[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY RESEARCH, ROSWELL PARK MEMORIAL INSTITUTE]

Closeness of Fit and Forces Involved in the Reactions of Antibody Homologous to the *p*-(*p'*-Azophenylazo)-benzoate Ion Group

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The extent of combination of rabbit antibodies homologous to the *p*-(*p'*-azophenylazo)-benzoate ion with various substances of related configuration was determined. Distinct specificity was observed in that the antibody combined most strongly with substances of closest approximation to the configuration of the homologous hapten. The active site on the antibody molecule was found to fit closely about the first (carboxyl-substituted) benzene ring and somewhat loosely about the second ring. The results suggest that the antibody makes contact with the face of the first benzene ring. The contribution to the free energy of interaction of the charge on the carboxylate ion is over 4.8 kcal./mole, indicating the presence of a positive charge in the antibody site adjacent to the carboxylate of the haptenic group. The contribution of the *p*-phenylazo group is 2.3 kcal. per mole. Thus, the uncharged portion of the haptenic group contributes a large share of the free energy of combination. The forces responsible are discussed. Further evidence was obtained supporting the theory that the annular nitrogen atom of pyridine is in a hydrated state when it participates in serological reactions.

It has been shown in a good many cases that the antibody formed against a haptenic group fits very closely about that group.[1-3] The antibody may act as though it were formed completely around the haptenic group, against one side, or against the face of the hapten.

Previous studies have also shown that antibodies formed against the long haptenic group, *p*-(*p'*-azophenylazo)-benzenearsonate (anti-R'_p antibodies), do not fit as closely about the group as do antibodies against the related, shorter *p*-azobenzenearsonate group (anti-R_p antibodies)[4,5]; *ortho* and *meta* substituents interfere in the combination of benzenearsonate with anti-R_p antibodies but not with anti-R'_p antibodies. The type of fit observed has been interpreted to indicate that the anti-R'_p antibody fits as a slit trench around the terminal benzenearsonate rest with one side open to accommodate substituents. Since we already have information concerning the fit of antibody against the *p*-azobenzoate group (anti-X_p antibody)[4,6] and know that antibody fits closely, we have now carried out a study of antibodies specific to the *p*-(*p'*-azophenylazo)-benzoate ion (anti-X'_p antibodies). The results provide information concerning closeness of fit and the forces involved in the interaction of antibody with the haptenic group.

Experimental Methods

Preparation of Haptens.—Methods of preparation and results of analyses of phenylazobenzoic acid and several of its derivatives are summarized in Table I. We are indebted to Dr. John H. Bryden for preparing several of these compounds while a graduate student at the California Institute of Technology. The nitroso compounds used as intermediates in these syntheses were made by reduction of the corresponding nitro derivative to a hydroxylamine with zinc dust in the presence of ammonium chloride or with ammonium sulfide; the product was oxidized with ferric chloride or sodium dichromate to give the nitroso compound, which was then recovered by steam distillation. Derivatives of nitrosobenzene, prepared in this manner, were coupled with *p*-aminobenzoic acid in glacial acetic acid. The azo compound which precipitated on standing was isolated as the sodium salt, then reconverted to the acid form. In those instances where the azobenzoic acid derivative was prepared by coupling a diazonium salt to a phenolic compound, the procedure was carried out in alkaline solution. The azo compounds prepared by either method were recrystallized at least twice from ethanol or an ethanol–water mixture.

The preparation of *p*-(*p'*-aminophenylazo)-benzoic acid has been described previously.[6]

The syntheses of 2-(4'-carboxyphenylazo)-8-amino-1-naphthol-3,6-disulfonic acid and of 2-(4'-nitrophenylazo)-8-amino-1-naphthol-3,6-disulfonic acid, hereafter referred to as "H Acid" azobenzoic acid, and "H Acid" azonitrobenzene, respectively, were carried out by coupling the appropriate benzenediazonium chloride to "H Acid" in alkaline solution. *p*-Cyanophenol was prepared by the method of Ahrens.[7] Other haptens were purchased from commercial sources and recrystallized to the correct melting point. The equivalent weight of each hapten containing an acidic group, including the phenols with $pK_a < 9$, was found to differ by less than 3% from the theoretical value.

Protein Antigens.—The antigen used for injection in the rabbits was prepared by diazotizing 2 g. of the sodium salt of *p*-(*p'*-aminophenylazo)-benzoic acid, acidifying and adding the mixture to 200 ml. of whole beef serum containing 15 g. of sodium carbonate. The antigen was purified by dialysis and extraction with cold methanol, followed by dialysis against saline–borate. The test antigen was prepared in a similar manner using 75 mg. of the *p*-(*p'*-aminophenylazo)-benzoate and 0.5 g. of crystallized ovalbumin. Coupling was carried out at *p*H 9.5 and the antigen was purified by dialysis against several changes of saline–borate solution.

Preparation of Antisera.—The method for obtaining and pooling antisera has been described previously.[8]

The γ-globulin fraction of the antiserum was prepared by the method of Kekwick[9] (3 precipitations with decreasing concentrations of sodium sulfate). The effectiveness of the method was checked by electrophoresis, at *p*H 8.6 in barbital buffer, of samples of serum before and after fractionation. Only one major peak, corresponding to γ-globulin, remained in the fractionated serum. No serum albumin or α-globulin, and only a trace of β-globulin was detectable. The amount of serum albumin remaining was less than one per cent. of the original content.

Reactions of Antiserum with Antigen and Hapten.—Solutions of haptens were prepared by dissolving in sodium hydroxide solution and back-titrating with HCl to *p*H 7.5–8.5; hapten solutions with buffering capacity in this region were adjusted to *p*H 8.0. Solutions were 0.16 *M* in sodium ion. Dilutions of the hapten solutions were made with 0.16 *M* sodium chloride; dilutions of the antigen were made with borate buffer of *p*H 8.0 and ionic strength 0.16.

The amount of antigen required to give optimum precipitation was determined. Then 1.0-ml. portions of antigen (optimum concentration), hapten and antibody solutions were added to a test-tube in that order; each of the components was at *p*H 8. The mixture was permitted to stand for one hour at 37°, then for five days at 3–5°. Pre-

(1) K. Landsteiner, "The Specificity of Serological Reactions," Revised Edition, Harvard University Press, Cambridge, Mass., 1945.

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

(3) D. Pressman, *Advances in Biol. and Med. Phys.*, **3**, 99 (1953).

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

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

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

(7) F. Ahrens, *Ber.*, **20**, part 2, 7952 (1887).

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

(9) R. A. Kekwick, *Biochem. J.*, **34**, 1248 (1940).

TABLE I

PREPARATION OF *p*-PHENYLAZOBENZOIC ACID DERIVATIVES

Compound	Method of prep.[a]	Results of analyses, % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
p-Phenylazobenzoic acid	A	69.01	69.43	4.46	4.53
p-(*p'*-Hydroxyphenylazo)-benzoic acid	B	64.46	64.64	4.16	4.07
p-(*p'*-Methylphenylazo)-benzoic acid	A	69.99	70.04	5.04	5.14
p-(*p'*-Aminophenylazo)-benzoic acid	C	64.72	64.98	4.60	4.80
p-(*o'*-Methyl, *p'*-hydroxyphenylazo)-benzoic acid	B	65.62	65.79	4.72	4.77
p-(*m'*-Methyl, *p'*-hydroxyphenylazo)-benzoic acid	B	65.62	65.73	4.72	4.61

[a] Method A, coupling of the appropriate nitroso compound with an aniline derivative. Method B, coupling of diazotized *p*-aminobenzoic acid with a phenol derivative. Details in text. Method C, coupling of diazotized *p*-aminobenzoic acid with aniline-ω-methylsulfonate, followed by hydrolysis in 1 *N* NaOH.

cipitates were centrifuged and washed three times with 5 to 10-ml. portions of 0.16 *M* sodium chloride solution. The amount of protein in the precipitate was estimated by a modified Folin procedure.[10] Control experiments were carried out in duplicate in which antigen and hapten, or antibody and hapten were incubated together. The mean value of the small blanks obtained was subtracted from the results of the analyses. Four to eight replicate experiments in which no hapten was present were performed in each series to serve as a basis for calculating the degree of inhibition. Each experiment, with a very few exceptions, was carried out in duplicate. The average mean deviation was 2.2%.

Under the conditions of these experiments, there was no change in the amount of precipitate found if the antigen was added to the mixture last.

Results

Data which indicate the effect of various substances on the combination of the antigen and antibody are given in Tables II to X. The same pool of antiserum was used throughout. The data in all tables except V and X were obtained in a single run.

TABLE II

EFFECT OF *ortho* DERIVATIVES OF BENZOATE ION ON THE PRECIPITATION OF ANTI-X'$_p$ ANTIBODY WITH X'$_p$-OVALBUMIN

Hapten (benzoate)	K'_0	$\Delta F°$rel. (cal.)	σ	Hapten concn., molar $\times 10^5$ 2.61	10.4	41.7	167	667
				Amount of precipitate[a]				
Unsubstd.	1.00	0	2.0	93	72	30	18	
o-Chloro	0.11	1220	1.5				67	30
o-Methyl	.030	1940	1.5				90	66
o-Iodo	.013	2400	2.5				91	74
o-Nitro[b]	<.01	>2500	~2.5					
o-Carboxy	<.01	>2500	..				102	90
o-Acetamino	.13	1130	2.0				61	26

[a] The amount of precipitate is reported as per cent. of the amount found in the absence of hapten, 244 μg. [b] Inhibition data in Table V.

From these data on the amounts of precipitate formed, values were obtained, by application of the theory of heterogeneous antisera,[11] for the hapten inhibition constant, K'_0, and the heterogeneity index, σ. Values of K'_0 are relative to that for benzoate, which is assigned the value $K'_0 = 1.00$. The corresponding value of the free energy of combination, relative to that of benzoate, is also given for each hapten.

The observed K'_0 values for the *o*-, *m*- and *p*-phthalates were divided by two to correct for the entropy contribution resulting from the symmetry of these compounds.

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

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

TABLE III

EFFECT OF *meta* DERIVATIVES OF BENZOATE ION ON THE PRECIPITATION OF ANTI-X'$_p$ ANTIBODY WITH X'$_p$-OVALBUMIN

Hapten (benzoate)	K'_0	$\Delta F°$rel. (cal.)	σ	Hapten concn., molar $\times 10^5$ 2.61	10.4	41.7	167	667
				Amount of precipitate[a]				
Unsubstd.	1.00	0	2.0	93	72	30	18	
m-Chloro	0.43	460	2.5			63	32	13
m-Methyl	.21	860	1.5			93	47	19
m-Iodo	.29	690	2.5			69	38	18
m-Nitro[c]	.12	1170	1.5					
m-Carboxy	.90[b]	60	2.5		63	31	13	
m-Acetamino[d]	1.3	−150	3.0		55	35	13	
3-Nitro, 4-chloro	0.41	490	2.0			62	32	10
3-Nitro, 5-chloro	0.070	1470	2.0				71	41

[a] The amount of precipitate is reported as per cent. of the amount found in the absence of hapten, 244 μg. [b] Corrected for symmetry contribution to K'_0; see Results. [c] Inhibition data in Table V. [d] Data obtained in a separate run in which the amounts of precipitate formed in the presence of 2.61×10^{-5}, 10.4×10^{-5} and 41.7×10^{-5} *M* benzoate (taken as standard) were 90, 66 and 26%, respectively, of the amount obtained in the absence of hapten (240 μg.).

Discussion

Closeness of Fit about the First Ring.—Information concerning closeness of fit about the first benzene ring can be obtained from data concerning the effect of *ortho* and *meta* substituents in that ring on the combining constant.

The results of substitution in the *ortho* position of a methyl, chloro, nitro, iodo, carboxy or acetamino group (Table II) constitute evidence for a very close fit of antibody about this part of the haptenic group, since in each case there is a large reduction in combining constant accompanying the substitution. This may come about through either of two steric effects. First, the substituent itself can prevent the close approach of the rest of the hapten to the antibody. Secondly, the fact that these substituents tilt the carboxylate group out of the plane of the benzene ring results in a new orientation of the carboxylate group; this may hinder combination with the antibody, since the latter was formed against the normal coplanar configuration of the benzoate ion.

The effect of substitution in the *meta* position (Table III) is similarly to decrease the combining power of benzoate with antibody, indicating a tight fit about this position. This is true of each substituent tested except the *m*-acetamino group.

A possible explanation for the apparently anomalous result of substitution of an *m*-acetamino group may lie in the fact that this group, which contains a chain of four atoms, can assume con-

TABLE IV

EFFECT OF *para* DERIVATIVES OF BENZOATE ION ON THE PRECIPITATION OF ANTI-X'$_p$ ANTIBODY WITH X'$_p$-OVALBUMIN

Hapten (benzoate)	K'_0	$\Delta F°$rel. (cal.)	σ	Hapten concn., molar × 10^5: 0.041	0.163	0.65	2.61	10.4	41.7	167
				Amount of precipitate[a]						
Unsubstd.	1.00	0	2.0				93	72	30	18
p-Fluoro	3.6	− 710	2.0				76	36	18	5
p-Chloro	5.3	− 920	3.0				66	33	21	7
p-Methyl	1.8	− 320	2.0				80	64	30	17
p-Bromo	5.4	− 930	2.0				71	31	14	5
p-Iodo	9.0	−1210	3.0				56	31	14	7
p-Amino	2.1	− 410	2.5				80	57	27	14
p-Hydroxy	4.7	− 850	2.5				71	41	18	9
p-Nitro[c]	1.8	− 320	2.0							
p-Carboxy	5.3[b]	− 920	3.0				55	28	12	6
p-Acetamino	1.6	− 260	2.5			95	84	66	34	
p-Phenyl	6.8	−1060	2.5			81	64	33	14	
p-Phenylazo	67	−2320	2.0	100	73	39	16			
p-(*p'*-Aminophenylazo)	70	−2350	1.5		73	35	12			
p-(*p'*-Methylphenylazo)	65	−2310	1.8		78	39	14			
p-(*p'*-Hydroxyphenylazo)	111	−2600	1.5	80	67	25				
p-(*o'*-Methyl-*p'*-hydroxyphenylazo)	81	−2430	2.0		75	36	14			
p-(*m'*-Methyl-*p'*-hydroxyphenylazo)	125	−2670	1.5		68	18	5			

[a] Amount of precipitate is reported as per cent. of the amount found in the absence of hapten, 244 μg. [b] Corrected for symmetry contribution to K'_0. [c] Inhibition data in Table V.

TABLE V

EFFECT OF NITRO DERIVATIVES OF BENZOATE ION ON THE PRECIPITATION OF ANTI-X'$_p$ ANTIBODY WITH X'$_p$-OVALBUMIN

Hapten (benzoate)	K'_0	$\Delta F°$rel. (cal.)	σ	Hapten concn., molar × 10^5: 10.4	41.7	167	667
				Amount of precipitate[a]			
Unsubstd.	1.00	0	2.2	70	32	16	
o-Nitro	<0.01	>2500	~2.5			99	88
m-Nitro	0.12	1170	1.5			57	22
p-Nitro	1.8	−320	2.0	58	25	9	
2,4-Dinitro	0.014	2360	2.0			90	72
2,5-Dinitro	.013	2400	2.0			92	73
3,5-Dinitro	.024	2060	3.0			77	59
2,4,6-Trinitro	<.01	>2500	~2.5			92	83

[a] The amount of precipitate is reported as per cent. of the amount found in the absence of hapten, 325 μg.

TABLE VI

ADDITIVITY OF EFFECTS OF SUBSTITUENTS ON COMBINATION OF HAPTEN WITH ANTI-X'$_p$ ANTIBODY

Hapten (benzoate)	K'_0 (obsd.)	K'_0 (calcd.)[a]
Unsubstd	1.00	
3-Nitro-4-chloro	0.41[b]	0.64 (0.12 × 5.3)
3-Nitro-5-chloro	.070[b]	.052 (0.12 × 0.43)
3,5-Dinitro	.024[c]	.014 (0.12 × 0.12)
2,4-Dinitro	.014[c]	<.018 (<0.01 × 1.78)
2,5-Dinitro	.013[c]	<.001 (<0.01 × 0.12)
2,4,6-Trinitro	<.01[c]	.000 (<0.01 × <0.01 × 1.78)

[a] Calculated on the assumption that the changes in free energy accompanying the substitutions of individual groups are additive; *i.e.*, that the K'_0 value equals the product of the individual K'_0 values. [b] Data from Table III. [c] Data from Table V.

figurations in which a large portion of the group extends into the space surrounding the *para* or azo-specific position. As will be shown below, substitution of any of the various groups tested for the hydrogen atom in the *para* position results in a greater release of free energy on combination; and in the case of the *m*-acetamino group, this effect may compensate for the steric interference associated with *meta* substitution. The other *meta*-substituted benzoates tested are smaller and little compensating interaction of this type is possible.

It is of interest that the carboxylate and nitro groups in the *meta* position have K'_0 values of 0.90 and 0.12, respectively. Since the two groups are nearly identical in size and configuration, the larger value for the carboxylate is probably due to charge interaction of the latter with antibody; especially since there is additional evidence (which will be discussed below) for the presence of a positive charge in the antibody site.

The general increase in free energy of combination associated with *ortho* or *meta* substitution indicates a tight fit of the antibody about the first benzene ring of the hapten; an increase in size of only 1.3 Å. (Cl substitution) causes an increase of 460 cal. in the case of *meta* substitution or 1220 cal. for *ortho* substitution.

Closeness of Fit Around the First Azo Group.— The effect of substitution for various groups in the *para* position of benzoate on the K'_0 value for combination with antibody is shown in Table IV. This position, of course, corresponds to that occupied by the azo group in the immunizing antigen. All the *para* substituents tested increase the constant. This is in marked contrast with the general decrease observed for substitution in the *ortho* or *meta* positions. It is in accordance with the fact that the antibody was formed to accommodate an azo group in this position and can therefore accommodate other substituents. The large relative combining constant of *p*-phenylbenzoate (K'_0 = 6.8) shows that the antibody is able to accommodate a rather large group in the azo-specific portion of the combining site.

By far the most effective haptens are the *p*-phenylazo derivatives of benzoate; this is consistent with the fact that these compounds are the most closely related structurally to the haptenic group on the immunizing antigen.

TABLE VII

SPECIFICITY OF ANTI-X'$_p$ ANTIBODY AGAINST THE CHARGED GROUP OF THE HAPTEN

Hapten	K'_0	$\Delta F°$rel. (cal.)	σ	Hapten concn., molar × 10^5 0.163	0.65	2.61	10.4	41.7	167	667
				Amount of precipitate[a]						
Benzoate	1.00	0	2.0			93	72	30	18	
Sulfate	<0.01	>2500	..							97
Benzenesulfonate	< .01	>2500	..							88
p-Arsanilate	< .01	>2500	..							87
Benzenephosphonate	< .01	>2500	..							106
p-Nitrophenolate	.23	810	3.0						48	24
"H Acid" *p*-azobenzoate	89	−2480	2.0	64	31	11	4			
"H Acid" *p*-azonitrobenzene	<0.01	>2500	..				86	87	92	89

[a] The amount of precipitate is reported as per cent. of the amount found in the absence of hapten, 244 μg.

TABLE VIII

EFFECT OF VARIOUS HAPTENS ON THE PRECIPITATION OF ANTI-X'$_p$ ANTIBODY WITH X'$_p$-OVALBUMIN

Hapten	K'_0	$\Delta F°$rel. (cal.)	σ	Hapten concn., molar × 10^5 2.61	10.4	41.7	167	667
				Amount of precipitate[a]				
Benzoate	1.00	0	2.0	93	72	30	18	
Acetate	<0.01	>2500	..					99
Phenyl acetate	<0.01	>2500	..					89
Cyclohexanecarboxylate	(0.02)	(2160)	(2.5)[b]					66
α-Naphthoate	0.031	1920	1.8				89	69
β-Naphthoate	2.0	− 380	2.5	81	54	30	14	
α-Thiophenate	1.3	− 150	2.5		66	39	16	55
Pyridine	<0.01	>2500	..					100

[a] The amount of precipitate is reported as per cent. of the amount found in the absence of hapten, 244 μg. [b] Assumed value of σ used for estimation of K'_0.

TABLE IX

EFFECT OF PYRIDINE CARBOXYLATE IONS ON THE PRECIPITATION OF ANTI-X'$_p$ ANTIBODY WITH X'$_p$-OVALBUMIN

Hapten	K'_0	$\Delta F°$rel. (cal.)	σ	Hapten concn., molar × 10^5 2.61	10.4	41.7	167	667
				Amount of precipitate[a]				
Benzoate	1.00	0	2.0	93	72	30	18	
Picolinate	0.028	1980	4.0		95	87	80	55
Nicotinate	0.38	530	2.0	95	90	65	35	
Isonicotinate	2.19	−430	2.5		59	28	14	5

[a] The amount of precipitate is reported as per cent. of the amount found in the absence of hapten, 244 μg.

It is probable that two factors contribute to the increase in combining constant attending the substitution of a larger group for the hydrogen atom in the *para* position. One is the greater polarizability and size of such a group as compared with the hydrogen atom, which would result in a greater van der Waals force of attraction. Secondly, a larger group may cause the displacement of more water from the surface of the antibody and hapten when combination takes place. The displacement of water would be expected to result in a decrease in free energy of the system in a manner analogous to the decrease of free interfacial energy which accompanies the breaking of an oil-in-water dispersion. This effect has been postulated as a major factor contributing to the energy of attraction of the uncharged portions of protein molecules.[12] Release of water—in this case, water bound by electrostatic interaction—also has been proposed as the explanation for entropy increases observed in the binding of ionic substances to serum albumin.[13,14]

(12) H. Eyring in "Symposium on the Mechanism of Enzyme Action," W. D. McElroy and B. Glass, editors, The Johns Hopkins Press, Baltimore, Md., 1954, pp. 10, 16.

(13) I. M. Klotz and J. M. Urquhart, THIS JOURNAL, **71**, 847 (1949).

(14) F. Karush, *ibid.*, **72**, 2705 (1950).

The K'_0 values for *para*-halogen substituted benzoates are in the order: fluoro < chloro < bromo < iodo. This is also the order of increasing polarizability and size of the halogen atom and is consistent both with increasing van der Waals attraction and water displacement. This direct correlation of K'_0 with size and polarizability in *para* substituents cannot readily be extended to groups containing more than one atom, probably because of the importance of the steric configuration of the group; however, each of the many *para*-substituted benzoates tested has a greater combining constant than benzoate itself.

As in the case of the *meta* substituents, the *para*-carboxylate has a larger K'_0 value, 5.3, than the *para*-nitro derivative (K'_0 = 1.9), and here again this difference may be attributed to charge effects.

Additivity of Steric Effects.—The disubstituted and trisubstituted benzoates are of special interest in connection with the study of closeness of fit. Table VI shows the observed combining constants together with values calculated from the combining constants of the corresponding monosubstituted benzoates on the assumption that the changes in free energy accompanying the substitution of more than one group are additive, *i.e.*, that the resultant relative combining constant is the product of the individual constants.

It can be seen that in general the agreement is quite good. The fact that there is agreement in the case of 3,5-dinitrobenzoate and 3-nitro-5-chlorobenzoate, in which substituents are present on both sides of the benzene ring is strong evidence that the combination is taking place through a fit against the face; it is only for a fit of this type that one would expect the steric effects of the two substituents to be additive. If combination occurred only along one side of the hapten, one would pre-

TABLE X

INHIBITION OF PRECIPITATION OF ANTI-X'$_p$ ANTIBODY BY PHENOLS AT pH'S 8 AND 9

Hapten[a]	Amount of precipitate[b] pH 8.0	pH 9.0	pK_a[c]	% dissociated pH 8.0 Calcd.[d]	Obsd.[e]	pH 9.0 Calcd.[d]	Obsd.[e]
Benzoate	18[a]	6	4.20	>99.9		>99.9	
Phenol	105	91	9.89	1	0	12	13
p-Cresol	111	..	9.81	1	0	14	10
p-Nitrophenol	25	28	7.00	91	91	99	100
p-Cyanophenol	50	46	7.95	53	57	92	94
p-Hydroxyacetophenone	90	73	7.84	59	54	94	93
p-Hydroxypropiophenone	94[a]	63	7.95	53	46	92	92

[a] Concentration of each hapten, 6.67×10^{-3} M, except for p-hydroxypropiophenone and benzoate at pH 8, which were tested at concentrations of 3.33×10^{-3} M and 1.67×10^{-3} M, respectively. [b] Amount of precipitate is reported as per cent. of the amount found in the absence of hapten; 240 μg. at pH 8, 235 μg. at pH 9. [c] pK_a value for benzoate taken from J. F. J. Dippy, *Chem. Revs.*, **25**, 151 (1939). pK_a values for phenols from J. M. Vandenbelt, C. Henrich and S. G. Vanden Berg, *Anal. Chem.*, **26**, 726 (1954). [d] Calculated from pK_a values. [e] To determine the degree of ionization, a solution of normal rabbit globulin in borate buffer, at approximately the same concentration as in the inhibition experiments, was adjusted to pH 8.0 or 9.0. A weighed amount of the phenol was added to a concentration of 5.0×10^{-3} M and the solution was back-titrated with standardized 0.01 N NaOH to the original pH. Owing to the buffering capacity of the protein and buffer, the uncertainty in these titrations was rather high, about 0.3 ml., which corresponds to an absolute error of 3% in the value for per cent. dissociated.

dict a greatly increased steric effect for a second substituent on the opposite side of the ring; because the antibody, in this case, could accommodate a single substituent by combining with the unsubstituted side of the ring, but could not readily accommodate a second *meta* substituent.

If combination occurred with the antibody completely surrounding the hapten, one would similarly expect an enhanced steric effect, because a close fit is indicated by the fact that even a single *meta* substituent causes a decrease in the combining constant. Therefore, if the antibody surrounded the hapten, a second *meta* substituent would show an enhanced steric effect since any looseness of fit is already taken up by the first *meta* substituent. And such an enhancement was not observed. This is in contrast to the X_p (anti-*p*-azobenzoate) system[6] in which the K'_0 values, relative to benzoate, for *m*-nitrobenzoate and 3,5-dinitrobenzoate were found to be 0.40 and 0.0044, respectively. The enhanced steric effect in the X_p system indicates a one-sided or surrounding fit.

One might alternatively explain the additivity of steric effects in the *meta*-positions on the assumption of flexibility of the antibody site, with each substituent contributing its own effect. However, the evidence cited above for a rigid antibody site in the X_p system shows that such flexibility is not the general case.

There is some lack of additivity in the case of 2,5-dinitrobenzoate, which shows a greater combining constant than that calculated. This is probably due to the known large steric effect of the *o*-nitro group, which twists the carboxylate out of the plane of the benzene ring. From the known specificity of the antibody for the carboxylate portion of the molecule, we would expect that the carboxylate would remain in its preferred orientation with respect to the antibody. This would result in a tilt of the benzene ring with respect to its normal orientation and might permit accommodation of the 5-nitro group, with a resultant increase in combining constant as observed.

Closeness of Fit about the Second Benzene Ring.—The fit about the second benzene ring appears to be much looser than that about the first. This conclusion is derived from a comparison of the results for the *o'*-methyl and *m'*-methyl derivatives of *p*-(*p'*-hydroxyphenylazo)-benzoate ion (Table IV). The *o'*-methyl substituent causes a small decrease in the standard free energy of combination (about 200 cal.) whereas the *m'*-methyl substituent increases the combining energy slightly (about 100 cal). The substitution of a methyl group in the *ortho* or *meta* positions of the *first* benzene ring causes increases of free energy of combination in both cases, and of much greater magnitude (Tables II, III.)

The contribution to ΔF°_{rel} of the azo group plus the second benzene ring, as shown by the data for *p*-phenylazobenzoate, is -2.3 kcal./mole. In view of the loose fit around the second ring, it seems reasonable to attribute a large part of this contribution to the azo group. This is supported also by the fact that the chloro group, which is no larger than the homologous azo group, and has the disadvantage of differing sterically from the azo group, causes a change in ΔF°_{rel} value of -920 cal., almost half as large as that of the entire *p*-phenylazo group.

Closeness of Fit around the Second Azo Grouping.—It is apparent (Table IV) that the fit around the second azo grouping is rather loose, since replacement of the *para*-hydrogen atom by amino or methyl does not change the combining constant appreciably; the introduction of a *para*-hydroxy group does increase the strength of combination slightly (by about 200 cal.).

Specificity of Antibody against the Charged Group.—The combining power of anti-X'$_p$ antibodies is found to be essentially zero with sulfate, benzenesulfonate, *p*-arsanilate and benzenephosphonate; these substances failed to inhibit precipitation even at a concentration of 6.67×10^{-3} M (Table VII). Ionized phenols, however, do combine with the anti-X'$_p$ antibody, *p*-nitrophenolate with a K'_0 value of 0.23. Data on other phenolates are in Table X.

The fact that benzenesulfonate, benzenephosphonate and *p*-arsanilate do not combine with the antibody, while phenolate ions and benzoate do, illustrates the specificity of this antibody with re-

gard to the nature of the charged group. It also constitutes evidence for a very tight fit around the carboxylate group of the hapten. Of the negatively charged substituents tested, only the O^- group is smaller than carboxylate and it is also the only other negatively charged group which permits effective combination with the anti-X'_p antibody.

Importance of the Benzene Ring in Juxtaposition to the Carboxylate Group.—The failure of acetate to combine with the antibody (Table VIII) indicates that the benzene ring is essential for measurable combination; the methyl group of acetate is not large enough, and does not contribute sufficiently to the van der Waals interaction to permit combination. Displacement of the phenyl from the carboxylate group by a $-CH_2-$ grouping, as in phenyl acetate, also decreases the combining affinity greatly, and provides further evidence that the configuration of the antibody site closely complements the contours of the benzoate ion.

Combination with Other Haptens.—The extent of combination of various other substances with this antibody is also shown in Table VIII. Saturation of the ring in benzoate to yield the cyclohexane carboxylate ion results in a configuration which combines much less effectively with anti-X'_p antibody. The increased thickness of the ring due to the saturation, the loss of coplanarity of the ring and carboxylate, and also the resultant loss of polarizability readily explain this result. The α- and β-naphthoate ions act essentially as (*ortho*, *meta*) and (*meta*, *para*) disubstituted benzoates, respectively. Owing to the high polarizability of naphthalene, both naphthoates would be expected to exhibit increased van der Waals interaction with the antibody as compared with benzoate. However, steric effects associated with *ortho* or *meta* substitution readily explain the low affinity of α-naphthoate for antibody (K'_0 = 0.031). In the case of β-naphthoate (K'_0 = 2.0), the adverse effect of that part of the second ring attached in the *meta* position is overcome by the contribution of the portion in the *para* position. A similar explanation was proposed earlier in this paper to account for the enhancing effect on K'_0 of the *meta*-acetamino group.

Pyridine, which lacks a negatively charged group, failed to combine with the antibody. The result for α-thiophenate, K'_0 = 1.3, shows that a five-membered ring containing a sulfur atom can be substituted for the benzene ring without adversely affecting the combining power. The results with pyridine carboxylates are discussed in the next paragraph.

Importance of Hydration of Pyridine Derivatives.—Table IX gives the K'_0 values for the three isomeric pyridine carboxylates. The K'_0 values are in the order *para* > *meta* > *ortho*. This is the same order as observed for each of the substituted benzoates tested. Also, the *ortho* and *meta* derivatives are less effective, while the *para* derivative is more effective than benzoate. Thus, the pyridine derivatives act as though the annular nitrogen atom had a group attached to it which interferes sterically with combination when present in the *ortho* or *meta* positions. In view of the fact that this nitrogen atom is strongly hydrated,[15] a simple explanation for the data is that the water of hydration can interfere with combination in a manner similar to that of a substituent group. Or, similarly, it may be necessary that the water of hydration be removed in order for combination to occur, in which case the energy required would appear as an increase in the free energy of combination. A similar effect, pointing to the importance of hydration as a determinant in specificity, has been observed in other antibody systems involving haptens with annular nitrogen atoms.[16-18]

Evidence for the Presence of a Positive Charge in the Antibody Site.—In order to estimate quantitatively the contribution to K'_0 of the negative charge in the haptenic group, the combining power of the antibody was measured with two haptens, "H acid" *p*-azobenzoate and "H Acid" *p*-azonitrobenzene. Since the nitro and carboxylate groups are nearly identical with respect to size, configuration and their coplanarity with the benzene ring, the relative combining powers of these two substances with antibody would indicate the effect of the charge of the benzoate ion on the combination. It was found that the "H acid" *p*-azobenzoate had a relative combining constant of 89 whereas the constant for the corresponding nitrobenzene derivative was less than 0.01, indicating a contribution of the negative charge of the carboxylate to the combining energy of over 4.8 kcal./mole. The great importance of a negative charge in the hapten indicates the presence of a positive charge in the antibody site adjacent to the position occupied by the carboxylate group of the hapten.

Knowing the difference in free energy, one can use the Schwarzenbach approximation[19] to estimate the equilibrium distance between the negative charge of the carboxyl group and the hypothetical positive charge in the antibody site. The observed difference in free energy of combination of the two "H acid" derivatives (> 4.8 kcal./mole) is found to correspond to a distance of less than 4.5 Å. between the negative charge of the hapten and a positive charge in the antibody site. This closely approximates the minimum distance of separation of charges (3.6 Å.) of an ammonium and a carboxylate group in contact.

Schwarzenbach's calculations, which give the effective dielectric constant in aqueous medium as a function of the distance between singly charged groups, are based on the ionization constants of model compounds. Thus, his calculations are related to free energies rather than heats of combination and his approximation includes the entropy changes resulting from the displacement of water from the charged groups on combination.

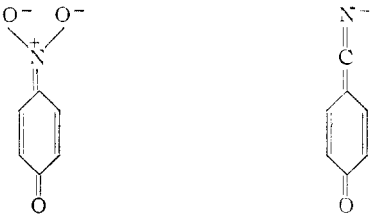
Further evidence for the presence of a positive charge in the antibody site is provided by the data in Table X, which show the results of experiments testing phenol and various *para*-substituted phenols as haptens. Owing to lack of sufficient serum only one concentration level was tested (in dupli-

(15) G. Briegleb, *Z. Elektrochem.*, **53**, 350 (1949).
(16) D. Pressman and L. Pauling, THIS JOURNAL, **71**, 2893 (1949).
(17) D. Pressman and M. Siegel, *ibid.*, **75**, 686 (1953).
(18) D. Pressman and M. Siegel, *ibid.*, **79**, 994 (1957).
(19) G. Schwarzenbach, *Z. physik. Chem.*, **A176**, 133 (1936).

cate) in many instances so that the form of this table differs from the others. Ionization constants of the haptens and the calculated per cent. dissociation at the *p*H of these experiments, 8.0 or 9.0, as well as experimental values determined in the presence of protein and buffer are listed in the last five columns. At *p*H 8, phenol and *p*-cresol, which are essentially un-ionized, do not combine with the antibody. *p*-Nitrophenol and *p*-cyanophenol which are ionized to the extent of 91 and 57%, respectively, show the greatest combining affinity while *p*-hydroxyacetophenone and *p*-hydroxypropiophenone, which are also ionized to a considerable degree, combine weakly. The latter compound was not tested at as high a concentration level as the other haptens because of its limited solubility at 5°.

In order to increase the degree of ionization, and to permit testing of *p*-hydroxypropiophenone at a higher concentration, experiments were carried out at *p*H 9.0. Because the amount of serum remaining was insufficient, *p*-cresol was omitted. At this *p*H all the substituted phenols listed in Table X are at least 90% dissociated while phenol itself is about 10% dissociated. All the substituted phenols inhibited precipitation more effectively than did phenol, which supports the results obtained at *p*H 8 in indicating that the ionized species is the important one for combination with antibody. Moreover, with one exception, each phenol was more effective at *p*H 9 than at *p*H 8, which is consistent with the greater ionization at higher *p*H. The exception is *p*-nitrophenol which was slightly more effective at *p*H 8. However, the increase in degree of ionization of this compound in going from *p*H 8 to *p*H 9 is small (99 *vs.* 91%). The results for the "H acids" and phenols are consistent in indicating that a positive charge is present in the antibody site.

Mode of Combination of *p*-Nitrophenol and *p*-Cyanophenol with Antibody.—A question of interest with regard to the combination of phenols with the antibody (Table X) is the reason for the considerably greater combining affinity of *p*-nitrophenol or *p*-cyanophenol as compared with *p*-hydroxyacetophenone and *p*-hydroxypropiophenone. At *p*H 9, the differences in degree of ionization are small. Also, the ketonic groups, owing to their larger size, would be expected to contribute as much as or more than the nitro or cyano groups to the van der Waals forces of attraction in the *para* position. It may well be that the nitro- and cyanophenols are stronger haptens because one of the important resonance forms of each of these compounds has the negative charge at the substituted (nitro or cyano) end of the molecule.[20]



This may permit them to combine in such a manner that the phenolate end of the molecule is in the *para*, or azo-specific, portion of the active site, while the nitro or cyano group, each of which bears a partial negative charge due to resonance, is in the carboxylate-specific position. Both the nitro and cyano groups are coplanar with the benzene ring and each is small enough to fit into the space occupied by the carboxylate. Thus they fulfill the steric requirements for combination. This is especially true of the nitro group which is nearly identical geometrically with carboxylate. It is not necessary, to be consistent with the inhibition data, that a large fraction of the net negative charge be on the nitro or cyano groups since these haptens, especially the latter, are less effective as inhibitors than benzoate. If the combination with antibody of *p*-nitrophenolate and *p*-cyanophenolate does occur as suggested here, *i.e.*, with the nitro or cyano group in the carboxylate-specific site, then the higher combining constants of these phenols, as compared with *p*-hydroxyacetophenone and *p*-hydroxypropiophenone, would be explained; for the negative charge on each of the former haptens would be brought into closer proximity with a positive charge in the antibody site, owing to the larger size of these groups as compared with O^-.

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(20) It has been proposed (G. W. Wheland, "The Theory of Resonance," John Wiley and Sons, New York, N. Y., 1944, p. 173) that the resonance structure of *p*-nitrophenolate shown above is largely responsible for its increased acid strength as compared with the *meta* isomer. That shown for the *p*-cyanophenolate should represent one of its stable resonance structures; the outer electron shell of each atom is complete and the affinity of a nitrogen atom for an electron is almost as great as that of oxygen.