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Summary

1. Casein has been separated into two fractions, α- and β-casein, which represent the two peaks in the electrophoretic pattern of casein at *p*H 7.

2. The fractions are not electrophoretically homogeneous under all conditions, but they have been purified so that neither fraction contains any of the other.

3. The electrophoretic behavior of casein and the existence of complex formation between α- and β-casein are discussed.

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The Serological Properties of Simple Substances. VIII. The Reactions of Antiserum Homologous to the *p*-Azobenzoic Acid Group

BY DAVID PRESSMAN, STANLEY M. SWINGLE, ALLAN L. GROSSBERG, AND LINUS PAULING

Our earlier studies of the reactions of antibodies with simple substances have for the most part been limited to antisera homologous to the *p*-azophenylarsonic acid group[1,2,3,4,5] and the *p*-(*p*-azophenylazo)phenylarsonic acid group[5,6]; recently we have investigated also the reactions of antiserum homologous to the *p*-azobenzoic acid group, with the results described in the present paper.[7] These investigations include quantitative studies of the specific precipitation of antiserum homologous to the *p*-azobenzoic acid group (hereafter called anti-X serum) by ovalbumin coupled with diazotized *p*-aminobenzoic acid (X-ovalbumin) and by the simple dihaptenic substance made by coupling diazotized *p*-(*p*-aminophenylazo)benzoic acid with chromotropic acid, of the effect of change of hydrogen-ion concentration on these precipitation reactions, and of the inhibition of precipitation by haptens. The data on inhibition by haptens have been interpreted in terms of the recently developed theory of heterogeneity of antisera.[5]

The inhibition by a great number of haptens of the precipitation of antisera homologous to the *p*-azobenzoic acid group with an azoprotein antigen was investigated qualitatively by Landsteiner[8] and Landsteiner and van der Scheer[9] and to a smaller extent by Hooker and Boyd.[10,11] That simple polyhaptenic substances can act as precipitating antigens was discovered by Landsteiner and van der Scheer,[12] who, however, did not include substances containing azobenzoic acid groups among those studied in this way. A study of these substances was first carried out by Hooker and Boyd,[11,13] who reported their failure to obtain precipitates with anti-benzoic acid sera and certain homologous polyhaptenic substances. In our work precipitation with anti-benzoic acid serum was observed for one of seven polyhaptenic substances studied.

Experimental Methods

Simple Test Antigens.—The polyhaptenic simple substances tested as precipitating antigens are listed in Table I. Their preparation is discussed in the following section.

Haptens.—The haptens used either have been described elsewhere[1,5] or were commercial products purified to the correct melting points.

Protein Antigens.—The immunizing antigens used for inoculation were prepared by coupling diazotized *p*-aminobenzoic acid with whole beef serum by the method of Landsteiner and van der Scheer.[14] Two preparations were made, with, respectively, 0.9 g. and 1.8 g. of *p*-aminobenzoic acid coupled with 200 ml. of serum and made up to 600 ml. These preparations were used interchangeably after it was found that they gave rise to antisera of similar titer.

The azoprotein test antigen was prepared by Mr. Carol Ikeda by diazotizing 0.03 g. of *p*-aminobenzoic acid and coupling the product with 2.0 g. of ovalbumin at *p*H 8. The antigen was dialyzed against distilled water for ten days, after which time the rate of passage of colored material through the membrane was negligible.

Antisera.—Antisera were prepared by injecting eight rabbits with beef serum coupled with *p*-aminobenzoic acid; the method was similar to that described previously for the preparation of anti-phenylarsonic acid sera.[1] The sera were pooled according to titer. A single pool of antiserum, which was obtained after nine months of inocula-

(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).

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

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

(7) An account of experiments carried out with mixtures of these antisera has already been published: L. Pauling, D. Pressman, and D. H. Campbell, *Science*, **98**, 263 (1943); THIS JOURNAL, **66**, 330 (1944).

(8) K. Landsteiner, *Biochem. Z.*, **104**, 280 (1920).

(9) K. Landsteiner and J. van der Scheer, *J. Exptl. Med.*, **54**, 295 (1931).

(10) S. B. Hooker and W. C. Boyd, *Proc. Soc. Exptl. Biol. Med.*, **47**, 187 (1941).

(11) S. B. Hooker and W. C. Boyd, *J. Immunol.*, **42**, 419 (1941).

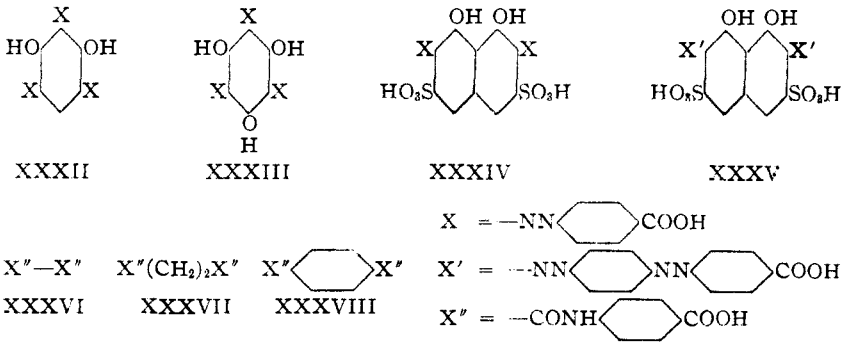
(12) K. Landsteiner and J. van der Scheer, *J. Exptl. Med.*, **56**, 399 (1932).

(13) W. C. Boyd, *ibid.*, **75**, 407 (1942).

(14) K. Landsteiner and J. van der Scheer, *ibid.*, **55**, 781 (1932).

TABLE I

SUBSTANCES USED IN PRECIPITATION TESTS



tion, was used in all of the experiments reported in this paper.

Reaction of Antiserum with Antigen and Hapten.—The reactants were mixed and permitted to stand for one hour at room temperature and over two nights at 5° unless otherwise stated. The precipitates were centrifuged and washed three times with 10-ml. portions of 0.9% sodium chloride solution. All dilutions of antigen and hapten were made with buffer solution of *p*H 8.5 unless otherwise noted. The buffer solutions were prepared by adding 0.16 *N* sodium hydroxide solution to 0.2 *M* boric acid in 0.9% sodium chloride solution.

Methods of Analysis.—The amount of protein in the precipitates was determined with the Folin–Ciocalteu protein reagent by the modification discussed elsewhere.[15] Determination of the amount of antigen in the precipitates was made with a photoelectric colorimeter on solutions of the precipitates in sodium hydroxide before the protein reagent was added.

Preparation of Substances.—XXXII, 1,3-Dihydroxy-2,4,6-tri-(*p*-azobenzoic acid)benzene; XXXIII, 1,3,5-trihydroxy-2,4,6-tri-(*p*-azobenzoic acid)benzene; and XXXIV, 1,8-dihydroxy-2,7-di-(*p*-azobenzoic acid)-3,6-disulfonic acid-naphthalene were prepared by coupling diazotized *p*-aminobenzoic acid in 30% excess at *p*H 8 to 9 with resorcinol, phloroglucinol, and chromotropic acid, respectively. The reaction mixtures were permitted to stand for one day at room temperature. The products were precipitated with hydrochloric acid and partially purified by repeated solution with sodium hydroxide and precipitation with hydrochloric acid followed by washing with acidulated water. Since diazotized *p*-aminobenzoic acid decomposes and couples with itself under the conditions of these preparations to yield a substance which is also soluble in alkali and insoluble in acid, it was necessary to remove as much of this material as possible. This impurity is soluble in hot 80% alcohol, and compound XXXII was purified by repeated extraction with 80% alcohol. From the ultraviolet absorption spectra of the decomposition product and the dyes, we estimate that not more than 10% of this impurity was present in any of the preparations. The observed carbon percentages are high except for XXXII, which was purified with alcohol. This substance gave a high carbon value before purification.

Anal. Calcd. for $C_{27}H_{18}O_9N_6$, XXXII: C, 58.5; H, 3.25. Found: C, 58.9, 59.0; H, 3.64, 3.56. Calcd. for $C_{27}H_{18}O_9N_6$, XXXIII: C, 56.8; H, 3.16. Found: C, 59.4, 59.7; H, 3.53, 3.58. Calcd. for $C_{24}H_{16}O_{12}N_4S_2$, XXXIV: C, 46.7; H, 2.60. Found: C, 48.5, 48.4; H, 3.11, 3.08.

***p*-(*p*-Aminophenylazo)benzoic acid** was prepared by coupling diazotized *p*-aminobenzoic acid with the theoretical amount of aniline-ω-methylsulfonate[16] and hydrolyzing the product for one and one-half hours at 90° in 1 *N* sodium hydroxide solution. The product was purified by precipitating three times at *p*H 6 and redissolving in base. The product of decomposition of diazotized *p*-aminobenzoic acid is, as mentioned previously, soluble at this *p*H. The amine was finally recrystallized twice from hot 80% alcohol as the sodium salt.

XXXV, 1,8-Dihydroxy-2,7-di-(*p*-(*p*-azophenylazo)benzoic acid)-3,6-disulfonic acid-naphthalene, was prepared by coupling diazotized *p*-(*p*-aminophenylazo)benzoic acid with chromotropic acid (mole ratio 2:1) in basic solution. Any monosubstituted chromotropic acid was removed by repeated extraction with an 80% acetone solution saturated with sodium chloride.

Anal. Calcd. for $C_{36}H_{24}O_{12}N_8S_2$: C, 52.5; H, 2.9. Found: C, 53.5, 54.0; H, 3.6, 4.1.

XXXVI, *p,p′*-Dicarboxyoxanilide; XXXVII, *p,p′*-dicarboxysuccinanilide; and XXXVIII, *p,p′*-dicarboxy-*p*-phthalanilide, were prepared by Dr. W. B. Renfrow, Jr. To a solution of 0.020 mole of recrystallized *p*-aminobenzoic acid in 50 ml. of hot dioxane which had been purified by refluxing with sodium metal and which contained 0.020 mole of pyridine there was added the appropriate acid chloride (0.009 mole in 15 ml. of dioxane), and the solution was boiled for fifteen minutes. The reaction mixture was then cooled, and the resultant precipitate, after two washings with water, was dissolved in 400 ml. of water containing 4 g. of potassium hydroxide and reprecipitated by acidifying to *p*H 3. The product was centrifuged and washed three times with water.

Anal. Calcd. for $C_{16}H_{12}O_6N_2$, XXXVI: C, 58.53; H, 3.68. Found: C, 57.40, 57.43, 57.73; H, 3.80, 4.00, 4.10. Calcd. for $C_{18}H_{16}O_6N_2$, XXXVII: C, 60.68; H, 4.50. Found: C, 59.77, 59.68; H, 4.45, 4.55. Calcd. for $C_{22}H_{16}O_6N_2$, XXXVIII: C, 65.34; H, 3.99. Found: C, 64.45, 64.34; H, 4.19, 4.21.

The Precipitation Reaction

Precipitation of Anti-X Serum with Polyhaptenic Simple Substances.—To 0.5-ml. portions of anti-X serum there were added 0.5-ml. portions of solutions of each of the substances listed in Table I. Concentrations of the substances ranging by factors of 3 from one part in 10,000 to one part in 270,000 were tested. Of the six substances only one, XXXV, gave visible precipitates with the antiserum. No precipitates were obtained under these conditions when normal serum was used in place of anti-X serum; nonspecific precipitation of normal serum and substance XXXV was, however, observed at concentrations of one part in 1000 or greater.

It has been observed that antisera homologous to the *p*-azophenylarsonic acid group in general give the largest amounts of precipitate with those polyhaptenic substances which have their phenylarsonic acid groups farthest removed from each other,[1] and that antisera homologous to the *p*-(*p*-azophenylazo)phenylarsonic acid group give precipitates only with substances in which the phenylarsonic acid groups are separated by at least three benzene rings.[6] These observations have been explained in terms of the steric interference of anti-

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

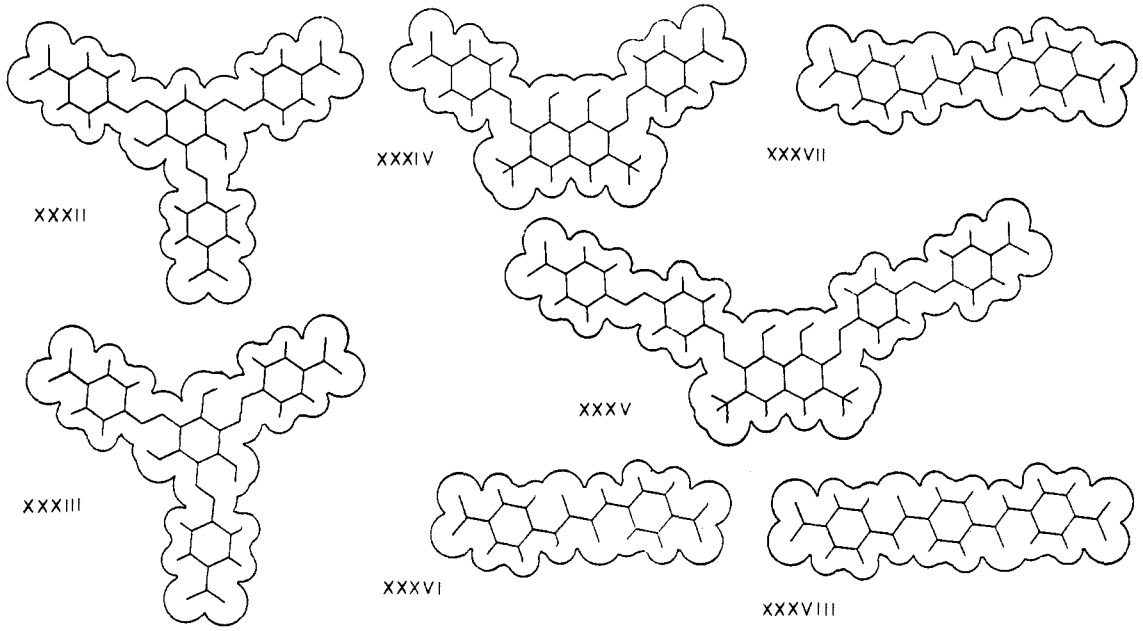


Fig. 1.—Scale drawing of molecules of substances used in precipitation tests.

body molecules clasping the haptenic groups of a small antigen molecule. The failure of substances XXXII, XXXIII, XXXIV, XXXVI, XXXVII, and XXXVIII to precipitate with anti-X serum may be explained in the same way: in all of these molecules (see Fig. 1) the benzoic acid groups are considerably closer together than in XXXV, which does precipitate with the antiserum. The fact that steric forces are here more effective in preventing precipitation than for the phenylarsonic acid system is presumably connected with the general weakness of the bonds formed by anti-benzoic acid antibodies.

TABLE II

EFFECT OF HYDROGEN-ION CONCENTRATION ON PRECIPITATION OF ANTI-X SERUM BY X-OVALBUMIN

Antigen in saline solution or buffer, 1 ml.; antiserum, 0.33 ml.; saline solution or buffer, 0.67 ml. In the absence of buffer antiserum and antigen solutions were adjusted to the *p*H indicated before mixing. Blanks of serum and saline solution or buffer at all *p*H values, 0 μg.

Initial *p*H	*p*H of supernate	Amount of antigen added, μg. 56	84	126	190	285
Buffer absent		Amount of protein precipitated, μg.[a]				
6.0	6.50	244	394	592	610	350
6.5	7.15	288	486	664	586	310
7.0	7.50	365	520	700	659	329
7.5	7.75	429	598	742	682	416
8.0	7.90	386	565	666	600	340
8.5	8.15	382	574	681	616	316
9.0	8.35	361	548	604	536	152
Buffer present						
6.0	6.54	241	412	549	529	230
7.0	7.25	381	539	665	561	172
8.0	8.05	365	530	605	535	211
9.0	8.90	328	474	586	448	6

[a] Averages of analyses in triplicate, with mean deviation ±2.9%.

Dependence of Amount of Precipitate on Hydrogen-Ion Concentration.—Data showing the effect of concentration of hydrogen ion on the amount of protein precipitated by X-ovalbumin and by substance XXXV with anti-X serum in the presence and the absence of borate buffer are given in Tables II and III.

The *p*H for maximum precipitation with X-ovalbumin as the test antigen (Table II) is about 7.7 (as measured on the supernatant solution) in the absence of borate buffer and about 7.3 in the presence of buffer. The amount of antigen giving the maximum amount of precipitate decreases slightly with increase in *p*H. The maximum amount of precipitate occurs for a somewhat larger amount of antigen in saline solution than in borate buffer, and the amount of the precipitate at the maximum is also somewhat larger. Similar effects of borate buffer have been observed in our previous studies.[2,6]

The *p*H value found for maximum precipitation of anti-X serum with antigen XXXV, about 9.2, is surprisingly high. The optimum antigen concentration changed very little over the *p*H range studied, 7.8 to 9.6. Tests were made in saline solution at only one *p*H value, 8.1, with results indicating that use of saline solution in place of borate buffer increases both the optimum antigen concentration and the amount of precipitate, as found also for X-ovalbumin.

The Composition of Precipitates of Antibody and Substance XXXV.—Values of amount of antigen XXXV, as well as of antibody, in the precipitates formed in this system were determined; these values are given in Table III, together with values of the ratio of the number of moles of antibody (assumed molecular weight

TABLE III

EFFECT OF HYDROGEN-ION CONCENTRATION ON THE PRECIPITATION OF ANTI-X SERUM WITH ANTIGEN XXXV

Antigen solution in buffer, 1.00 ml.; antiserum, 0.75 ml.; saline solution or buffer, 0.25 ml. Blank of antiserum and buffer, 0.0 μg. A, amount of antigen in precipitate (μg.); average of triplicate analyses with mean deviation ±2.0%; B, amount of antibody in precipitate (μg.); average of triplicate analyses with mean deviation ±1.8%; C, ratio of moles of antibody to moles of antigen.

Initial pH	pH of supernate	Amount of antigen added, μg. 7.8	15.6			31.2			62.5			125			250		
		B	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
7.7	7.8	40	2.7	385	0.73	5.6	757	0.70	6.4	805	0.62	6.7	718	0.55	7.4	653	0.46
8.0	8.1	53	2.9	482	.85	6.3	884	.72	7.5	914	.63	8.3	911	.56	10.0	894	.46
8.5	8.45	54	3.6	500	.71	7.1	896	.65	8.3	946	.59	8.8	923	.54	10.0	903	.45
9.0	8.75	56	4.7	519	.58	8.6	983	.59	10.0	1012	.52	10.6	977	.48	11.6	923	.41
9.3	9.1	56	3.9	535	.71	6.9	984	.73	9.4	1042	.57	9.4	964	.53	10.1	913	.43
9.5	9.2	41	4.0	468	.60	8.4	879	.54	9.1	875	.50	9.4	837	.47	9.4	748	.41
10.0	9.6	8	2.0	185	.48	4.4	434	.50	4.5	400	.45	3.7	301	.42	2.8	201	.36
8.1[a]	8.1	71	4.8	558	.60	9.3	1017	.56	11.5	1113	.50	13.0	1094	.43	14.9	1057	.36

[a] Run made with use of saline solution in place of buffer.

157,000) to the number of moles of antigen. This ratio decreases with increase in the amount of antigen used, as was observed also for phenylarsonic acid systems.[3]

The fact that the mole ratio is less than unity shows that the antibody molecules have an effective valence greater than two; the structural significance of this has been discussed elsewhere.[3]

Evidence indicating that the antigen is not precipitated non-specifically even at the highest concentrations used in the series reported in Table III is provided by an experiment carried out to test this possibility. One milliliter of solution of antigen XXXV, containing 250 μg., and 1 ml. of ovalbumin solution were added to 1 ml. of anti-ovalbumin serum. In the resulting precipitate of ovalbumin and anti-ovalbumin, which contained about 1 mg. of protein, there was found only about 0.2 μg. of substance XXXV. The *p*H of the supernatant solution was 8.0.

Inhibition of Precipitation by Haptens

Data showing the effect of various haptens (over fifty in number) on the precipitation of anti-X serum with antigen XXXV and with X-ovalbumin are given in Table IV. These data were interpreted with the aid of the extended quantitative theory developed in the preceding paper of this series,[5] which is based on the assumption that the heterogeneity of the antiserum can be described by an error-function distribution in the free energy of interaction of antibody and hapten in competition with antigen. Application of this theory leads to the evaluation of two constants for each antibody–antigen–hapten system: one of these constants, K_0', is an average hapten inhibition constant, representing the average bond strength of antibody and hapten relative to that of antibody and antigen, and the other constant, σ, is an index of the effective heterogeneity of the antiserum. Values of K_0' and σ for the haptens with each of the two antigens are given in the table.

The reference point for K_0' has been taken as $K_0' = 1.00$ for benzoic acid as hapten. This choice of reference point differs from that made for the phenylarsonic acid systems described in our earlier papers, for which the value $K_0' = 1.00$ was assigned to certain *p*-substituted phenylarsonic acids; the new choice increases all values of K_0' by a factor of about 10.

The heterogeneity of the anti-X serum, as indicated by the values of σ in Table IV, is about the same as that of the anti-R and anti-R′ sera previously investigated.[5] The values of σ tend to be somewhat larger in the precipitation of X-ovalbumin than in that of antigen XXXV, although the difference may not be greater than the probable error (about ±0.5) of the assigned values. For each antigen a trend of the values of σ may be discerned: in general σ increases as K_0' decreases. An explanation of this effect, which was observed also with anti-R and anti-R′ sera, has been proposed.[5]

The values of K_0' given in Table IV range between 37.8 and 0.0003. The smaller values, below about 0.05, have little quantitative significance, except as to order of magnitude; most of the larger values we estimate to be reliable to within about ±20%. It has been found[5] that corresponding values of K_0' for two pools of antiserum or for the same pool of antiserum with two different antigens usually agree to within ±30%, but that larger differences occasionally occur, corresponding to a factor of 2 or more. The values for antigen XXXV and X-ovalbumin in Table IV show only rough agreement; about one-half of the larger pairs of values disagree by more than 30%, and for a few haptens the disagreement is very large (as for β-naphthoic acid, with $K_0' = 2.1$ and 10.7). The significance of this lack of agreement is not clear.

One very interesting difference in behavior of antigen XXXV and X-ovalbumin was noted: whereas the precipitation of anti-X serum with X-ovalbumin was inhibited to some extent by every one of the haptens studied, those haptens which produced only a small inhibition with the protein antigen gave increased amounts of precipitates with antigen XXXV. This effect is shown

TABLE IV

EFFECT OF HAPTENS ON THE PRECIPITATION OF ANTI-X SERUM WITH ANTIGEN XXXV AND X-OVALBUMIN

With antigen XXXV: antigen solution, 1.25 ml. (55 μg.); antiserum, 0.75 ml.; hapten solution, 1.00 ml. With X-ovalbumin: antigen solution, 1.67 ml. (160 μg.); antiserum, 0.33 ml.; hapten solution, 1.00 ml. One hour at room temperature and over two nights at 5°.

		Antigen XXXV		X-ovalbumin		Moles of hapten added, × 10^8											
						1.7	5	15	40	200	1000	1.7	5	15	40	200	1000
						Amount of precipitate with Antigen XXXV[b]						Amount of precipitate with X-ovalbumin[b]					
	$K_A \times 10^{5}$[a]	K_0'[c]	σ[d]	K_0'[c]	σ[d]												
p-Acetaminobenzoic acid	5.2	38.	1.0	24.	2.6	432	88	9				699	505	285			
m-Acetaminobenzoic acid	8.5	0.37	2.5	0.57	2.0	956	888	887	720	420	165				780	593	52
o-Acetaminobenzoic acid	23	0.104	3.5	.25	3.5	950	961	885	752	621	385				777	642	389
p-(*p*-Hydroxyphenylazo)-benzoic acid		11.3	1.0	21.9	2.0	793	489	69				727	530	185			
p-(*p*-Aminophenylazo)-benzoic acid		10.4	1.5	17.0	2.0	760	520	137				791	572	810			
p-Nitrobenzoic acid	37.6	12.8	2.0	11.5	3.0	679	442	171				785	613	435			
m-Nitrobenzoic acid	32.1	0.12	2.8	0.40	2.2	1002	998	956	850	620	338				820	594	231
o-Nitrobenzoic acid	671	(0.0009)	(4)	0.0055	(5)	1005	981	1000	991	975	900				929	883	800
p-Methoxybenzoic acid	3.4	7.4	2.0	7.3	2.5	756	585	275				841	720	524			
o-Methoxybenzoic acid	8.1	0.0086	4.0	0.060	4.0	928	897	906	900	835	704				839	766	587
m-Iodobenzoic acid	14.1	.42	2.5	0.99	2.0	970	931	849							721	382	0
p-Bromobenzoic acid	10.7	4.0	1.5	5.0	2.5	847	775	450				915	772	591			
m-Bromobenzoic acid	15.4	0.28	2.0	1.25	2.0	965	914	859	824	486	78				646	358	21
o-Bromobenzoic acid	140	0.060	2.5	0.074	2.8	991	955	915	900	763	459				987	800	551
p-Methylbenzoic acid	4.2	3.6	1.5	2.6	4.0	918	757	485				862	766	645			
m-Methylbenzoic acid	5.4	0.22	3.0	0.66	2.0	975	914	845	780	527	229				743	519	75
o-Methylbenzoic acid	12.3	.0086	4.0	0.084	4.0	973	961	946	970	860	710				864	755	540
p-Chlorobenzoic acid	10.5	3.2	2.5	3.7	2.8	851	726	503				908	791	634			
m-Chlorobenzoic acid	14.8	1.34	2.0	0.79	3.5	922	882	698				900	890	802			
o-Chlorobenzoic acid	114	0.16	2.2	0.20	3.0	964	924	894	835	592	222				867	684	392
β-Naphthoic acid	68	2.10	1.0	10.7	3.0	925	892	661	316	19	5	787	645	433			
α-Naphthoic acid	20	(0.0025)	(4)	0.18	4.0	984	1040	970	920	915	841				800	655	432
p-Hydroxybenzoic acid	2.9	1.95	2.5	1.14	3.0	896	790	610				951	890	794			
m-Hydroxybenzoic acid	8.3	0.45	3.0	0.43	3.5	913	870	816				950	922	860			
o-Hydroxybenzoic acid	105	0.29	2.0	0.46	2.5	976	909	851	781	486	111				798	570	236
p-Fluorobenzoic acid	7.2	1.87	2.0	1.16	4.0	900	835	633				927	836	790			
p-Phthalic acid	31.,1.5	2.8	2.0	7.3	2.0	906	754	534				894	795	496			
m-Phthalic acid	29.,2.5	0.85	2.5	0.83	5.0	972	870	749				875	818	724			
o-Phthalic acid	130.,0.39	(0.0003)	(4)	0.0024	(5)	1010	1030	1028	971	960	955				914	881	856
p-Aminobenzoic acid	1.2	1.06	2.0	0.88	3.0	895	840	745	545	134	5	940	920	819			
m-Aminobenzoic acid	1.6	0.50	2.0	.33	2.2	955	910	840	698	346	54				837	631	271
o-Aminobenzoic acid	1.0	2.6	1.5	1.45	4.0	956	810	580				875	830	710			
Benzoic acid	6.3	1.00	1.5	1.00	2.5			816	571	133	9	945	919	807	735	398	51
o-Benzoylbenzoic acid	37.			0.0032	(5)	1010	1010	994	1030	1140	1235				926	898	834
o-N-Phenylaminobenzoic acid		(0.039)	1.5	0.43	3.0	985	974	975	991	905	589				803	535	289
2-Hydroxy-3-naphthoic acid		0.39	1.5	1.76	1.7	982	938		808	330	73				640	214	34
2,4-Dihydroxybenzoic acid	51.6	0.39	2.5	0.51	2.0	955	915	845	725	334	275				803	561	165
2-Hydroxy-3-nitrobenzoic acid	1570	(0.015)	2.5	.17	3.5	985	980	969	925	890	674				856	678	452
2-Hydroxy-5-nitrobenzoic acid	890	(0.0050)	(4)	.19	3.0	980	1030	1016	1060	1019	764				897	683	418
2,4-Dinitrobenzoic acid	3850	0.041	3.0	.060	4.0	1000	1005	942	921	777	530				882	785	580
3,5-Dinitrobenzoic acid	160			.0044	(5)	1010	990	1031	1055	1139	1160				937	898	818
Naphthalic acid				.0007	(5)	970	946	925	1010	1050	1128				965	965	920
Trimesic acid		0.0003	(4)	.0004	(5)	982	1000	1010	986	995	955				960	960	939
2-Hydroxy-3,5-dinitrobenzoic acid				.026	6	1028	1028	1042	1115	1310	1290				830	745	621
2,4,6-Trinitrobenzoic acid		(0.0018)	(4)	.034	5.0	986	1018	1038	915	970	850				875	765	626
2,4,6-Trimethylbenzoic acid	3.75			.0030	(5)	1000	984	969	1035	1075	1168				914	886	841
Tetrachlorophthalic acid				.0042	(5)	1045	1015	1055	1071	1185	1238				920	879	820
β-Phenylpropionic acid	2.2			.0055	(5)	1024	1030	1030	986	1031	1094				926	889	795
Phenylacetic acid	4.9			.0016	(5)	1011	1030	1038	935	974	1080				926	920	878
Cyclohexanecarboxylic acid	1.3			.0016	(5)	975	995	1015	985	1005	1049				970	939	837
p-(*p*-Hydroxyphenylazo)-phenylsulfonic acid				.12	3.5				1062	1171					883	717	
Phenylsulfonic acid		.0010	(4)	.0008	(5)	948	978	968	955	900	968				960	939	908
p-(*p*-Hydroxyphenylazo)-phenylarsonic acid		.0086	(4)	.0015	(5)				954	848					945	921	
Phenylarsonic acid		.0003	(4)	.0005	(5)				992	967					952	950	934
Sodium sulfate		.0003	(4)	.0010	(5)				975	970	950				960	911	934
Sodium phosphate		.0012	(4)	.0003	(5)				982	947	880				950	966	955

[a] Values of acid dissociation constants are for the most part as given by J. F. J. Dippy, *Chem. Rev.*, **25**, 151 (1939), or as recorded in Beilstein. [b] The amounts of precipitate are in parts per mille of the amounts in absence of hapten: 730 μg. for antigen XXXV in the sequence of tests at the three lowest hapten concentrations (*p*H of supernates 8.6–8.7), 800 μg. for antigen XXXV in the other sequence of tests (*p*H 8.4–8.5), and 976 μg. for X-ovalbumin (*p*H 8.4–8.5). Blanks of antiserum and buffer, 0 μ g. All values are averages of triplicate analyses, with mean deviation ±2%. [c] Values in parentheses are considered to be unreliable because of the possibility of interference by the enhancement effect. [d] Values of σ given in parentheses were assumed.

by about a dozen haptens in Table IV; a striking example is 2-hydroxy-3,5-dinitrobenzoic acid, which causes a 30% increase in the amount of precipitate. Still greater enhancement was observed for other substances (normal fatty acids), and the phenomenon has been found to occur also for non-arsonic acid haptens with anti-R serum and simple antigens containing *p*-(*p*-azophenylazo)-phenylarsonic acid groups. A report on this effect will be published later.

For several haptens in Table IV the value of K_0' for antigen XXXV is reported to be smaller than that for X-ovalbumin by a factor between 10 and 100. It seems likely that the data for antigen XXXV are perturbed by the enhancement effect, and that the true values of the inhibition constant for these haptens are larger than the values given in the table, which we have accordingly placed in parentheses.

The Effect of Single Substituents on the Inhibition Constant of Benzoic Acid.—In general, the para-substituted benzoic acids are better inhibitors than benzoic acid itself, and the meta-substituted and ortho-substituted benzoic acids are poorer.

For the substituents NO_2, Br, Cl, CH_3, COOH, and CH_3CONH the dependence of the inhibition constant on position in the ring is $p > m > o$; however, for the amino group the order $o > p > m$ is observed both for antigen XXXV and for X-ovalbumin. The hydroxyl group seems to be intermediate in behavior; the normal order $p > m > o$ is observed for antigen XXXV, and the order $p > o > m$ for X-ovalbumin.[17] With antisera homologous to the *p*-azophenylarsonic acid group we have found[4,5] the order $p > m > o$ to hold for the groups studied, NO_2, CH_3, and NH_2; the anomalous behavior of the *o*-amino group is not shown in the phenylarsonic acid system. A further discussion of this matter is given below.

The change of the substituent from the para to the meta position decreases K_0' by a factor which ranges from about 100 for the strongest groups (acetamino, nitro) to about 3 for the weakest, and the change from the meta to the ortho position causes a further decrease by a factor of the order of magnitude of 10, except for the anomalous cases of the hydroxyl group and the amino group.

The order of activity of groups in the para position in benzoic acid, as given by the average values of K_0' for the two antigens XXXV and X-ovalbumin, is the following: $CH_3CONH > OHC_6H_4NN > NH_2C_6H_4NN > NO_2 > CH_3O > Br > Cl > CH_3 > COOH > OH > F > H > NH_2$. (The value of K_0' for phthalic acid is divided by 2, for the purpose of this comparison, as a correction for the contribution of the symmetry number to the entropy.) This is essentially the same as the order of activity of these substituents in the para position in phenylarsonic acid in inhibiting the precipitation of anti-R serum,[4,5] except that for the R system the nitro group is the most effective substituent and the hydroxyl and amino groups precede the carboxyl group.

These observations are in part easily interpretable in terms of the structure of the molecules and the nature of the intermolecular forces which are operative.[18] The para-substituted benzoic acids are stronger haptens than benzoic acid itself because the groups in the para position interact with the antibody (which is in this respect complementary to a para-azo group) more strongly than does the small and only feebly polarizable hydrogen atom. The acetamino and phenylazo groups in particular would be expected to be very effective; on the other hand, the strength of the nitro group is surprising, as is also the weakness of the carboxyl group and the amino group.

The effect of substitution in the meta position seems to be almost entirely steric; the antibody presumably is made to fit rather closely round a hydrogen atom in this position, and any larger group gives rise to steric hindrance which decreases the value of K_0' from 1.0 to about 0.5. The only exceptions among the values for meta-substituted benzoic acids in Table IV are *m*-chlorobenzoic acid with antigen XXXV and *m*-bromobenzoic acid with X-ovalbumin, for which the values $K_0' =$ 1.34 and 1.25, respectively, are reported. In the inhibition of precipitation of anti-R serum, meta substitution by the nitro group in phenylarsonic acid increases the value of K_0' by a factor of about 4. The fact that in the benzoic acid system steric hindrance of meta substituents is more pronounced than in the phenylarsonic acid system suggests that the anti-X antibodies fit their haptenic group more closely than the anti-R antibodies fit theirs. It has previously been inferred from data on hapten inhibition that the fit of anti-R antibodies is closer than that of anti-R′ antibodies.[5,6] The inverse correlation of the closeness of fit with the strength of the bond between haptenic group and antibody (the strength of the bond, as shown by the precipitating power of the antisera, increases in the order X, R, R′) is interesting in light of the prediction[19] of an inverse correlation between specificity of interaction and the strength of antigen–antibody bonds.

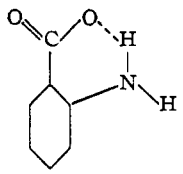
Most substituent groups in the ortho position in benzoic acid produce a decrease in K_0' to a value considerably below 0.1, presumably through strong steric hindrance. The strikingly exceptional behavior shown by the amino group, as mentioned above, is displayed in diminished amount by the hydroxyl group, the N-phenylamino group, and the acetamino group, but not by the methoxyl group. This suggests that the characteristic feature of molecular structure which is responsible for the phenomenon is a hydrogen bond between the

(17) Landsteiner and van der Scheer[3] reported that with their anti-*p*-azobenzoic acid serum and an X-protein antigen the order of inhibition was $p > o > m$ for hydroxy and amino substitution in benzoic acid, and $p > m > o$ for the groups NO_2, Br, Cl, and CH_3.

(18) L. Pauling, D. H. Campbell, and D. Pressman, *Physiol. Rev.*, **23**, 203 (1943).

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

group in the ortho position (which provides the hydrogen atom) and the ionized carboxyl group. The way in which this structural feature achieves its effect is, however, not clear.



Values of the acid dissociation constant of several of the haptens are given in Table IV; there is no significant correlation between them and the values of K_0'. This result shows that the factors which determine the magnitude of the attraction of the hapten ions for the antibody are not the same as those which determine the magnitude of the attraction of the hapten ions for hydrogen ion. The lack of correlation between values of the acid dissociation constant and K_0' was not clear in systems involving anti-R and anti-R′ sera, since in these cases the arsonic acid haptens are dissociated to different extents at the hydrogen-ion concentrations used, whereas in the systems involving anti-X sera the haptens are completely dissociated.

The Effect of Two or More Substituents.—It might be expected that normally, when no significant interaction between the groups occurs, the effect of two or more substituent groups on the free energy of interaction of a substituted benzoic acid with antibody would be the sum of the free energy effects of the groups separately, and that accordingly the value of K_0' for the polysubstituted acid would be equal to the product of the values (on the scale with $K_0' = 1$ for benzoic acid) for the monosubstituted acids. It is seen from Table V that values calculated in this way agree moderately well with the observed values for the disubstituted benzoic acids; only very rough agreement is found for the trisubstituted acids, for which the observed values are unreliable. The only pronounced discrepancy in Table V is shown by 3,5-dinitrobenzoic acid; comparison with 2-hydroxy-3,5-dinitrobenzoic acid suggests that the observed value is too small by a factor of 10 or more.

The very small values (0.0003, 0.0004) observed for trimesic acid (1,3,5-benzenetricarboxylic acid) are surprising in comparison with the values 0.85 and 0.83 for *m*-phthalic acid.

TABLE V

HAPTEN INHIBITION CONSTANTS FOR DISUBSTITUTED BENZOIC ACIDS

Substituents	Antigen XXXV Calcd.[a]	Antigen XXXV Obs.	X-ovalbumin Calcd.[a]	X-ovalbumin Obs.
2,4-Dihydroxy	0.57	0.39	0.52	0.51
2-Hydroxy-3-nitro	.035	(.015)	.18	.17
2-Hydroxy-5-nitro	.035	(.005)	.18	.19
2,4-Dinitro	(.012)	.041	.063	.060
3,5-Dinitro			.16	.004

[a] The product of the observed values for the corresponding monosubstituted benzoic acids. Values in parentheses are uncertain.

Naphthoic Acids.—The values of K_0' observed for α-naphthoic acid (0.18) and β-naphthoic acid (2.10 and 10.7) are seen to be reasonable when these acids are considered as 2,3 and 3,4-disubstituted benzoic acids, respectively, and the expected large van der Waals attraction of the naphthalene residue with the contiguous groups of the antibody is borne in mind.

The comparison of 2-hydroxy-3-naphthoic acid and β-naphthoic acid is interesting; the *o*-hydroxyl group changes K_0' by the factor 0.2, which is shown to be reasonable by the value for *o*-hydroxybenzoic acid.

The very small value of K_0' for naphthalic acid (naphthalene-1,8-dicarboxylic acid) is presumably the result of steric effects similar to those usually caused by an ortho substituent in benzoic acid, and presumably associated with non-coplanarity of the carboxyl groups and the aromatic ring.

Inhibition by Polyhaptenic Substances.—The substances XXXII, XXXIII, XXXIV, XXXVI, XXXVII, and XXXVIII, which failed to give precipitates with the anti-X serum, were found to be very effective in inhibiting the precipitation of anti-X serum with antigen XXXV and with X-ovalbumin; values of K_0' for these substances, as determined in a series of experiments with antigen XXXV to be reported in detail later, are about 120, 170, 60, 50, 110, and 100, respectively.

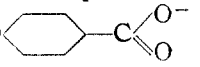
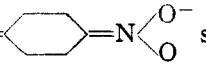
Other Haptens.—All other haptens which we tested were found to be poor inhibitors.

Cyclohexanecarboxylic acid was found to interact only very weakly with our antiserum (Landsteiner and van der Scheer[9] and Hooker and Boyd[10] observed sufficient inhibition of precipitation with their sera to be observable qualitatively); the difference in strength of this hapten and benzoic acid may be due in part to steric effects, resulting from the difference in shape of the two molecules, and in part to the smaller van der Waals interactions of the saturated ring than of the aromatic ring. The same factors are responsible for the very small inhibiting powers of phenylacetic acid and β-phenylpropionic acid, as well as of the fatty acids from acetic acid to *n*-heptanoic acid (which were tested, but are not included in Table IV).

Phenylsulfonic acid and phenylarsonic acid and their *p*-(*p*-hydroxyphenylazo) derivatives exert a small inhibitory effect, about one one-thousandth as great as that of the corresponding benzoic acids. The fact that inhibition occurs may be attributed to the presence of a negatively charged group attached to a benzene ring; the smallness of the effect in comparison with benzoic acid is due to the different shape and increased size of the sulfonic acid or arsonic acid group and the carboxyl group. The azo compounds are in general better inhibitors than the acids from which they are derived,

which provides further evidence that the azo group contributes to the interaction with the antibody.

A small amount of inhibition was observed for sulfate ion and for phosphate ion.

The similarity in shape of the *p*-hydroxybenzoate ion  and the *p*-nitrophenolate ion  ,  suggested that *p*-nitrophenol might be an effective inhibitor for the anti-benzoic acid system at *p*H 8; experimental test, however, showed that it is not.

This investigation was carried out with the aid of a grant from The Rockefeller Foundation. We wish to thank Professor Dan H. Campbell for advice during the course of the work and Dr. W. B. Renfrow, Jr., and Mr. Carol Ikeda for the preparation and analysis of several substances.

Summary

A quantitative study has been made of the serological properties of anti-X serum, prepared by injecting rabbits with beef serum coupled with diazotized *p*-aminobenzoic acid. It was found that this antiserum gives specific precipitation with a simple substance containing two *p*-azobenzoic acid groups (substance XXXV), made by coupling diazotized *p*-(*p*-aminophenylazo)benzoic acid to chromotropic acid, but not with any one of six other polyhaptenic substances, in which the haptenic groups are closer together than in XXXV. The antiserum also precipitates X-ovalbumin, made by coupling *p*-aminobenzoic acid with ovalbumin.

The dependence on the *p*H of the system of the amount of precipitate formed by anti-X serum with XXXV and with X-ovalbumin was studied.

The effect of each of 56 haptens in inhibiting precipitation was also investigated, and the data were interpreted with use of a quantitative theory based on the assumed heterogeneity of the antiserum. It was found that the order of activity of the haptens is nearly the same for the two precipitating antigens, XXXV and X-ovalbumin. The order of activity of groups in para-substituted benzoic acids is $CH_3CONH > OHC_6H_4NN > NH_2C_6H_4NN > NO_2 > CH_3O > Br > Cl > CH_3 > COOH > OH > F > H > NH_2$. In general the meta-substituted benzoic acids are weak inhibitors, and the ortho-substituted benzoic acids are still weaker, presumably as the result of steric hindrance. An exception is *o*-aminobenzoic acid, which exerts a strong inhibiting action, which may be connected with the presence of the hydrogen bond between the amino group and the carboxyl group. The effects of two or more substituents in benzoic acid are roughly additive in the free energy of interaction with antibody.

The precipitation of antigen XXXV with anti-X serum was found to be enhanced by moderate amounts of weak haptens, including substances other than substituted benzoic acids. This phenomenon was not observed for X-ovalbumin.

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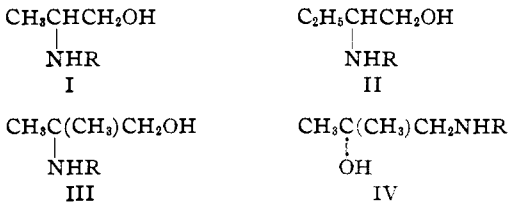
[CONTRIBUTION FROM THE CHEMICAL LABORATORIES OF BRYN MAWR COLLEGE AND COLUMBIA UNIVERSITY]

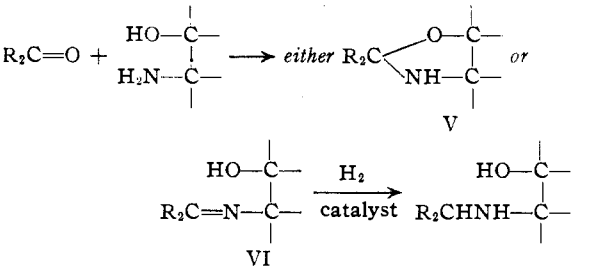
Monoalkylaminopropanols and Butanols and their Esters

BY EVELYN M. HANCOCK[1] AND ARTHUR C. COPE

In previous papers[2] practical methods have been described for preparing and esterifying 2-alkylaminoethanols and 1-alkylamino-2-propanols. This paper describes the preparation and esterification of 2-alkylamino-1-propanols (I), 2-alkylamino-1-butanols (II), 2-alkylamino-2-methyl-1-propanols (III) and 1-alkylamino-2-methyl-2-propanols (IV).



Each of the aminoalcohols was prepared by condensing a ketone (or an aldehyde) with an aminoalcohol containing a primary amino group and hydrogenating, the two steps being carried out either simultaneously or successively.



Steric hindrance of either the carbonyl or the amino group slowed these reactions. The hydrogenation of ketones with 2-amino-2-methyl-1-pro-

(1) Sharp and Dohme Research Associate.

(2) (a) Cope and Hancock, THIS JOURNAL, **64**, 1503 (1942); (b) **66**, 1448 (1944); (c) **66**, 1453 (1944).