[CONTRIBUTION FROM THE SECTION OF IMMUNOCHEMISTRY OF THE SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH AND FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, NO. 1939]

The Closeness of Fit of Antibenzoate Antibodies About Haptens and the Orientation of the Haptens in Combination[1-3a]

BY DAVID PRESSMAN,[3b] MALCOLM SIEGEL AND LUTHER A. R. HALL

RECEIVED JUNE 30, 1954

From the inhibitory action of the chloro-substituted phenylazobenzoates on the specific precipitation of antibodies formed against benzoate ions, information has been obtained about the closeness of fit about hapten of the previously uninvestigated portion of the antibody complementary region. With antibodies to the *o*-azobenzoate ion, the fit about the benzene ring was closest in the 3- and 4-positions and least close in the 6-position. With antibodies to the *m*-azobenzoate ion, the fit about the benzene ring was closest in the 4-position, least close in the 5-position and intermediate in the 2- and 6-positions. For those cases where two orientations for the combination of hapten with antibody are possible, such as with the chlorobenzoates, the relative distribution has been calculated. For example *m*-chlorobenzoate, which has been considered to combine with anti-*m*-azobenzoate antibodies with the chlorine in the azospecific site of the antibody, is calculated to combine to the extent of 10% in the non-preferred orientation.

In previous studies on the interaction with simple substances of antibodies prepared against aromatic ring compounds, there often was an ambiguity in the determination of closeness of fit of antibody around the haptenic group. The uncertainty in the determined closeness of fit resulted from the possibility that the hapten could fit into the complementary region of the antibody molecule in more than one orientation. For example, in the case of antiserum specific to the *m*-azobenzenearsonate ion,[4] it was not possible to determine whether an *ortho*-substituted benzenearsonate ion combined with the antibody with the *ortho* substituent adjacent to the antibody region which had been formed complementary to the position of attachment of the azo linkage, or *para* to that region. Figure 1 (A and B) shows two possible orientations for combination of *o*-chlorobenzenearsonate ion with the combining site of an antibody against the *m*-azobenzenearsonate group. Only two orientations are considered possible, since it is known from inhibition studies that antibody specificity is directed against the arsonate group, and that the benzene rest also is essential for combination with this antibody.[5] The combining region of the antibody can presumably only accommodate the benzene with faces oriented in a fixed direction, and the arsonate group can fit closely only in the one region formed complementary to the arsonate group of the immunizing antigen.

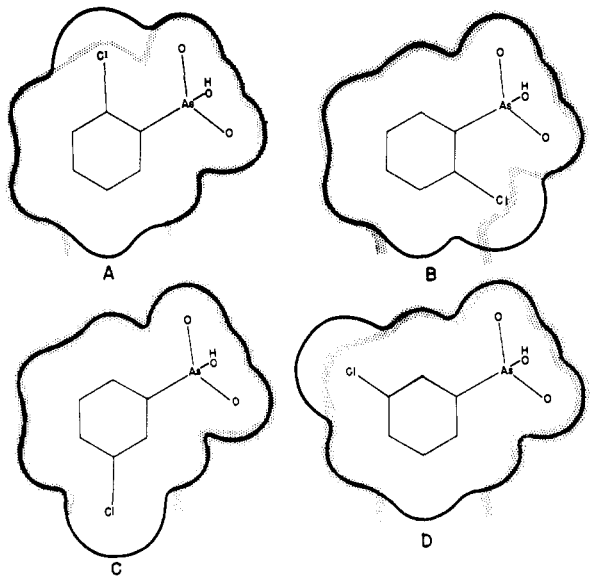

Fig. 1.—Orientation of haptens in antibody specific region: A and B, orientation of *o*-chlorobenzenearsonate ion with antibody against *m*-azobenzenearsonate group; C and D, orientation of *m*-chlorobenzenearsonate ion with antibody against *m*-azobenzenearsonate group.

The interaction of this antibody with a *meta*-substituted compound has always been considered as occurring with the substituent occupying the position which had been formed against the azo group of the immunizing hapten (Fig. 1C). The effect of a single *meta* substituent with the other orientation (Fig. 1D) was unknown. There could be no ambiguity with the *para*-substituted compounds.

In order to obtain more precise information about the fit of antibody around haptens, (*p'*-hydroxyphenylazo)-benzoates and (*p'*-hydroxyphenylazo)-chlorobenzoates were prepared, and their interaction with antibodies against *o*-, *m*- and *p*-azobenzoate ions determined. Since the azo group and the carboxylate group of the hapten most certainly occupy the positions corresponding to those of the azo and carboxylate groups of the homologous immunizing antigen, the hapten molecule is firmly oriented by combination at two regions of the specific site of the antibody. The chloro substituent is therefore definitely fixed with respect to the surface of the antibody and detailed information can now be obtained about the fit of antibody about the various positions of substitution on the benzoate ion.

Material and Methods

Haptens.—Many of the simple benzoic acid derivatives in this study were commercial preparations recrystallized to the correct melting point and acidic equivalent weight.

(1) This research was supported by a grant from the Rockefeller Foundation and Contract #AT(30-1)-910 of the Atomic Energy Commission.

(2) The results were presented before the Division of Biological Chemistry during the National Meeting of the American Chemical Society in Los Angeles, California, March, 1953.

(3) (a) Part of the work reported was carried out by Luther Hall in partial fulfillment of the requirements for the degree of Master of Science. (b) Roswell Park Memorial Institute, Buffalo 3, N. Y.

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

(5) D. Pressman, A. B. Pardee and L. Pauling, *ibid.*, 67, 1602 (1945).

TABLE I

SYNTHESIS OF CHLOROAMINOBENZOIC ACIDS AND OTHER INTERMEDIATES

Starting material	Intermediate	Yield, %	Meth-od[r]	M.p., °C. Found	M.p., °C. Reptd.	Equiv. wt. Calcd.	Equiv. wt. Found[a]	
2-Amino-3-nitrotoluene	2-Chloro-3-nitrotoluene	85	1	21.5	21.5[b]			
	2-Chloro-3-nitrobenzoic acid	55	2	183	185[c]	201.6	206.0	±0.2
	2-Chloro-3-aminobenzoic acid	60	3	156–158	158[d]	171.5	171.3	1.1
2-Amino-4-nitrotoluene	2-Chloro-4-nitrotoluene	85	1	57.6	65[e]	...	...	...
	2-Chloro-4-nitrobenzoic acid	40	2	140.9	140–142[f]	201.6	205.2	0.4
	2-Chloro-4-aminobenzoic acid	53	3	212–213	213[g]	171.5	172.1	1.1
2-Amino-5-nitrotoluene	2-Chloro-5-nitrotoluene	93	1	40	44[h]	...	...	...
	2-Chloro-5-nitrobenzoic acid	43	2	162–164	165[i]	201.6	191.8	
	6-Chloro-3-aminobenzoic acid	20	3	186–187	188[j]	171.5	172.9	5.1
6-Chloro-2-nitrotoluene	6-Chloro-2-nitrobenzoic	42	2	162–164	161[k]	201.6	205.7	0.5
	6-Chloro-2-aminobenzoic acid	29	3	146–147	146–147[l]	171.5	168.2	0.2
4-Chloro-2-nitrotoluene	4-Chloro-2-nitrobenzoic acid	46	2	141–143	140–143[m]	201.6	203.5	0.4
	4-Chloro-2-aminobenzoic acid	55	3	234–235	235–236[l]	171.5	170.0	0.1
3,5-Dinitrobenzoic acid	5-Amino-3-nitrobenzoic acid	66	4	205–207	208[n]	...	...	...
	5-Chloro-3-nitrobenzoic acid	23	1	146–147	147[n]	201.6	202.2	1.1
	5-Chloro-3-aminobenzoic acid	78	3	230 dec.	216[n]	171.5	172.8	
m-Chlorobenzoic acid	3-Chloro-2-nitrobenzoic acid	5	5	236–238	235[o]	201.6	203.0	0.2
	3-Chloro-2-aminobenzoic acid	78	3	189–190	187–188[p]	171.5	171.6	
m-Chlorobenzoic acid	5-Chloro-2-nitrobenzoic acid	42	5	136–138	137–138[o]	201.6	205.6	0.5
	5-Chloro-2-aminobenzoic acid	62	3	204–205	204[q]	171.5	165.4	0.1

[a] Average of duplicate analyses. [b] A. F. Holleman, *Rec. trav. chim.*, **27**, 456 (1908). [c] A. F. Holleman and B. R. de Bruyn, *ibid.*, **20**, 209 (1901). [d] A. F. Holleman and G. L. Voerman, *ibid.*, **21**, 57 (1902). [e] F. Ullman and C. Wagner, *Ann.*, **355**, 359 (1907). [f] J. B. Cohen and D. McCandlish, *J. Chem. Soc.*, **87**, 1271 (1905). [g] G. F. Kunckell and A. Richartz, *Ber.*, **40**, 3395 (1907). [h] H. Goldschmidt and M. Hönig, *ibid.*, **19**, 2443 (1886). [i] H. Hübner, *Ann.*, **222**, 166 (1884). [j] P. Griess, *Ber.*, **19**, 316 (1886). [k] A. G. Green, and T. A. Lawson, *J. Chem. Soc.*, **59**, 1019 (1891). [l] P. Cohn, *Monatsh.*, **22**, 473 (1901). [m] J. B. Cohen and H. P. Armes, *J. Chem. Soc.*, **89**, 458 (1906). [n] H. Hübner, *Ann.*, **222**, 67 (1884). [o] H. Hübner and M. Ulrich, *ibid.*, **222**, 95 (1884). [p] B. R. Baker, R. E. Shaub, J. P. Joseph, F. J. McEvoy and J. H. Williams, *J. Org. Chem.*, **17**, 141 (1952). [q] R. Dorsch, *J. prakt. Chem.*, [2], **33**, 50 (1886). [r] Methods: 1, decomposition of the diazonium salt with cuprous chloride[a]; 2, oxidation with potassium permanganate[a,t]; 3, low pressure hydrogenation with a platinum oxide catalyst[s]; 4, reduction with ammoniacal hydrogen sulfide[n]; 5, nitration with fuming nitric acid.[o] [s] H. Gilman and A. H. Blatt, "Organic Syntheses," Coll. Vol. I, John Wiley and Sons, Inc., New York, N. Y., 1941. [t] H. Gilman and A. H. Blatt, *ibid.*, Coll. Vol. II, 1943.

TABLE II

(*p'*-HYDROXYPHENYLAZO)-CHLOROBENZOIC ACIDS

Substituted benzoic acid	M.p., °C.	Analyses, % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
3-Chloro-2-(*p'*-hydroxyphenylazo)	187–189	56.4	56.6	3.26	3.40
4-Chloro-2-(*p'*-hydroxyphenylazo)	245–246	56.4	56.2	3.26	3.27
5-Chloro-2-(*p'*-hydroxyphenylazo)	231–233	56.4	55.4	3.26	3.42
6-Chloro-2-(*p'*-hydroxyphenylazo)	194–195	56.4	56.6	3.26	3.37
2-Chloro-3-(*p'*-hydroxyphenylazo)	219–221	56.4	56.6	3.26	3.49
4-Chloro-3-(*p'*-hydroxyphenylazo)[a]	268–271	56.4	56.4	3.26	3.26
5-Chloro-3-(*p'*-hydroxyphenylazo)	239–241	56.4	56.2	3.26	3.47
6-Chloro-3-(*p'*-hydroxyphenylazo)	244–246	56.4	56.6	3.26	3.42
2-Chloro-4-(*p'*-hydroxyphenylazo)	213–215	56.4	56.6	3.26	3.46
o-(*p'*-Hydroxyphenylazo)[b]	207.8–208.2	64.4	65.1	4.14	4.51
m-(*p'*-Hydroxyphenylazo)[b]	230–232	64.4	64.1	4.14	4.06
p-(*p'*-Hydroxyphenylazo)[c]	277–279 dec.	64.4	64.5	4.14	4.35

[a] The intermediate 4-chloro-3-aminobenzoic acid was prepared by Mr. Dan Rice. [b] Prepared by Dr. A. Recsei. [c] Prepared by Dr. J. H. Bryden.

The method of preparation of the (*p'*-hydroxyphenylazo)-benzoic acids and of the (*p'*-hydroxyphenylazo)-chlorobenzoic acids is summarized below. In Table I are listed the starting materials and intermediates obtained in the preparation of the intermeditae chloroaminobenzoic acids. These acids were subsequently diazotized and coupled with phenol. Coupling of the diazotized acids was carried out at *p*H 9 with a 10-fold excess of phenol. Excess phenol was removed by extraction with ether from the neutralized reaction mixture. Residual ether was removed by boiling. The highly colored azobenzoic acids were precipitated at *p*H 2 and purified by recrystallization from aqueous alcohol. Melting points and analyses of the products are listed in Table II.

Protein Antigens.—The antigens used for injection were prepared by coupling the diazotization product of 125 mg. of *o*-, *m*- or *p*-aminobenzoic acid with 2 g. of regenerated lyophilized beef serum at *p*H 9.5. After standing overnight at 3–5°, azoproteins were dialyzed against borate buffered saline until colorless dialysates were obtained.

The test antigens were prepared similarly, by coupling 250 mg. of ovalbumin[6] at *p*H 10 with the diazonium salt from 55 mg. of aminobenzoic acid. After standing overnight at 3–5°, the azoproteins were dialyzed exhaustively against many portions of cold borate buffered saline.

Preparation of Antisera.—Antisera were prepared by methods similar to those described previously.[7] The rabbits were bled five days after the last of a series of injections. High-titer sera from rabbits with each antigen were pooled, and the globulin fraction for each pool was separated by precipitating three times at a 1.75 *M* ammonium sulfate con-

(6) The ovalbumin used was the crystallized Armour preparation.

(7) D. Pressman, J. H. Bryden and L. Pauling, THIS JOURNAL, 67, 1219 (1945).

TABLE III

EFFECT OF HAPTENS ON THE PRECIPITATION OF ANTI-X_0 GLOBULIN WITH X_0-OVALBUMIN

Anti-X_0 globulin, 0.50 ml., X_0-ovalbumin in borate buffer, 0.50 ml. (250 μg. protein); hapten in saline, 0.50 ml. One hour at room temperature and 3 days at 3–5°.

Hapten	Hapten concn., molar × 10^5										
	2.6	5.3	10.6	21.3	42.7	83.3	167	667	K'_0	σ	$\Delta F_{rel.}$
	Amount of precipitate[a]										
Benzoate					630		520	260	1.00	3.5	0
o-Chlorobenzoate		880		670		440			2.9	2.5	−580
m-Chlorobenzoate					680		540	230	1.0	3.5	0
p-Chlorobenzoate					770		620	400	0.45	3.5	430
o-(*p'*-Hydroxyphenylazo)-benzoate		570		370		190			19	3.5	−1700
3-Chloro-2-(*p'*-hydroxyphenylazo)-benzoate		810		490		260			7.1	2	−1100
4-Chloro-2-(*p'*-hydroxyphenylazo)-benzoate	860		690		370				7.0	2	−1100
5-Chloro-2-(*p'*-hydroxyphenylazo)-benzoate		610		490		240			11	4	−1300
6-Chloro-2-(*p'*-hydroxyphenylazo)-benzoate		620		410		210			15	3.5	−1500
p-Fluorobenzoate					820		610	360	0.63	2.5	240
p-Bromobenzoate					770		650	410	.38	3.5	520
p-Iodobenzoate					760		620	370	.54	3	330

[a] The amount of precipitate is reported in parts per mille of the amount present in the absence of hapten; 272 μg. These values are averages of triplicate analyses with mean deviation of ±4%.

TABLE IV

EFFECT OF HAPTENS ON THE PRECIPITATON OF ANTI-X_m GLOBULIN WITH X_m-OVALBUMIN

Anti-X_m globulin, 1.00 ml. (20.2 mg. protein), X_m-ovalbumin in borate, 1.00 ml. (183 μg. protein); hapten in saline, 1.00 ml One hour at room temperature and three days at 3–5°.

Hapten	Hapten concn., molar × 10^5										
	3.3	6.7	13	26	53	104	208	417	K_0'	σ	$\Delta F_{rel.}$
	Amount of precipitate[a]										
Benzoate			780		750		410		1.00	4	0
o-Chlorobenzoate				900		690		530	.30	4	680
m-Chlorobenzoate	920		840		580				1.8	2.5	−300
p-Chlorobenzoate				810		740		470	0.41	4	480
m-(*p'*-Hydroxyphenylazo)-benzoate		540		330		80			17	2.5	−1600
2-Chloro-3-(*p'*-hydroxyphenylazo)-benzoate			770		520		220		2.8	2.5	−560
4-Chloro-3-(*p'*-hydroxyphenylazo)-benzoate			770		630		310		1.9	2.5	−350
5-Chloro-3-(*p'*-hydroxyphenylazo)-benzoate	730		590		410				5.9	4	−980
6-Chloro-3-(*p'*-hydroxyphenylazo)-benzoate			800		540		200		2.5	2	−500

[a] The amount of precipitate is reported in parts per mille of the amount present in the absence of hapten; 216 μg. All values are averages of triplicate analyses with mean deviation of ±5%.

TABLE V

EFFECT OF HAPTENS ON THE PRECIPITATION OF ANTI-X_p GLOBULIN WITH X_p-OVALBUMIN

Anti-X_p globulin, 1.00 ml.; X_p-ovalbumin in borate buffer, 1.00 ml. (200 μg.); hapten in saline, 1.00 ml. One hour at room temperature and three days at 3–5°.

Hapten	Hapten concn., molar × 10^5										
	1.7	3.3	6.7	13	26	53	104	208	417	K'_0	σ	$\Delta F_{rel.}$
	Amount of precipitate[a]											
Benzoate				820		600		280		1.00	2.5	0
o-Chlorobenzoate					890		730		530	0.17	3	960
m-Chlorobenzoate			900		600		300			1.8	2	−300
p-Chlorobenzoate		860		620		390				2.8	2.5	−560
p-(*p'*-Hydroxyphenylazo)-benzoate		510		170		20				23	1.5	−1700
2-Chloro-4-(*p'*-hydroxyphenylazo)-benzoate	840		680		370					5.4	2.5	−940

[a] The amount of precipitate is reported in parts per mille of the amount present in the absence of hapten; 227 μg. All values are averages of triplicate analyses with mean deviation of 4%.

centration.[8] This procedure is known to separate the globulins effectively from the albumins.[9]

Reactions of Antisera and Analysis.—Equal volumes of antigen, antisera and hapten were mixed and incubated for about one hour at room temperature, and then allowed to stand for 3 days at 3–5°. A concentration of test antigen diluted with borate buffer (*p*H 8)[10] was used which yielded a maximum amount of precipitate. The precipitates were washed 3 times with 8 ml. of saline, dissolved in 2.5 ml. of 1 *M* NaOH and analyzed by a modified Folin procedure.[11]

Hapten stock solutions were prepared by dissolving a weighed quantity of hapten in the calculated quantity of 0.16 *M* NaOH solution and adjusting the *p*H to 7–9. Dilutions were made with 0.16 *M* NaCl.

Results

The extent of combination of hapten with antibody was determined by the ability of the hapten to inhibit the precipitation of the antibenzoate antibodies with the hapten homologous ovalbumin antigen. In each case the amount of antigen used was that which gave the greatest amount of precipitate. As in other antigen–antibody systems, the antiserum gave the largest amount of precipitate with an optimal amount of antigen, and less

(8) H. N. Eisen and D. Pressman, *J. Immunol.*, **64**, 487 (1950).

(9) D. Pressman and M. Siegel, THIS JOURNAL, **75**, 686 (1953).

(10) D. Pressman, D. H. Brown and L. Pauling, *ibid.*, **64**, 3015 (1942).

(11) D. Pressman, *Ind. Eng. Chem., Anal. Ed.*, **51**, 357 (1943).

with either more or less antigen. The *p*H was kept at 8, since the carboxylic acids are completely dissociated at this hydrogen ion concentration. Data on the effect of hapten on the amount of precipitate obtained from the reaction of the globulin fraction of antiserum against *o*-azobenzoate ion (anti-X_0 globulin) with the optimal concentration of X_0-ovalbumin are given in Table III; data for the X_m system (anti-X_m globuln and X_m-ovalbumin) in Table IV; data for the X_p system in Table V. Values for the hapten inhibition constant K_0' and the heterogeneity index σ, obtained on application of the theory of heterogeneous antisera,[12] are listed. There are also listed values for the relative free energy of combination of antibody with hapten (*i.e.*, the value relative to the combination of antibody with the unsubstituted benzoate ion). The determinations were carried out with the globulin fraction of the whole serum, avoiding the complication of binding of the hapten by serum albumin.

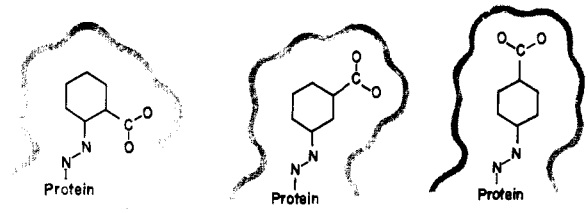

Fig. 2.—Van der Waals outline of the haptens used in the preparation of antibodies.

Discussion

Closeness of Fit of Antibenzoate Antibodies around the Azobenzoate Group.—Figure 2 shows the van der Waals outline of the three haptens used in the preparation of the antibodies. The antibodies act as though they fit closely around these outlines. In Fig. 3 the van der Waals outline of the inhibiting hapten (heavy black line) is superimposed on that of the immunizing hapten (shaded line). The necessity of orienting the inhibiting hapten so that carboxylate group, benzene ring and azo group are planar and coincide with the orientation of the immunizing hapten fixes the chlorine substituents unambiguously with reference to the complementary region of the antibody. Where the van der Waals outline of the inhibiting hapten overlaps that of the immunizing hapten, there is visual indication of possible steric hindrance. Values for the relative free energy of combination (with respect to unsubstituted benzoate ion) are also given.

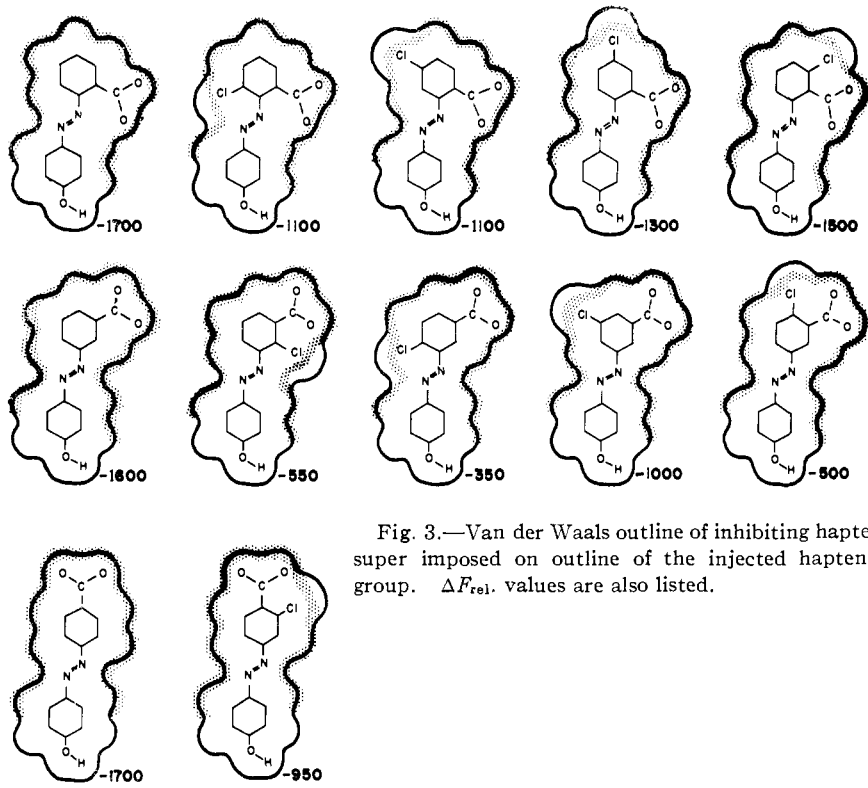

Fig. 3.—Van der Waals outline of inhibiting hapten super imposed on outline of the injected haptenic group. $\Delta F_{rel.}$ values are also listed.

Figure 4 summarizes the effect of chlorine substituents in various positions on the free energy of combination of the (*p'*-hydroxyphenylazo)-benzoate rest with antibody. These substituent values are obtained as the difference in the relative free energies of combination of the unsubstituted (*p'*-hydroxyphenylazo)-benzoate and the chlorosubstituted azo derivative. A large free energy contribution indicates that antibody fits closely around the position of substitution (and thus the substituent exerts a large steric effect). In the anti-*o*-azobenzoate antibodies (anti-X_o system) the closest fit of the antibody around the benzene ring is in the 3- and 4-positions, and there is little interference with combination by a substituent in the 6-position (*ortho* to the carboxylate group). In the X_m- and X_p-systems there is a very close fit around the benzene ring in the position *ortho* to the carboxylate. This difference in effect in the X_o-system on the one hand and the X_m and X_p on the other may well be due to the *ortho*-chloro substituent twisting the carboxylate group out of the plane of the benzene ring. In the X_m- and X_p-systems the large effect of the *ortho* chloro substituent may be due to this twisting of the carboxylate group of the inhibiting hapten so that it no longer fits well into an antibody site which had been formed against the coplanar carboxylate grouping of the immunizing antigen. In the case of the anti-X_0 antibodies, the antibodies were formed against the *o*-azobenzoate in which the carboxylate group was already tilted out of the plane of the benzene ring. The

(12) L. Pauling, D. Pressman and A. L. Grossberg, THIS JOURNAL, **66**, 784 (1944).

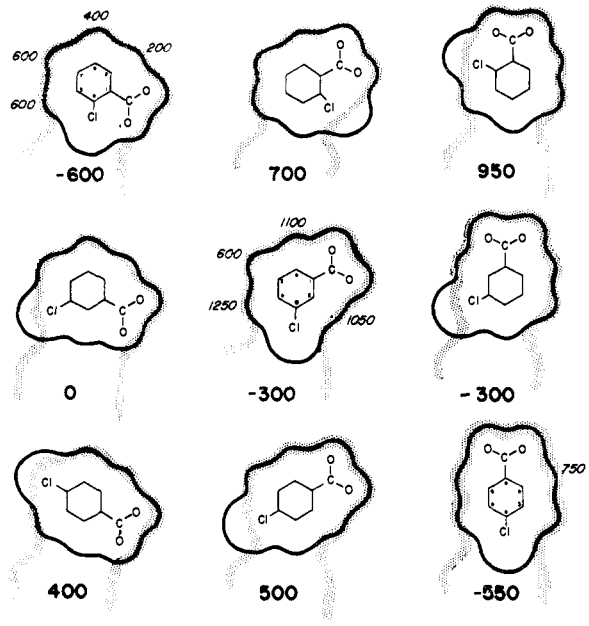

Fig. 4.—The effect of chlorine substituents on the combination of benzoate ion with antibody. The small numbers around the van der Waals outline of the benzene ring summarize the effect of a chlorine substituent in that position on the free energy of combination of (*p'*-hydroxyphenylazo)-benzoates with antibody. The large numbers under each outline represent the relative free energy of combination of the outlined inhibiting hapten with the antibody. The first column represents the X_o-system; the second, X_m; the third, X_p.

presence of a chloro group in the 6 (*ortho* position) has no further effect on tilting, and thus exerts only a small effect on combining constant.

The haptenic group is joined to the protein of the immunizing antigen through an azo linkage. In view of the large protein moiety on the other side of the azo linkage one might not expect a close fit of the antibody about the azo group. However, in the case of the X_o- and X_m-systems, when substituents were placed *ortho* to the azo linkage of the inhibiting haptens, large effects were observed on the combination of hapten with antibody. The 3-chloro-2-(*p'*-hydroxyphenylazo)-benzoate was the poorest inhibitor for the X_o-system and the 2- and 4-chloro-3-(*p'*-hydroxyphenylazo)-benzoates were very poor inhibitors in the X_m-system. There must be a very tight fit of antibody about the azo linkage of the unsubstituted hapten for substituents in these positions adjacent to the azo linkage to exert such a large influence.[13]

Orientation of Chlorobenzoate Ions in Combination with Antibody.—In Fig. 4 outlines for the simple chlorobenzoates are superimposed on the pertinent region of the outline of the immunizing hapten (X_o, X_m, X_p). The relative free energy of combination of the chlorobenzoates is listed below each figure, and at each site of substitution of a chloro group the free energy contribution attributable to the substituent in that position on the azobenzoate is also listed. In combining with antibody the carboxylate group orientation for these compounds is determined by the carboxylate complementary region of the antibody. The *o*-chloro group must occupy one of two possible *ortho* positions[14]; the *m*-chloro group, one of two possible *meta* positions[14]; and the *p*-chloro, the single possible *para* position.

In those cases where the substituent can occupy the region of the antibody complementary to the azo group of the immunizing hapten, the hapten combines very strongly, as often has been described previously.[4,15] Thus *o*-chlorobenzoate ion is the strongest inhibitor with anti-X_o serum, *m*-chlorobenzoate with anti-X_m, etc. However, even though combination with the chlorobenzoate in one orientation is favored here, there still may be some contribution of the second orientation.

An estimate of the contribution of the less favored orientation can be made from the effect of the chlorine substituent on the free energy of combination of the (*p'*-hydroxyphenylazo)-chlorobenzoate (Fig. 4). In the case of *o*-chlorobenzoate combining with anti-X_o antibody, the replacement of the hydrogen by a chlorine in the unfavored position (the 6-position) causes a change of 200 calories per mole in free energy of combination. However, there is a change of -1000 calories per mole in the favored position. The -1000 calories per mole is obtained from the sum of -600 calories per mole (the observed relative free energy change of *o*-chlorobenzoate as compared to benzoate ion in combining with antibody) plus a statistical term -400 calories per mole (RT ln 2) which corrects for the fact that the unsubstituted benzoate ion can fit in the antibody complementary region in two ways (with one face of the benzene ring up or with the opposite face up) while the chlorobenzoate in the favored position can fit in only one way. The difference in free energy of combination in the favored and unfavored orientations, 1200 calories per mole, represents a factor of 10 in distribution. Similarly, in the combination of *m*-chlorobenzoate with anti-X_m antibody the replacement of a hydrogen by a chlorine in the unfavored position (5-position) causes a change in the free energy of combination of 600 calories per mole as determined above, and a change of -700 calories in the favored position (-300 observed plus a -400 due to the statistical term). The difference, 1300 calories per mole, again represents a factor of 10 in distribution between the favored and unfavored orientations.

The values of the contributions of the less favored orientations as calculated above are minimum values since the value used for the effect of a chlorine substituent in the less favored orientation was taken from the value observed when the substitution was made on the azobenzoate rest. The effect of a chlorine substituent on the combination of benzoate with antibody is generally less than the effect of the substituent on the combination of the azobenzoate

(13) This may indicate that the antibody is formed against an antigen which has the hapten lying along the surface of the protein. Then the positions adjacent to the azo group would be more exposed for interaction with antibody than when the hapten extends perpendicularly from the surface of the antigen.

(14) Only one position is shown in Fig. 4.

(15) K. Landsteiner, "The Specificity of Serological Reactions," Harvard University Press, Cambridge, Mass., 1947.

with the same antibody as shown in Table VI. Accordingly, there is a looser fit between chlorobenzoates and antibody than between azochlorobenzoates and antibody. The reason for this is that whereas the chlorosubstituent only interferes with the interaction of antibody with the carboxylate and the benzene ring in the simple benzoate case, it also interferes with the interaction with the azo group and the second ring in the case of the azohapten.

TABLE VI

FREE ENERGY CONTRIBUTION OF CHLORO SUBSTITUENTS

System	Benzoate	$\Delta F_{rel.}$, cal. Obsd.	Calcd.[a]
X_o	*m*-Cl	0	500[b]
	p-Cl	400	600
X_m	*o*-Cl	700	1050[c]
	p-Cl	500	1250
X_p	*o*-Cl	950	750

[a] Calculated as the difference between the relative free energies of combination with antibody of the azobenzoate and the corresponding chloro derivative. [b] Statistical average of the values for the effect of the 3-chloro and the 5-chloro group taking into consideration the relative distribution of the two possible orientations. [c] Statistical average of the values for the effect of the 2-chloro and the 6-chloro group.

The looser fit makes it difficult to predict a favored orientation in those cases where the substituent cannot occupy the region of the antibody complementary to the azo group of the immunizing hapten, *i.e.*, in the combination of *o*-chlorobenzoate with X_m-antibodies or *m*-chlorobenzoate with X_o-antibodies. However, the data in Table VI, together with Fig. 4, show the greatest reduction in free energy of combination from the calculated value occurs for those cases where it is possible for the chlorobenzoate to shift so that either the chlorine substituent or the benzene ring itself can shift toward the antibody "hole" formed about the azo group of the injected antigen. Thus *p*-chlorobenzoate shows a reduction of ΔF of 750 calories with anti-X_m antibody. Here it can shift as described above to decrease steric effects. With anti-X_o antibody, however, no such rotation can occur and the reduction in ΔF is only 200 calories. The relatively large reduction for *m*-chlorobenzoate in the X_o system and *o*-chlorobenzoate in the X_m system indicate that some shift takes place with these compounds also. Nothing can be stated as to a preferred orientation of the chlorine group since either the chlorine group or the benzene ring can be displaced toward the azo complementary region of the antibody depending on whether combination takes place with the chlorine or the benzene in the position adjacent to the azo-complementary region.

In the combination of *o*-chlorobenzene with anti-X_p antibody there is no chance of shift into the azo specific region and the free energy is not decreased.

Closeness of Fit of Anti-X_o Antibodies around the 4-Position.—Since there is no ambiguity about the orientation of the *p*-chlorobenzoate ion in its combination with the anti-X_o antibody, a comparison was made of the effect of fluorine, chlorine, bromine and iodine in the *para* position on the combination of benzoate ion with anti-X_o antibody. The data are presented in Table III. All of these substituents decrease the combination of benzoate ion with anti-X_o antibody. The decrease becomes larger as the van der Waals radius increases from fluorine to bromine, reaching a maximum decrease with bromine (ΔF_{rel} 520 cal.). However, iodine which is even larger than bromine is less effective in decreasing combination. This greater combination of the iodobenzoate must be due to the greater van der Waals interaction of the iodine. Evidence that the greater van der Waals attraction of an iodine may counterbalance the steric effect of its greater radius has been obtained with antibodies against the 4-azophthalate ion.[16] There the combining constant of the tetrahalophthalate ion with antibody was in the order Cl > I > Br.

Acknowledgment.—We wish to thank Richard Clarke and Jacob Planinsek for assistance in this work.

BUFFALO, NEW YORK

(16) D. Pressman and L. Pauling, THIS JOURNAL, **71**, 2893 (1949).