Where spherites form within a minute or so (see (c) and (d)) double refraction, when detectable under the conditions used, has a very short time (less than 0.01 second) indicating a fair concentration of short fibrils. This favors spherite formation for, as shown in Fig. 1a, the spherites resulting from this type of treatment are small, compact, and show well defined polarization crosses.

For purposes of comparison fibrils having lengths of 10, 20, 40 and 100 thousand ångström units have relaxation times of 0.03, 0.2, 1.39 and 18.8 seconds. These values were calculated from equation 13, page 511 of Cohn and Edsall,[9] assuming $\beta = 70 \times 10^{-8}$ cm., $\eta = 0.01$ poise and $T = 300°$.

(9) Cohn and Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943.

Summary

In acid solution insulin may be modified to form highly asymmetric fibrils. The aggregation of fibrils into spherites in which the fibrils are radially oriented accounts for the visible heat precipitate of insulin. The rate of spherite formation increases with increasing hydrogen ion concentration, protein concentration, neutral salt concentration, temperature and fluidity. In the absence of salts the acid anion has a pronounced effect on fibril and spherite formation.

Fibril formation precedes spherite formation. Spherite formation, favored by a high concentration of short fibrils, is absent under those conditions which lead, initially, to low concentrations of very long fibrils.

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The Reactions of Antiserum Homologous to the *p*-Azophenyltrimethylammonium Group[1]

BY DAVID PRESSMAN, ALLAN L. GROSSBERG, LELAND H. PENCE, AND LINUS PAULING

A great amount of information about the nature of serological reactions has been obtained through experiments on the properties of antisera produced by animals on injection of artificially conjugated proteins, especially azoproteins. This work, carried out during the past quarter of a century by Landsteiner and his collaborators[2] and by other investigators, has dealt mainly with the reactions of antisera with azoproteins and simple substances containing negatively charged haptenic groups (azophenylarsenate, azobenzoate, etc.) or neutral groups (azophenyl, etc.). The only serological study of positively charged haptenic groups which has been reported is that of Haurowitz and his collaborators,[3] who prepared antiserum by injecting rabbits with an azoprotein containing the *m*-azophenyltrimethylammonium group, which was made by the reaction of sheep serum globulin with diazotized trimethyl-(*m*-aminophenyl)-ammonium ion; this antiserum was found to precipitate the immunizing azoprotein and also similar azoproteins made from bovine serum globulin and ovalbumin, and the precipitation was found to be inhibited by a simple dihaptenic substance, di-(*m*-azophenyltrimethylammonium)-tyrosine.

(1) The Serological Properties of Simple Substances. XII. For no. XI of this series see D. Pressman, A. B. Pardee, and L. Pauling, THIS JOURNAL, **67**, 1602 (1945).

(2) K. Landsteiner and L. Lampl, *Biochem. Z.*, **86**, 343 (1918); K. Landsteiner, "The Specificity of Serological Reactions," Charles C. Thomas, Springfield, Ill., 1936.

(3) F. Haurowitz, K. Sarafyan, M. M. Yenson, S. Berkol, and P. Schwerin, *Rev. Fac. Sci. Univ. d'Istanbul*, **A5**, 1 (1940); F. Haurowitz, K. Sarafyan, and P. Schwerin, *J. Immunol.*, **40**, 391 (1941); F. Haurowitz, *ibid.*, **43**, 331 (1942).

Extending our studies of the serological properties of simple substances, we have now prepared an antiserum homologous to a positively charged haptenic group, the *p*-azophenyltrimethylammonium group, and have studied its reactions with a large number of substances. The antiserum used (called anti-A_p serum in the rest of this paper) was made by injecting rabbits with sheep serum coupled with diazotized trimethyl-(*p*-aminophenyl)-ammonium chloride. Studies were made of the precipitation of this antiserum by two azoproteins containing the same haptenic group, A_p-ovalbumin and A_p-horse serum albumin, of the inhibition of precipitation in these systems by a score of haptens, and of the effect of change of hydrogen-ion concentration on these reactions.

Experimental Methods

Protein Antigens.—The immunizing antigen used for inoculating the rabbits was made by diazotizing three portions of trimethyl-(*p*-aminophenyl)-ammonium chloride hydrochloride weighing 0.10, 0.24, and 0.43 g., respectively, coupling these at *p*H 8.0 to 8.5 and 5° with three 67-ml. portions of sheep serum, and finally mixing the three preparations, on the assumption that such a mixture would cover the range of highest antigenicity. When the mixture was brought to *p*H 4.6 only a slight amount of precipitate formed. The *p*H was brought to 7 and the solution was dialyzed against saline solution.

Test antigens were made from crystallized hen ovalbumin and from crystallized horse serum albumin by reaction with diazotized trimethyl-(*p*-aminophenyl)-ammonium chloride hydrochloride. Preparations 1 and 2 of A_p-ovalbumin were made by coupling 0.1-g. and 0.45-g. portions of the diazotized amine at *p*H 9 with 0.8 and 5.0 g. of ovalbumin, respectively. The antigens were purified by precipitating twice at *p*H 4.9, redissolving each time at

*p*H 9, and finally dialyzing against saline solution. Each precipitation at *p*H 4.9 rendered much of the material insoluble at *p*H 9. The loss thus incurred was about 80%.

Preparation 3 of A_p-ovalbumin was made by coupling 0.2 g. of the diazotized amine with 1 g. of ovalbumin at *p*H 9. The solution was dialyzed against borate buffer of *p*H 8. About 70% of the total protein precipitated during the dialysis. The clear supernate was used.

The A_p-horse serum albumin test antigen was prepared by diazotizing 1.0 g. of the amine and coupling with 5 g. of crystallized horse serum albumin. The solution was dialyzed against saline. The antigen was twice precipitated at *p*H 4.5 and redissolved at a higher *p*H. No insoluble material was formed by this treatment.

The protein concentrations of these antigens were determined by Kjeldahl analysis.

Preparation of Antisera.—Antisera were obtained and pooled in a manner similar to that described for anti-R sera.[4] Four different pools (A, B, C, and D) were used in this work.

Simple Haptens.—The simple haptens used were either the commercial products (with the correct melting point) or compounds prepared in these Laboratories as described in the following section.

The Reaction of Antigen, Antiserum, and Hapten.—The reactants were mixed and permitted to stand for one hour at room temperature and overnight at 5° for experiments with A_p-ovalbumin as the antigen and over two nights at 5° for experiments with A_p-horse serum albumin. The precipitates were centrifuged, washed three times with 10-ml. portions of saline solution, and analyzed by our standard method.[5]

The sera and solutions of antigens and haptens were brought to the desired *p*H values with hydrochloric acid or sodium hydroxide, and dilutions were made with borate buffers of the same *p*H values. These buffers were made by adding 0.16 *N* sodium hydroxide solution to a solution 0.2 *M* in boric acid and 0.16 *N* in sodium chloride.

Preparation of Compounds

Trimethylphenylammonium iodide was prepared by treating dimethylaniline with excess methyl iodide: m. p. obs., 217.5–218.5° with sublimation; reported,[6] 218°.

Trimethyl-(*o*-tolyl)-ammonium iodide was prepared similarly from *o*-toluidine: m. p. obs., 208–210°; reported,[7] 209°.

Trimethyl-(*m*-tolyl)-ammonium iodide was prepared similarly from *m*-toluidine: m. p. obs., 187–188°; reported,[8] 177°.

Trimethyl-(*p*-tolyl)-ammonium iodide was prepared similarly from *p*-toluidine: m. p. obs., 204.5–205°; reported,[9] 216–220°.

Trimethyl-(β-naphthyl)-ammonium iodide was prepared similarly starting with dimethyl-(α-napthyl)-amine: m. p. obs., 161.5–163.5° dec.; reported,[10] 164° dec.

Trimethyl-(*p*-aminophenyl)-ammonium chloride hydrochloride was prepared by acetylating *p*-aminodimethylaniline with acetic anhydride and sodium acetate, methylating with methyl iodide in methanol, replacing iodide ion with chloride ion by means of silver chloride, hydrolyzing with hydrochloric acid, and crystallizing from methanol: m. p. obs., 220° dec.; reported,[11] 219° dec.

Trimethyl-(*p*-(*p*-hydroxyphenylazo)-phenyl)-ammonium chloride was prepared by adding diazotized trimethyl-(*p*-aminophenyl)-ammonium chloride hydrochloride to a tenfold excess of phenol in sodium carbonate solution. *Anal.*[12] Calcd. for $C_{15}H_{18}ON_3Cl$; Cl, 12.2. Found: Cl, 12.3, 12.4.

Triethylphenylammonium iodide was prepared by treating diethylaniline with excess ethyl iodide in methanol: m. p. obs. 125–127°. *Anal. by Volhard titration:* Calcd. for $C_{12}H_{20}NI$: I, 41.6. Found: I, 41.5.

Trimethyl-(*p*-aminobenzyl)-ammonium chloride hydrochloride was prepared by the method of Reilly and Drumm.[11] The substance softened to a glass at 193–195° and melted with dec. at 254–265° as reported.[11]

Triethyl-(*p*-acetaminobenzyl)-ammonium iodide was prepared by a method similar to that used for the trimethyl compound by Reilly and Drumm[11]: m. p. 196.5–197.5°. *Anal.*[12] Calcd. for $C_{15}H_{25}ON_2I$: I, 33.7. Found: I, 33.8, 33.9.

Trimethylphenylarsonium iodide was prepared by the method of Bertheim[13] for arsenobenzene prepared by the method of Binz, *et al.*[14]: m. p. obs., 245–248°; reported, 248°.

1-Amino-3,6-disulfonic acid-7-(*p*-azophenyl-trimethylammonium)-8-hydroxynaphthalene was prepared by diazotizing 0.005 mole of trimethyl-(*p*-aminophenyl)-ammonium chloride hydrochloride and coupling with 0.0055 mole of "H-acid" in sodium carbonate solution. The dye was salted out with 10 volumes of saturated ammonium sulfate solution. The solid was extracted four times with 700-ml. portions of hot alcohol. The dye was precipitated with three liters of ether. It was purified by dissolving in 40 ml. of water and precipitating with hydrochloric acid solution at *p*H 0.8. The dye was washed with acetone before drying.

1-Amino-3,6-disulfonic acid-7-azobenzene-8-hydroxynaphthalene, 1-amino-3,6-disulfonic acid-7-(*p*-azo-*t*-butylbenzene)-8-hydroxynaphthalene, and **1-amino-3,6-disulfonic acid-7-(α-azonaphthalene)-8-hydroxynaphthalene** were prepared by coupling 0.10 mole of the corresponding diazotized amine with 0.11 mole of "H-acid" in sodium carbonate solution. The products were precipitated at *p*H 1 to 2. The α-azonaphthalene substance and the *p*-azo-*t*-butylbenzene substance were crystallized from alcohol at *p*H 8 and are presumably the disodium salts. The azobenzene substance was crystallized from alcohol at *p*H 2 and is presumably the monosodium salt. The products were freed of sodium chloride by repeated washing with acetone until the test for chloride ion with silver nitrate was negative.

The Effect of Hydrogen-Ion Concentration on the Precipitation of Anti-A_p Serum and Azoprotein Test Antigens

Data are given in Table I on the amount of precipitate formed by anti-A_p (pool B) and A_p-ovalbumin at several values of the hydrogen-ion

TABLE I

EFFECT OF HYDROGEN-ION CONCENTRATION ON THE PRECIPITATION OF ANTI-A_p SERUM AND A_p-OVALBUMIN

Antigen solution (prep. 3), 1.5 ml.; antiserum (pool B), 0.375 ml.; buffer, 2.625 ml. Antiserum and antigen solutions were adjusted to the *p*H indicated before mixing.

Initial *p*H	*p*H of supernate	Amount of antigen added, μg. 228	455	910	1820	3640
		Amount of protein precipitated, μg.[a]				
6.0	6.3–6.4	267	(371)	473	692	1017
7.0	7.1–7.15	287	372	394	483	209
8.0	8.0–8.1	264	317	388	386	42
9.0	8.95	260	317	373	326	20

[a] Averages of triplicate analyses, with mean deviation ± 2%. Duplicate analyses in parentheses.

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

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

(6) R. W. D. Preston and H. O. Jones, *J. Chem. Soc.*, 1942 (1912).

(7) J. von Braun, *Ber.*, **49**, 1107 (1916).

(8) J. von Braun and O. Kruber, *ibid.*, **46**, 3474 (1913).

(9) E. Wedekind, *ibid.*, **35**, 773 (1902).

(10) L. Landshoff, *ibid.*, **11**, 645 (1878).

(11) J. Reilly and P. J. Drumm, *J. Chem. Soc.*, 871 (1935).

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

(13) A. Bertheim, *Ber.*, **47**, 273 (1914).

(14) A. Binz, H. Bauer, and A. Hallstein, *ibid.*, **53**, 427 (1920).

concentration (pH 6.0 to 9.0), and similar data are given in Table II for anti-A_p serum (pool C) and A_p-horse serum albumin.

It is seen that for each system the amount of precipitate increases steadily with decreasing pH, whereas with systems involving negatively charged haptenic groups an optimum pH, usually about pH 8, is observed.[15,16,17] The behavior of the A_p systems may be attributed to the high isoelectric points of the azoprotein test antigens, which may well be increased by one pH unit above the values (4.5 to 5.0) for the unconjugated proteins by the attached basic groups; at low pH values the decreased electrostatic repulsion resulting from decrease in the electric charges on the molecules would permit precipitation of molecules of antibody and antigen through only weak specific forces of attraction, which otherwise would not cause precipitation. This effect is especially pronounced when a very large amount of azoprotein is added at low pH. For A_p-horse serum albumin the optimum zone remains constant at about 300 μg. of antigen at pH 9, 8, and 7, and shifts to 820 μg. at pH 6; and a similar effect is indicated for A_p-ovalbumin.

TABLE II

EFFECT OF HYDROGEN-ION CONCENTRATION ON THE PRECIPITATION OF ANTI-A_p SERUM WITH A_p-HORSE SERUM ALBUMIN

Antigen solution, 1.50 ml.; antiserum (pool C), 0.75 ml.; buffer, 2.25 ml. Antiserum and antigen solutions were adjusted to the pH indicated before mixing.

Initial pH	pH of supernate	Amount of antigen added, μg. 51	103	205	410	820	1640
		Amount of protein precipitated, μg.[a]					
6.0	6.5	156	377	548	662	717	655
7.0	7.3	175	417	571	586	494	178
8.0	8.1	183	385	521	543	407	49
9.0	9.0	98	286	467	470	217	14

[a] Averages of triplicate analyses, with mean deviation $\pm 2\%$.

Parallel experiments were carried out with normal serum and each of the two azoproteins; no precipitates were obtained, even at pH 6.

The Inhibition of Precipitation by Haptens

Data showing the effect of various haptens in inhibiting the precipitation of anti-A_p serum with A_p-ovalbumin at pH 7.7–7.8 are given in Table III. These data were interpreted with the aid of the theory of heterogeneous antisera[18]; the values found for the hapten inhibition constant K_0' and the heterogeneity index σ are given in the table. Similar data for hapten inhibition of the precipitation of anti-A_p serum with A_p-horse serum albumin at pH 6 and 8 are given in Table IV. Data are included in Table IV for some very weak haptens (alkyl substituted ammonium ions, and haptens with neutral haptenic groups); further experiments were made with these haptens, with the results reported in Tables V and VI.

TABLE III

EFFECT OF HAPTENS ON THE PRECIPITATION OF ANTI-A_p SERUM WITH A_p-OVALBUMIN

Antigen solution (prep. 1), 1.0 ml. (585 μg.); antiserum (pool A), 1.0 ml.; hapten solution, 1.0 ml.

Hapten—trimethylammonium ion	K_0'	σ	Moles of hapten added $\times 10^8$ 2	10	40	50	200	1000
			Amount of precipitate[a]					
p-(p-Hydroxyphenylazo)-phenyl	4.6	1.5	711	234		23		
α-Naphthyl	2.20	2.0	792	435		127		
p-Tolyl	1.21	1.0	974	668		156		
m-Tolyl	1.00	1.5	1075	685		234		
o-Tolyl	0.80	2.0	1000	692		338		
Phenyl	1.00	1.3	987	704		214		
p-Aminophenyl	0.85	1.0	1078	753		256		
p-Aminobenzyl	.46	1.5	1110	841		471		
Other haptens								
Trimethylphenylarsonium ion	.50	0.8	1124	942		435		
Triethyl-(p-acetaminobenzyl)-ammonium ion	.38	2.5			552		221	114
Triethylphenylammonium ion	.25	1.5			708		296	127
Tetramethylammonium ion	.023	2.5			1087		744	477
Tetraethylammonium ion	.096	2.5			809		416	247

[a] The amounts of precipitate are in parts per mille of the amount in the absence of hapten, 308 μg. pH of supernates 7.7–7.8. Values are averages of triplicate analyses, with mean deviation $\pm 2\%$.

A greater hapten concentration was required at pH 6 than at pH 8 for a comparable degree of inhibition.

The phenomenon of hapten inhibition is seen from the data in the tables to be essentially the same for these systems, with a positively charged haptenic group, as for the systems previously reported, with negatively charged haptenic groups. The haptens which resemble the haptenic group of the immunizing azoprotein in structure exert a strong inhibitory effect, whereas those haptens which are less closely related in structure exert only a weak effect or none at all. Some of the weak haptens were found to give increased precipitation[17]; this effect, enhancement of precipitation by weak haptens, will be discussed in a later paper.

The values found for the heterogeneity index σ lie for the most part within the customary range 1.0 to 3.0.

The three sets of values for the hapten inhibition constant K_0' given in Tables III and IV (relative to the value $K_0' = 1.00$ for the phenyltrimethylammonium ion) agree to within about

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

(16) D. Pressman, J. T. Maynard, A. L. Grossberg, and L. Pauling, *ibid.*, **65**, 728 (1943).

(17) D. Pressman, S. M. Swingle, A. L. Grossberg, and L. Pauling, *ibid.*, **66**, 1731 (1944).

(18) L. Pauling, D. Pressman, and A. L. Grossberg, *ibid.*, **66**, 784 (1944).

TABLE IV

EFFECT OF HAPTENS ON THE PRECIPITATION OF ANTI-A_p SERUM WITH A_p-HORSE SERUM ALBUMIN

Antigen solution, 2.25 ml. (274 μg.); antiserum (pool C), 0.75 ml.; hapten solution, 1.5 ml. Antiserum and antigen solutions were adjusted to *p*H 6.0 or 8.0 before mixing.

Hapten-trimethylammonium ion	K_0' at *p*H 6	K_0' at *p*H 8	σ at *p*H 6	σ at *p*H 8	Moles of hapten added × 10^8 23.5	47	94	188	375	750	1500	5.9	Moles of hapten added × 10^8 11.8	23.5	47	94	188	375	750	1500
					Amount of precipitate at *p*H 6[a]								Amount of precipitate at *p*H 8[a]							
p-(*p*-Hydroxyphenylazo)-phenyl	4.0	4.8	2.0	2.0	481[b]	185							(387)[c]		72					
p-Acetaminophenyl	1.75	2.15	1.5	2.0	759	282		62				721		388		73				
α-Naphthyl	1.38	2.02	3.5	2.5	639	421		240				700		436		181				
p-Tolyl	1.21	1.45	2.0	2.0	758	440		142					645		336		47			
m-Tolyl	0.59	0.86	2.0	2.0	835	625		286						607		297		34		
o-Tolyl	0.87	1.05	1.5	2.5	843	545		170						562		295		30		
Phenyl	1.00	1.00	2.0	2.0	800	(493)		147						576		252		17		
p-Aminophenyl	0.87	0.93	2.5	2.5	805	476		263						595		291		36		
p-Acetaminobenzyl	.72	.71	3.0	3.0	758	534		320						614		384		70		
p-Aminobenzyl	.48	.63	3.5	3.0	772	616		391							523		295		104	
Other haptens																				
Trimethylphenylarsonium ion	.48	.48	2.0	2.5	944	662		350							592		295		30	
Triethyl-(*p*-acetaminobenzyl)-ammonium ion	.27	.34	2.5	3.0		745		481		183					615		398		175	
Triethylphenylammonium ion	.16	.24	3.0	3.0	861		680		451						691		438		222	
Tetramethylammonium ion	.061	.052	1.5	2.5		1020		829		493						825		604		331
Tetraethylammonium ion	.085	.097	2.5	2.5		851		712		430						704		472		222
"H-acid"-*p*-azo-*t*-butylbenzene		.064		3.0				1030	1000	(955)						910		550		(324)
"H-acid"-azobenzene		.0082		2.5				1090	1148	1240						920		879		691
"H-acid"-α-azonaphthalene							1070	1050	1280						879		810		821	

[a] The amounts of precipitate are in parts per mille of the amounts in the absence of hapten: 437 μg. at *p*H 6 (*p*H of supernates 6.7–6.8), and 532 μg. at *p*H 8 (*p*H of supernates 8.1). Blanks of antiserum and buffer, 7 μg. at *p*H 6 and 5 μg. at *p*H 8. Values are averages of triplicate analyses, with mean deviation ±2%; duplicate analyses in parentheses. [b] Also 790 at 5.9. [c] Also 688 at 2.95.

TABLE V

EFFECT OF WEAK HAPTENS ON THE PRECIPITATION OF ANTI-A_p SERUM WITH A_p-OVALBUMIN

Antigen solution (prep. 2), 1.0 ml. (750 μg.); antiserum (pool A), 1.0 ml.; hapten solution, 1.0 ml. Antiserum and antigen solution were adjusted to *p*H 6 before mixing.

Hapten (ammonium ion)	K_0'	σ	Moles of hapten added × 10^8 2	10	40	50	200	1000
			Amount of precipitate[a]					
Trimethyl-α-naphthyl	1.32	4	900	750		540		
Trimethylphenyl	1.00	4	880	780		590		
Tetraethyl	0.096	6			790		650	500
Tetramethyl	.043	6			840		730	580
Triethyl	.010				830		(790)	940
Trimethyl	.004				940		930	790
Diethyl	.001				970		970	900
Dimethyl					970		1010	950
Ethyl					970		960	980
Methyl					980		970	1010

[a] The amounts of precipitate are in parts per mille. Average amount of precipitate in the absence of hapten, 488 μg. Blank of serum and saline, 0 μg. *p*H of supernates, 6.3–6.5. Averages of triplicate analyses, with mean deviation ±2.0%, duplicate analyses in parentheses.

20%. The averaged values for the trimethylarylammonium ions are the following:

	K_0'
p-(*p*-Hydroxyphenylazo)-phenyl	4.7
p-Acetaminophenyl	1.95
α-Naphthyl	1.87
p-Tolyl	1.29
m-Tolyl	0.82
o-Tolyl	0.91
Phenyl	1.00
p-Aminophenyl	0.88

The order of effectiveness of the para substituents is that previously observed for anti-R_p, anti-R_p', and anti-X_p sera.[16,17,18]

The fact that substitution of a methyl group in the ortho position of the benzene ring decreases the value of K_0' only slightly indicates that there is appreciable looseness of fit of hapten and antibody. Such a looseness of fit would correspond to a radial dilatation of the antibody molecule of about 0.8 Å.[19]

It is interesting that substitution by methyl in the ortho position increases the value of K_0' over that of the meta substituted compound in Table IV. This lack of steric effect permits the high polarizability of the naphthyl group (the difference in mole refraction, $R_{naphthyl}-R_{phenyl}$, is 18.3 cm.^{3}) to be reflected in the high value K_0' = 1.87 for the hapten containing the α-naphthyl group. A similar large value of K_0' for hapten containing the α-naphthyl group is also shown by anti-R_p' serum, whereas the values are very small for anti-R_p and anti-X_p sera.

Coulomb Interaction of Antibody and Hapten.—It has been generally believed that an important part of the force of attraction between an antibody molecule and an electrically charged homologous haptenic group is the Coulomb attraction of the electrical charge of the haptenic group and a complementary electrical charge of opposite sign located in the combining region of the antibody. No evidence has hitherto been

(19) L. Pauling and D. Pressman, THIS JOURNAL, 67, 1003 (1945).

TABLE VI

EFFECT OF HAPTENS WITHOUT A POSITIVE CHARGE ON THE PRECIPITATION OF ANTI-A_p SERUM WITH A_p-HORSE SERUM ALBUMIN

Series B was run six months after series A. Antigen solution, 1.50 ml., (330 μg., series A; 400 μg., series B); antiserum (pool D), 0.50 ml.; hapten solution, 1.0 ml.

			Moles of hapten added × 10^8												
Series A	K'	σ	0.25	0.49	0.98	1.95	3.9	7.8	15.6	31.3	62.5	125	250	500	1000
			Amount of precipitate[a]												
Phenyltrimethylammonium ion	1.00	2.5						880		721		453			
"H-acid"-*p*-azophenyltrimethylammonium ion	3.8	2.5				880		710		477					
"H-acid"-*p*-azo-*t*-butylbenzene	0.23	2.5									845		545		353
"H-acid"-azobenzene															784
"H-acid"-α-azonaphthalene														760	
Series B															
Phenyltrimethylammonium ion	1.00	2				986		895		675		359		207	
"H-acid"-*p*-azophenyltrimethylammonium ion	4.9	2.5		981		900		694		409		226			
p-(*p*-Hydroxyphenylazo)-phenyltrimethylammonium ion	12.4	2.5	985		876		642		(374)		171				
"H-acid"-*p*-azo-*t*-butylbenzene	0.34	2									832		480		281
"H-acid"-azobenzene	.058	3.5									845		770		610
"H-acid"											941		918		776

[a] The amounts are tabulated as the fractions per mille of the amount precipitated in the absence of hapten, 722 μg. in series A and 487 μg. in series B. Blanks of serum and buffer, 3 μg. in series A and 7 μg. in series B. *p*H of supernates 8.0. Values are averages of triplicate analyses, with mean deviation ±2%; duplicate analyses in parentheses.

advanced regarding the position of the complementary charge in the antibody.

In order to obtain information on this point hapten-inhibition experiments were carried out with two substances, "H-acid"-*p*-azo-*t*-butylbenzene and "H-acid"-*p*-azophenyltrimethylammonium ion; the precipitation reaction studied was that between A_p-horse serum albumin and anti-A_p serum. The data obtained are given in Table VI, together with data for some related haptens.

The haptenic groups in these two substances are very closely similar in size, shape, and electric polarizability; they differ significantly in that one haptenic group is electrically neutral and the other group is positively charged. Accordingly the van der Waals attraction and steric interaction of antibody with these two haptenic groups would be very nearly the same, and the difference in values of K_0' for haptens containing these groups is to be attributed to the Coulomb attraction of the positive charge of the ammonium ion group and a complementary negative charge in the combining region of the antibody.

The ratio of the values of K_0' for "H-acid"-*p*-azophenyltrimethylammonium ion and "H-acid"-*p*-azo-*t*-butylbenzene was 16.5 in one experiment and 14.4 in the other.[20] The average value, 15.5, may be used to evaluate the Coulomb interaction energy between the charged haptenic group and the antibody. The difference in standard free energy of combination of the antibody with these similar charged and uncharged haptenic groups is accordingly $RT \ln 15.5 = 1510$ cal./mole ($T \cong 278°$A.). This energy value may be identified with the expression Ne^2/Dr (e = electronic charge, D = effective dielectric constant, r = distance between charges) and a value of r obtained. For the effective dielectric constant of water recourse may be made to the function obtained by Schwarzenbach.[21] The value thus found for r is 7.0 Å.

This value is especially interesting because it is close to the smallest value which is structurally possible. The positive charge of the phenyltrimethylammonium ion may be considered to be at the center of the nitrogen atom (that is, the charge is effectively spherically symmetrical about this point). The radius of this ion (to the surface of the methyl groups) is[22] 3.5 Å. The minimum distance of approach of a negative charge to the surface of the antibody is the radius of an oxygen atom, 1.4 Å. Hence the minimum value of r which could occur is 4.9 Å. The fact that the value calculated from the hapten inhibition data is only 2.1 Å. greater than this minimum value shows that the complementary negative charge is close to the surface of the antibody at the place where it fits around the trimethylammonium group; it is not unlikely that this charge is carried by a carboxyl ion side-chain which constitutes the surface layer of the antibody at this place.[23]

(20) It may be seen from Table VI that a considerable change (about 50%) occurred in the relative values of K_0' for the "H-acid" haptens and the reference hapten trimethylphenylammonium ion during the six-months period between the two experiments reported in this table. The ratio found for one of the "H-acid" haptens and the reference hapten with a different antigen-antibody system (Table IV, *p*H 8) is also different. These differences may be due to differences in the interaction of the antibodies with the "H-acid" residue.

(21) G. Schwarzenbach, *Z. physik. Chem.*, **A176**, 133 (1936). Schwarzenbach's function (valid at 20°) may be approximated over the range of values $5 < r < 10$ Å. by an effective dielectric constant $D = 6r - 11$, with r in ångström units.

(22) L. Pauling, "The Nature of the Chemical Bond," 2nd ed., Cornell University Press, Ithaca, New York, 1940, pp. 164 and 189.

(23) The foregoing calculation and conclusions are based on the assumption that a single negative charge in the antibody provides the electrostatic attraction for the charged haptenic group.

Inhibition by the Trimethylphenylarsonium Ion and the Triethylphenylammonium Ion.—The observed rather strong combination of anti-A_p serum with the trimethylphenylarsonium ion (Tables III and IV) demonstrates the close similarity of structure of the quaternary cations of arsenic and nitrogen.

The trimethylphenylarsonium ion and the triethylphenylammonium ion are larger than the trimethylphenylammonium ion by 0.48 and 0.99 Å., respectively, and thus because of steric effects would be expected to combine less strongly with anti-A_p serum than this ion. This effect is observed; the values of K_0' for these larger ions are 0.49 and 0.22, respectively.

Haptens Containing the Benzyl Group.—The average values of K_0' for the three haptens containing benzyl instead of phenyl, from Tables III and IV, are the following:

	K'	
Trimethyl-(*p*-acetaminobenzyl)-ammonium ion	0.72	(1.87)
Trimethyl-(*p*-aminobenzyl)-ammonium ion	.52	(0.88)
Triethyl-(*p*-acetaminobenzyl)-ammonium ion	.33	(0.22)

The values in parentheses are those for the corresponding phenyl compounds. The decrease by about 50% shown by the first two haptens on replacement of phenyl by benzyl is the expected steric effect of replacing the homologous haptenic group by a larger group. On the other hand, the increase in K_0' shown by triethyl-*p*-acetaminobenzylammonium ion is surprising: possibly the dilatation of the antibody by the three ethyl groups is great enough to permit the benzyl group to replace the phenyl group without additional strain; the van der Waals attraction of the added methylene group would then lead to an increase in K_0' of about the magnitude observed.

Alkylammonium Ions.—Hapten inhibition data for methyl- and ethyl-substituted ammonium ions are given in Tables III, IV, and V. (Data closely similar to those in Table V were also obtained with A_p-horse serum albumin as precipitating antigen.) Only a few of the ions show a significant amount of inhibition at the concentrations studied. The replacement of an ethyl group (or a methyl group) by a hydrogen atom results in a decrease of K_0' to about one-tenth its value (Table V). This effect is somewhat greater than that predicted from the change in polarizability of the ions.

The greater value of K_0' for the tetraethylammonium ion than for the tetramethylammonium ion is probably due to the greater van der Waals attraction of the antibody for the ethyl group; because of the looseness of fit of the antibody the steric effect of the larger group is not determinative.

Haptens without a Positive Charge.—In Table VI are data concerning inhibition by several haptens without a positive charge. "H-acid"-azobenzene exhibits a value of K_0' only 20% as great as the sterically homologous "H-acid"-*p*-azo-*t*-butylbenzene. "H-acid"-α-azonaphthalene inhibits somewhat better than the azobenzene substance, presumably through its greater van der Waals forces.

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Summary

A quantitative study has been made of the reactions of anti-A_p serum, prepared by injecting rabbits with sheep serum treated with diazotized trimethyl-(*p*-aminophenyl)-ammonium ion, in order to obtain information about the properties of antibodies homologous to a positively charged haptenic group. The precipitation reactions of this antiserum with the azoproteins A_p-ovalbumin and A_p-horse serum albumin are similar to those of homologous antisera and antigens containing negatively charged haptenic groups, except that for the A_p-system there is an unusual increase in the amount of precipitate when the *p*H is changed from 8 or 9 to 6; this increased ease of precipitation is attributed to the shift in isoelectric point of the antigen caused by the added positively charged groups.

The power of various haptens, mainly substituted phenyltrimethylammonium ions, to inhibit the precipitation reactions was found to depend on the structure of the haptens in essentially the same way as for systems involving negatively charged haptenic groups.

The ratio of inhibiting powers of two similar haptens, one containing a trimethylammonium-ion group and the other the uncharged tertiary butyl group, is such as to indicate the presence of a complementary negative charge very near the surface of the combining region of the antibody.

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