effect.

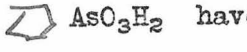
2.  inhibits anti NN  to about the same extent as  inhibits anti -NN  . This indicates that the effect is not entirely steric.

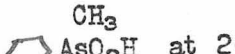
3. Methyl arsonic acid, which has both the groups  and  , inhibits anti -NN  fairly well, but anti NN  rather poorly.

4. Steric effects could be tested by using  and  .

5. Substitution of P for As leads to no appreciable change in the haptenic strength. However, a change of Sb for As causes the compound to have no inhibiting effect. This can be correlated with the previously noted fact that  is really (3 ) $2H_2O$ with two easily removable waters.

Summary

1. Some compounds similar to  have been studied for inhibiting action.

2. K_A for  at $25.5^\circ = 2.1 \times 10^{-6}$.

References

(1) Erlenmeyer and Berger, Biochem. Z., 255, 429 (1932).

(2) Haurowitz and Breinl, Z. Physiol. Chem., 214, 111 (1933).

(3) Michaelis, Ber., 6, 819 (1873).

(4) Christiansen, "Organic Derivatives of Antimony", New York, Chemical Catalog Co., 1925.

(5) Quick and Adams, J.A.C.S., 44, 811 (1922).

(6) Bertheim, Ber., 48, 352 (1915).

(7) Winmill, J. Chem. Soc., 101, 723 (1912).

(8) Steinkopf and Schewn, Ber., 54, 2795 (1921).

(9) Pressman and Brown, J.A.C.S., 65, 540 (1943).

(10) Pressman, Ind. Eng. Chem., Anal. Ed., 15, 357 (1943).