[CONTRIBUTION FROM THE SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH]

The Binding of Simple Substances to Serum Proteins and its Effect on Apparent Antibody–Hapten Combination Constants[1,2]

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The extent of binding to normal serum proteins has been determined for many of the substances used previously in the determination of antibody–hapten combining constants, K'_0. In those cases where binding was significant, the values of K'_0 were corrected for the effect of serum binding. Where the extent of serum binding was not readily evaluated, new K'_0 values were determined using the albumin-free globulin fraction of the antiserum. Most of the correlations previously made of the effect of the structure of haptens on their combination with antisera are still valid.

In previous reports concerning the relative extent of combination of various simple substances with antibody prepared in rabbits,[3-12] the relative combining constants were determined from measurements of the effect of varying concentrations of the simple hapten on the amount of specific precipitate obtained with an antiserum and the hapten homologous antigen. However, serum components (albumins) are known to bind simple substances,[13a] and it has been pointed out from this Laboratory that if binding takes place, the apparent relative antibody–hapten combining constant would be affected.[13b,14] The effect of binding of the hapten to serum proteins can only be important for those substances of which an appreciable fraction is bound to the serum proteins at the hapten concentrations required for the determination of the hapten–antibody combination constant. Haptens which combine strongly with antibody (inhibit specific precipitation at low concentration) are most sensitive to an albumin binding effect since the fraction of hapten bound is most extensive at low hapten concentrations. If such haptens, which have high values of K'_0, bind strongly to serum proteins, the true value of K'_0 will be greater than the previously calculated apparent value (uncorrected for serum binding).

In order to correct any published relative antibody–hapten combination constants which might be in error because of such binding, we have determined the extent of binding to normal serum proteins of many of the haptens used previously. We are reporting the results of the binding measurements and the corrections for the hapten inhibition constants for the anti-X_p, anti-R_0, anti-R_m, anti-R_p, anti-R'_p, and anti-A_p systems (antisera to *p*-azophenylcarboxylate, *o*-, *m*-, *p*-azophenylarsonate, *p*-(*p'*-azophenylazo)-phenylarsonate and *p*-azophenyltrimethylammonium groups, respectively).

For the haptens in the anti-S_p and anti-X_{mp} systems (antisera to 4-azosuccinanilate and 4-azophthalate groups), since the binding to serum proteins could not be measured conveniently, we have redetermined the combining constants with antibody using the albumin-free globulin fraction of the appropriate antiserum. We have shown that such a globulin fraction of normal rabbit serum does not bind simple substances.[15] The results are given in terms of K'_0, the relative antibody–hapten combination constant with the unsubstituted hapten as reference.

Experimental

Materials.—The substances used for binding and hapten inhibition measurements were those described previously.[3-12,15,16]

The normal rabbit serum used was regenerated from lyophilized pooled sera. For regeneration, 8.25 g. of solid was made up to 100 ml. with distilled water. One pool of regenerated sera was used for all the binding measurements with arsonic acids. Different preparations were used in the other experiments. The regenerated sera contained 63–66 mg. of protein per ml. as determined by Nessler analysis. The methods of preparation of antisera and antigens were the same as those used for similar preparations described previously.[6,11,12]

Binding to Serum by Equilibrium Dialysis.—The arsonic acids, succinanilic acids, substituted "H-acids" and the other colored substances used in the binding measurements were dissolved in sufficient sodium hydroxide for neutralization, adjusted to *p*H 8 ± 1 and diluted to the appropriate volume with saline. For the arsonates binding to serum was determined by dialyzing the arsonate between 10 ml. of the buffered serum solution inside a $^{23}/_{32}$-in cellophane sausage casing and 10 ml. of saline contained in a 30-ml. vial. The dialyses were carried out in duplicate at each arsonate concentration with the dialyzable ion added inside the bag for one run and outside for the duplicate, to provide a check on the attainment of equilibrium. The solution inside the bag was composed of 3 ml. of serum and 7 ml. of borate buffer, or 3 ml. of serum, 6 ml. of buffer and 1 ml. of

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(3) D. Pressman, D. H. Brown and L. Pauling, THIS JOURNAL, **64**, 3015 (1942).

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

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

(6) D. Pressman, S. M. Swingle, A. L. Grossberg and L. Pauling, *ibid.*, **66**, 1781 (1944).

(7) L. Pauling and D. Pressman, *ibid.*, **67**, 1003 (1945).

(8) D. Pressman, J. H. Bryden and L. Pauling, *ibid.*, **67**, 1219 (1945).

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

(10) D. Pressman, A. L. Grossberg, L. H. Pence and L. Pauling, *ibid.*, **68**, 250 (1946).

(11) D. Pressman, J. H. Bryden and L. Pauling, *ibid.*, **70**, 1352 (1948).

(12) D. Pressman and L. Pauling, *ibid.*, **71**, 2893 (1949).

(13) (a) For a review see A. Goldstein *J. Pharm. Exp. Therapy*, **95**, 102 (1949). (b) The hapten–antibody combination constant, K'_0, has been calculated previously by use of the total concentration instead of the free concentration of hapten present. Binding of the hapten to the normal serum proteins results in a reduction of the free concentration of hapten available for combination with antibody. K'_0 calculated on the basis of total concentration of hapten may therefore be in error.

(14) F. Karush, *ibid.*, **72**, 2705 (1950); see also J. R. Marrack, in Sumner's and Myrback's "The Enzymes," Vol. I, Academic Press, Inc., New York, N. Y. 1950, p. 353.

(15) D. Pressman and M. Siegel, unpublished work.

(16) Radioiodine was obtained from the U. S. Atomic Energy Commission.

arsonate. The outer solution was either 9 ml. of saline and 1 ml. of arsonate or 10 ml. of saline.

The substituted "H-acids," other azo-compounds and succinanilate ion diluted with borate buffer were dialyzed in a similar fashion against a solution of normal serum (1 part serum and 5 parts saline). As a check on the attainment of equilibrium, the highly colored "H-acids" were dialyzed in two directions.

The vials were rocked 5 times per minute for 48 hours in a cold room maintained at 3–5°. Arsenic content or optical densities of the solutions both inside and outside the dialysis bags were determined.

Arsenic Analysis.—The arsenic content of each solution was determined by a modification of the method of Magnuson and Watson.[17] This modification increased the sensitivity of the method and yielded lower and more reproducible blanks. It consisted of the concentration of the final blue color from the dilute aqueous solution into a small volume of *n*-butanol by quantitative extraction.

For analyses, 3 ml. of the sample from each phase at the lower arsonate concentration, or 0.2 ml. for the higher concentration, was digested with 2 ml. of 36 *N* sulfuric acid and 6 drops of 12 *N* nitric acid in a micro-Kjeldahl flask. The mixture was heated to fuming, 6 drops of 12 *N* nitric acid was added and subsequently 6 drops of 30% hydrogen peroxide, followed by heating to fuming after each addition. To assure complete removal of nitric acid, which would interfere with subsequent color development, 2-ml. portions of water were boiled off three times. The resulting colorless solution was transferred quantitatively with 8 ml. of wash liquid (5 vol. of water and 3 vol. of 36 *N* sulfuric acid) to the bulb of the distilling apparatus described by Magnuson and Watson. The distilling head and a dropping funnel were connected to the flask which was then lowered onto the heating cone. When boiling had started and steam had begun to condense in the bottom of the trap, 5 ml. of water was added to the trap through the top of the still and the condenser placed so that the condensate would pass down the capillary. Two ml. of 30% potassium bromide was blown from the dropping funnel into the flask. The distillate was drained from a stopcock fused to the base of the trap into a 50-ml. Folin–Wu digestion tube. The trap was rinsed twice with 3-ml. portions of water and the rinsings added to the distillate. To ensure adequate acidity 1 ml. of 1 *N* HCl was added to the digestion tube, followed by 2 ml. of the molybdate and 2 ml. of the hydrazine reagent described by Magnuson and Watson. The contents of each tube were adjusted to about 35 ml., thoroughly mixed, heated for 10 minutes in a 90–100° water-bath, then cooled. The blue color which developed was extracted quantitatively by shaking the solution with 9.0 ml. of *n*-butanol. The optical density of the butanol phases was measured in 1-cm. cells in the Beckman spectrophotometer at the absorption maximum, 815 mμ. The optical density of the color developed with arsenic acid standards was reproducible within 2% over the region measured (5–25 γ arsenic in 5-γ intervals) and yielded a linear plot of optical density *vs.* concentration.

Spectral Analysis.—The concentration of colored substances, or of substances absorbing light in the ultraviolet, was measured in 1-cm. cells in the model DU Beckman spectrophotometer. The wave length at which the optical density was measured was determined by preliminary measurements of spectral curves of the absorbing substances alone, serum alone and absorbing substances mixed with varying concentrations of normal serum. For most substances a wave length was selected at which absorption by normal serum was small, and at which no spectral shift was observed (optical density of mixture of dye and serum equaled the sum of the individual optical densities). Of the "H-acids" measured, only the positively charged ion (probably because of its weak binding) showed no spectral shift; the others showed spectral shifts with isobestic points in a region where light absorption by normal serum was small. All of these dyes were found to obey Beer's law in the concentration range measured, 4×10^{-6} *M* to 10^{-4} *M*. The light absorption by serum also obeyed Beer's law over the range measured, $1/2$ to $1/6$ normal serum concentration.

The optical density of succinanilate ion was of necessity measured at a wave length where there is marked serum absorption. The succinanilate ion concentration inside the dialysis bag was determined by the differences in optical density at 240 mμ between the inner and outer phases, both diluted 1:50 with borate buffer. A correction was made for the small amount of light-absorbing material which dialyzed out of the serum. Because of the high background due to serum absorption, succinanilate binding was also determined by the difference in concentration of the outer phase before and after dialysis. At these concentrations of succinanilate ion no significant amount is bound to the dialysis bag, and all the material removed from the outer phase may be assumed to have migrated to the inner phase. Identical binding values were obtained for the binding of succinanilate when measured by these two methods.

Binding at Varying Serum Concentration.—Three dialysis bags containing 10 ml. of serum of $1/3$, $1/6$ and $1/9$ original concentration (diluted with borate buffer) were placed in a bottle containing 30 ml. of 2×10^{-4} *M* radiolabeled *p*-iodobenzoate[15,16] in saline. The bottle was rocked in the cold room for 48 hours. After the attainment of equilibrium, 1-ml. aliquots from each of the protein phases together with 1 ml. of 1 *N* sodium hydroxide were placed in planchets. The planchets were dried under a battery of infrared lamps and the activity in each planchet counted under a thin window Geiger tube. Three 1-ml. aliquots were transferred from the outer phase to separate planchets and 1 ml. of 1 *N* sodium hydroxide added to each. To bring the total protein concentration in an aliquot from the outer phase to the same value as in an aliquot from an inner phase appropriate amounts of serum were added to each. Each of the outer phase planchets were dried and counted as above. The difference in number of counts between inner phase and outer phase planchets (measured at the same serum concentration) was a measure of the concentration of bound radioactive *p*-iodobenzoate in the serum phase. The outer phase radioactivity was a measure of unbound *p*-iodobenzoate both in the serum phase and in the outer aqueous phase. The ratios of bound to free iodobenzoate in the three dialysis bags were 6.9, 3.5 and 2.4, and these ratios divided by the relative serum concentration were the same, 1.0. Within experimental error, the bound concentration was proportional to the serum concentration at the same free concentration of *p*-iodobenzoate ion.

Reaction of Antiserum Globulin with Antigen and Hapten.—Hapten solutions were prepared by dissolving the hapten in 1 *N* sodium hydroxide, adjustment to *p*H 8 and dilution with saline. Antigen dilutions were made with borate buffer of *p*H 8. The reactants were mixed and permitted to stand for three nights at 5°. The precipitates were centrifuged, washed three times with 10-ml. portions of 0.9% sodium chloride and dissolved in sodium hydroxide. Protein analysis was by the modified Folin–Ciocalteu procedure.[18]

Fractionation of Antiserum.—The globulin fraction of anti-X_p, anti-S_p and anti-X_{mp} sera was separated from the whole antisera by fractionation three times with ammonium sulfate.[19] The ability of the resulting fraction to bind methyl orange was measured. The three times precipitated globulin fractions were found to bind less methyl orange than a 1:30 dilution of whole normal serum, indicating that 3% or less of the original protein responsible for binding remains in the globulin fraction.

Results and Discussion

The Effect of Binding of Hapten to Serum Proteins on Hapten–Antibody Combination Constant.—That there is an effect of the normal whole serum proteins on the antibody combination constant is shown by the experiment of Table I. The effect of hapten in different concentrations on the precipitation of anti-X_p antibody with X_p-ovalbumin (ovalbumin substituted with *p*-azobenzoate groups) was determined using the albumin-free globulin fraction of the antiserum diluted with borate buffer in one case and with normal serum in the other. The amount of antigen used was that which gave optimum precipitation with the antibody. In the absence of hapten, the optimum concentration of antigen and the optimum amount of precipitate did

(17) H. J. Magnuson and E. B. Watson, *Ind. Eng. Chem., Anal. Ed.*, **16**, 339 (1944).

(18) D. Pressman, *ibid.*, **15**, 357 (1943).

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

TABLE I

EFFECT OF NORMAL SERUM ON THE HAPTEN INHIBITION OF SPECIFIC PRECIPITATION OF ANTI-X_p ANTIBODIES

The albumin-free globulin fraction of anti-X_p serum (1 ml., 20 mg.) in borate buffer was mixed with either 1 ml. of borate buffer of *p*H 8.0 or with 1 ml. of normal rabbit serum and added to a mixture of 1 ml. of X_p-oval (30 μg.) in borate buffer and 1 ml. of hapten solution in saline. All values are the average of triplicate analyses with mean deviation ±6%.

Benzoate ion	K'_0	σ	Hapten concn.,[a] molar × 10^4 0.3	0.7	1.3	2.6	5.3	10.5	21
			Amount of precipitate[b]						
Buffer added									
Unsubstituted	1.00	2.5			830		600		320
p-Chloro	4.9	2.5		690		400		180	
p-Acetamino	9.6	4			380		200		70
p-Nitro	7.9	3.5		570		300		160	
p-Iodo	19	2.5	540		350		120		
Serum added									
Unsubstituted	1.00	2.5			860		610		390
p-Chloro	3.7	2.5		760		510		240	
p-Acetamino	10	3			430		310		140
p-Nitro	5.2	3		650		470		260	
p-Iodo	5.0	3	790		650		265		

[a] Final concentration of hapten. [b] The amounts of precipitate are in parts per mille of the amount of precipitate in the absence of hapten, 268 γ in the presence of borate and 267 γ in the presence of serum.

not appear affected by the presence of whole serum. The presence of whole serum had but slight effect on the inhibition of precipitation by benzoate ion. Benzoate is 16% bound to whole serum proteins under conditions in this experiment, so that the free concentration is 84% of the total concentration. On the other hand, the inhibition of precipitation by *p*-iodobenzoate is markedly affected. An appreciably larger concentration of *p*-iodobenzoate was required to produce the same degree of inhibition with serum present than in its absence. The relative hapten–antibody combining constant, K'_0, is only 5 in the presence of serum and 19 in the absence of serum. These values are what would be expected from the known extent of binding of *p*-iodobenzoate to serum proteins. K'_0 is, for practical purposes, the ratio of the concentration of the reference ion required for half inhibition to the concentration of hapten required for half inhibition. K'_0 corrected will equal

$$K'_{0\,\text{apparent}} \times \frac{(\%\ \text{reference free})}{(\%\ \text{hapten free})}$$

The iodobenzoate is 19% free (81% bound to serum) at the concentration of half inhibition. A corrected value K'_0 of $5 \times 84\%/19\% = 22$ is obtained for the case where serum is present. This is in excellent agreement with the experimentally observed value of 19 obtained from inhibition studies of the globulin fraction where no serum binding takes place. The extent of combination of *p*-chlorobenzoate and *p*-nitrobenzoate was affected to an intermediate extent indicating degrees of binding between those of benzoate and *p*-iodobenzoate. The extent of combination of *p*-acetaminobenzoate was slightly greater in the presence of serum, indicating that *p*-acetaminobenzoate is bound less strongly to the albumin than is the reference benzoate ion.

The Correction of Previously Reported Constants for Serum Binding Effects.—The corrected relative hapten–antibody combination constant, K'_0, was obtained by the same calculation described previously[5] with the exception that the concentration of the unbound hapten was used rather than the total concentration (bound and unbound). The free concentration of the hapten was calculated from the total hapten concentration, the serum concentration in the hapten inhibition experiments and the data for serum-binding of haptens. Since the extent of binding to serum was measured to within 5–10%, the correction of the previously determined constants does not decrease their precision significantly.

The fraction of substance bound varies with serum concentration. The concentration of substance bound to serum is proportional to the concentration of the serum when the concentration of the free ion is kept constant. This relationship can be derived from equilibrium considerations and was verified by experiments involving the binding of radiolabeled *p*-iodobenzoate ion to rabbit serum (see Experimental). Since the same serum concentration was not used in all hapten inhibition experiments as in the binding measurements, correction was made for the different serum concentrations. Thus, the effect of the binding by serum becomes less important the more dilute the serum used in the hapten inhibition studies.

At a fixed serum concentration the fraction of hapten bound at a particular hapten concentration was determined by approximation from the plot of free ion concentration/bound ion concentration *vs.* free ion concentration for three points which had been determined by equilibrium dialysis measurements.[20]

Since the haptens studied are charged ions, there are slight Donnan effects incurred in the determination of the extent of binding. These tend to decrease the concentration of the hapten in the protein phase when they are negative ions and to increase the concentrations of the haptens for the cases where the hapten has a positive charge. This effect would account for a 2% difference in concentration of free ion in the two phases for a hapten bearing a single negative or positive charge. The correction is at maximum 4% for ions with two charges. These Donnan effects are negligible within the limits of our accuracy.

The *p*-Azobenzoate System (Anti-X_p System).[6]—Values for the binding to serum of several carboxylate ions have been determined previously.[15] These values were used in the recalculation of the antibody–hapten combination constants in Table II. The correction in K'_0 for most substances was slight. The greatest correction was a factor of 1.9 in K'_0 for the highly bound *p*-(*p'*-aminophenylazo)-benzoate. Although many haptens for which the binding was not measured may also be strongly bound, it is still possible to obtain significant values for K'_0 by estimating the degree of binding by serum. At low values of K'_0, where the hapten

(20) This plot would be a straight line according to the simple binding theory involving the equation

$$\frac{1}{r} = \frac{K}{n[a]} + \frac{1}{n}$$

as given by I. M. Klotz, F. M. Walker and R. B. Pivan, THIS JOURNAL, 68, 1486 (1946).

TABLE II

CORRECTION OF K'_0 VALUE IN ANTI-X_p SYSTEM

Compound	Free concn., $M \times 10^6$	Hapten binding: Found for 1/9 serum concn. Total concn., $M \times 10^6$	Found for 1/9 serum concn. % bound	Calcd. for 1/9 serum concn. Total concn., $M \times 10^6$	Calcd. for 1/9 serum concn. % bound	Total concn. at 50% inhibition: Anti-gen XXXV, $M \times 10^6$	Total concn. at 50% inhibition: X_p-oval $M \times 10^6$	Per cent. bound at 50% inhibition: Anti-gen XXXV	Per cent. bound at 50% inhibition: X_p-Oval	K'_0: Reported previously[6] Anti-gen XXXV	K'_0: Reported previously[6] X_p-Oval	K'_0: Cor. for protein binding Antigen XXXV	K'_0: Cor. for protein binding X_p-Oval
Benzoate	6.8	12	43.8	8.6	20	17	40	16	12	1.00	1.00	1.00	1.00
	96	110	16.0	102	6								
	490	510	2.4	500	1								
m-Toluate	5.6	13	58.5	8.1	31	70	70	12	12	0.22	0.66	0.21	0.66
	91	120	24.2	101	10								
	480	520	7.4	493	3								
p-Toluate	6.4	13	49.0	8.5	25	5	10	27	24	3.6	2.6	4.1	3.0
	89	120	26.2	99	11								
	460	540	14.2	485	5								
α-Naphthoate	4.5	14	68.8	7.7	41		200		13		0.18		0.18
	80	130	38.3	97	17								
	440	560	20.7	479	8								
Salicylate	45	55	17.7	48	7		100		5		0.46		0.43
p-(*p'*-Hydroxy-phenylazo)-benzoate	23	62	62	36	36	1.5	2	44	43	11.3	21.9	17.0	33.8
	414	554	25	460	10								
p-(*p'*-Amino-phenylazo)-benzoate	3.8	15.2	75	7.6	50	2	2	53	53	10.4	17.0	18.6	31.8
	57	140	58	84	32								

concentration at half inhibition is high, the per cent. bound by serum is low, especially since the anti-X_p serum was used at one-ninth original concentration. By taking the values of binding for the very strongly bound *p*-iodobenzoate as the maximum which might be expected, maximum values for the error due to binding may be determined for various values of K'_0. *p*-Iodobenzoate exists free in serum of one-ninth concentration to the extent of 96, 89, 74 and 47% at total concentrations of 10^{-2}, 3×10^{-3} and 3×10^{-4} *M*, respectively. These concentrations correspond to the concentration for 50% of precipitation for haptens with K'_0 values of 0.03, 0.1, 0.3 and 1 inhibition. Since the reference benzoate ion is free to the extent of 84% in its effective region, values of K'_0 previously reported as less than 0.03 will be 12 to 16% too low, as 0.1 will be 5 to 16% too low, as 0.3 will be from 16% too low to 13% too high, as 1 will be 16% too low to 78% too high. Thus, the values of K'_0 previously reported as below 0.3 are correct within 16% whether bound or not. Values of K'_0 up to 1 may be in greater error but a corrected value must lie below 2.

TABLE III

BINDING OF ARSONATES AT *p*H TO RABBIT SERUM OF 0.3 ORIGINAL SERUM CONCENTRATION

A, total concentration of arsonate in protein solution ($M \times 10^5$); B, extent of binding (% of amount present) with deviation from average for dialysis in both directions; C, binding constant for first ion with albumin[25] ($k \times 10^{-3}$)

Arsonate ion	A	B	C
o-(*p'*-Hydroxyphenylazo)-phenyl	1.8	38 ± 9	3.5
	86	25 ± 4	
m-(*p'*-Hydroxyphenylazo)-phenyl	2.3	70 ± 1	14.2
	90	34 ± 0	
p-(*p'*-Hydroxyphenylazo)-phenyl	2.1	58 ± 4	8.2
	91	35 ± 3	
p-(*p'*-Aminophenylazo)-phenyl[a]	1.9	39 ± 1	3.7
p-(*p'*-Aminobenzoylamino)-phenyl	1.7	18 ± 1	1.2
	81	14 ± 4	
p-(*p'*-Nitrobenzoylamino)-phenyl	1.8	29 ± 4	2.3
	84	22 ± 4	
p-Iodophenyl	1.7	25 ± 0	1.9
	79	9 ± 1	
α-Naphthyl	1.7	26 ± 7	2.0
	81	16 ± 4	
β-Naphthyl	2.0	50 ± 0	5.9
	86	25 ± 4	

The following arsonate ions were bound to an extent of less than 10% at 10^{-5} and 10^{-3} *M* total concentration.

Phenyl	2,4-Dimethylphenyl
p-Acetaminophenyl	2,4-Dinitrophenyl
o-Aminophenyl	*p*-Hydroxyphenyl
m-Aminophenyl	*o*-Methylphenyl
p-Aminophenyl	*m*-Methylphenyl
p-Benzoylaminophenyl	*p*-Methylphenyl
p-Benzyl	*o*-Nitrophenyl
p-Bromophenyl	*m*-Nitrophenyl
p-Carboxyphenyl	*p*-Nitrophenyl
o-Chlorophenyl	Methyl
m-Chlorophenyl	Arsonate
p-Chlorophenyl	*p*-Aminophenylmethyl
2,4-Dichlorophenyl	

[a] Insoluble at 10^{-3} *M*.

The Phenylarsonate Systems.[3-5,7,9]—In the determination of the extent of binding of arsonates to serum proteins, we have used serum at 0.3 original concentration (very near the highest serum concentration used in the hapten–antibody studies) and have measured the binding of haptens both at the highest concentration used in the hapten–antibody studies, about 10^{-3} *M*, and at a low concentration, about 10^{-5} *M*. The extent of binding is reported in Table III. The determination of binding of less than 10% was not precise since the small binding

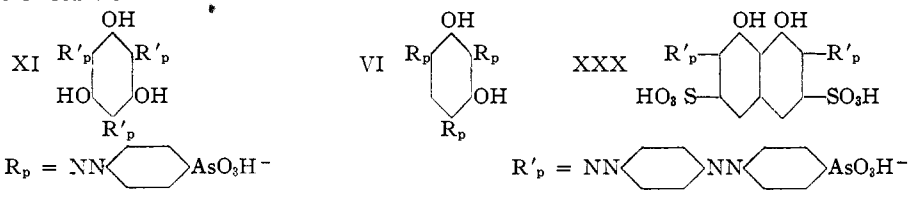
TABLE IV

RELATIVE HAPTEN–ANTIBODY COMBINING CONSTANTS CORRECTED FOR SERUM BINDING

Antisera homologous to the *p*-azophenylarsonate group, and to the *p*-(*p'*-azophenylazo)-phenylarsonate group (anti-R_p and anti-R'_p sera, respectively).[a] A, Anti-R_p serum and antigen VI, Table VI, ref. 3; B, anti-R_p serum and antigen XXX, Table I, ref. 5; C, anti-R_p serum and R'_p-ovalbumin, Table I, ref. 5; D, anti-R_p serum and antigen XI, Table III, ref. 5; E, anti-R'_p serum and antigen XXX, Table VI, ref. 4; F, anti-R'_p serum and antigen XXX, Table II, ref. 5; G, anti-R'_p serum and R'_p-ovalbumin, Table II, ref. 5.

Arsonate ion	A	B	C	D	E	F	G
Phenyl	1.00	1.00	1.00	1.00	1.00	1.00	1.00
p-(*p'*-Aminophenylazo)-phenyl	7.2	8.9	13.4		53	27	26
p-(*p'*-Hydroxyphenylazo)-phenyl	10.3	10.6	14.0	6.3	35	34	30
o-Nitrophenyl	0.9	0.69	0.71	0.50	5.9	2.4	1.8
m-Nitrophenyl	3.2	3.4	3.7	0.89	7.0	3.8	3.6
p-Nitrophenyl	6.8	11.1	16.5	3.6	5.7	3.3	4.4
o-Methylphenyl	0.24	0.16	0.25	0.30	3.0	1.25	1.00
m-Methylphenyl	1.6	1.6	1.4	1.0	2.2	1.3	1.1
p-Methylphenyl	3.6	3.7	4.0	1.3	2.0	2.5	2.7
o-Aminophenyl	0.22	0.20	0.28	0.50	0.31	0.43	0.63
m-Aminophenyl	1.04	0.93	1.00	0.80	.44	0.80	0.69
p-Aminophenyl	1.4	1.4	1.4	1.4	.89	1.1	1.0
p-Chlorophenyl	3.7	5.2	5.5		2.8	2.6	2.9
p-Bromophenyl	3.6	5.9	6.5		3.5	3.3	3.1
p-Iodophenyl	5.2	7.1	8.4		5.5	6.1	6.8
p-Carboxyphenyl	1.0	1.4	1.4		0.74	1.1	0.9
p-Hydroxyphenyl	2.0	1.6	1.6		1.4	1.4	1.2
p-Acetaminophenyl	5.0	7.3	8.6	2.6	7.0	3.6	3.3
p-Benzoylaminophenyl	3.1	4.6	5.7	2.4	6.3	5.4	4.8
p-(*p'*-Aminobenzoyl)-phenyl	4.2	4.2	6.4		9.4	8.2	7.0
p-(*p'*-Nitrobenzoyl)-phenyl	5.3	3.5	8.5		12.4	6.2	6.1
α-Naphthyl	0.75	0.50	0.42	0.27	16.9	4.8	2.9
β-Naphthyl	4.8	3.8	4.8	1.5	5.1	5.2	3.9
1,4-Aminonaphthyl	0.33	0.64	0.64		12.6	4.8	3.2

[a] The antigens used are



was determined as the difference between two large numbers representing the arsenic concentrations inside and outside the dialysis bag. Substances bound less than 10% are grouped together.

The relative combining constants for hapten and antibody as determined previously with protein antigens for the anti-R_0,[7] anti-R_m,[7] anti-R_p,[3,5,7] anti-R'_p,[4,5] systems have all been corrected for the hapten binding effects and the corrected K'_0 values are in Tables IV and V. For most constants little or no correction was required. For a few, such as those of the azophenylarsonates and the naphthylarsonates, correction factors as high as 2 or even greater were required.

Corrected values are also given for the combination of antibody with hapten using simple multihaptenic substances as precipitating antigen.[21] None of the values of K'_0 listed in reference 9 requires correction, with the possible exception of the value for phenylphosphonate ion. Since serum binding was not determined for this substance and the value of K'_0 was high, any binding would increase this value.

The *p*-Azophenyltrimethylammonium Ion System (anti-A_p System).[10]—The extent of binding of several of the haptens used in the studies concerning anti-Ap antibodies is shown in Table VI. The binding of *p*-(*p'*-hydroxyphenylazo)-phenyltrimethylammonium ion to serum proteins is very low, being of the order of only 21% even at low concentrations. The phenyltrimethylarsonium ion at low concentration is also essentially unbound by serum.[22] From these two bindings it may be concluded that the binding of ions containing a positive charge is very low, particularly since the corresponding *p*-(*p'*-hydroxyphenylazo)-substituted benzoate and phenylarsonate ions are strongly bound.

Since the bindings are so low, the values of K'_0 as reported previously are essentially correct. The value of K'_0 as previously reported for those few substances which bind are given in Table VI along with the values corrected for serum binding. Be-

(21) When multihaptenic substances are used as a precipitating antigen, there may be a factor of the antigen itself being albumin-bound. However, since the antigen concentration was kept constant for each set of data, the system serum-bound antigen, free antigen and antibody-precipitated antigen is probably constant for all the haptens studies in any one set at the same degree of inhibition of precipitation unless the hapten is present in such high concentration and binds so strongly that it displaces serum-bound antigen, changing the characteristics of the system. Such antigen displacement may explain the increased amounts of precipitate obtained with some hapten–anti-hapten systems when a simple heterohaptenic substance is added. See D. Pressman and A. L. Grossberg, *Science*, **101**, 2619 (1945), and also ref. 6.

(22) Determined by equilibrium dialysis and analysis of both phases for arsenic content.

TABLE V

RELATIVE HAPTEN–ANTIBODY COMBINING CONSTANTS CORRECTED FOR SERUM BINDING

Antisera homologous to the *o*-, *m*- and *p*-azophenylarsonate ion groups (anti-R_o, -R_m, -R_p sera, respectively), original data in ref. 7

Arsonate ion	System: Anti-R_o R_o-oval	Anti-R_o R_m-oval	Anti-R_m R_m-oval	Anti-R_p[a] R_m-oval	Anti-R_p R_p-oval
	Relative constant				
Phenyl	1.00		1.00	1.00	1.00
o-(*p*'-Hydroxyphenylazo)-phenyl	12	11	0.51	0.41	0.14
m-(*p*'-Hydroxyphenylazo)-phenyl	0.40	1.3	6.5	11	3.8
p-(*p*'-Hydroxyphenylazo)-phenyl	0.007	0.08	0.85	41	13
o-Nitrophenyl	5.8	15.5	0.64	0.76	0.60
m-Nitrophenyl	0.54	1.3	5.5	2.5	1.5
p-Nitrophenyl	0.064	0.23	0.35	9.7	5.3
o-Methylphenyl	3.5		.51	0.40	0.21
m-Methylphenyl	0.87		.98	1.4	0.78
p-Methylphenyl	0.17		.25	3.2	1.9
o-Chlorophenyl	8.2	5.9	.89	0.66	0.30
m-Chlorophenyl	1.6	2.2	1.3	1.9	1.3
p-Chlorophenyl	0.77	1.05	0.40	4.0	2.2
o-Aminophenyl	1.4	2.5	0.55	0.46	0.26
m-Aminophenyl	0.42	1.7	1.05	1.0	0.56
p-Aminophenyl	0.27	0.9	0.44	1.3	1.23
α-Naphthyl	2.2		.46	1.4	0.52
β-Naphthyl	0.17		.71	12	6.0
2,4-Dinitrophenyl	.17		.32	2.6	1.88
2,4-Dimethylphenyl	.33		.18	1.0	0.53
2,4-Dichlorophenyl	.91		.78	2.1	0.83

[a] Recalculated to K'_0 = 1.00 for phenylarsonate.

to be accordingly RT ln 8 = 1150 cal. per mole. Then, by a calculation involving the Schwarzenbach[23] formula for the dielectric constant of water, the value for the distance between the positive trimethylammonium group and the oxygen of a carboxylate ion (assuming that the antibody negative charge involved is on a carboxylate ion) is 2 to 3 Å., depending on whether the positive ion approaches the carboxylate from the side, Fig. 1a, or "head on," Fig. 1b. When the approach is "head on," the distance between the positive charge on the nitrogen and the negative charge distributed equally on both oxygens is 8 Å. When the approach is from the side, the positive charge is 7 Å. from the charge of the nearer oxygen and 9.5 Å. from that of the more distant.

The value of 2 Å. between the van der Waals surface of the positive and negative groups reported previously was calculated for the "head on" approach.

The larger distance, 3 Å., now obtained is large enough to accommodate a molecule of water of hydration on one of the ions taking part in the combination.

The 4-Azosuccinanilate System (Anti-S_p System).[8,11]—Binding measurements with *p*-(*p*'-hydroxyphenylazo)-succinanilate ion and with succinanilate ion are shown in Table VII. It can be seen that substituted succinanilate ions are appre-

TABLE VI

BINDING TO RABBIT SERUM OF SUBSTANCES IMPORTANT IN ANTI-A_p SYSTEM

Extent of binding at 1/3 original serum concn.

Substance	Free concn., molar × 10^5	Total hapten concn., molar × 10^5	Bound, %	K'_0: Corrected for binding A[a]	Corrected for binding B[b]	Reported previously[10] A[a]	Reported previously[10] B[b]
HO—C_6H_4—N=N—C_6H_4—$N(CH_3)_3^+$	8.8	11.1	21		13		12.4
	90	108	17				
$C_6H_5As(CH_3)_3^+$	1.5	1.5	0				
	70	70	0				
"H-acid"—N=N—C_6H_4—$N(CH_3)_3^+$	1.5	1.7	10	4.0	5.2	3.8	4.9
	15.6	17	8				
	158	170	5				
"H-acid"—N=N—C_6H_4—$C(CH_3)_3$	0.34	5.7	94	0.48	0.68	0.23	0.34
	9.8	54	82				
	180	310	42				
"H-Acid"—N=N—C_6H_5	1.1	3.1	65				
	20	39	48				
	290	360	19				
"H-acid"—N=N—$C_{10}H_7$	0.17	5.7	97				
	7.9	56	86				
	240	370	35				

[a] Table VI, series A, ref. 10. [b] Table VI, series B, ref. 10.

cause of the high serum binding of the *t*-butyl substance, its combining constant with the antibody is greater than previously considered. The calculation made previously of the distance of a negative ion in the specific combining site of anti-A_p antibody from the positive center of the combined hapten, through a consideration of the relative combining powers of the *t*-butyl and trimethylammonium compound, is thus in error. The average ratio of the corrected values of K'_0 for these two haptens is 8. The Coulomb interaction energy between the charged haptenic group and the antibody comes out

ciably bound to serum proteins and thus the values of K'_0 as reported previously may be in error, especially in those cases where K'_0 is high. Therefore, values of K'_0 were redetermined for all haptens which had previously shown values of K'_0 greater than 0.15[24] using the albumin-free globulin fraction of a pool of anti-S_p serum. The new values of K'_0 and of the heterogeneity index, σ^5, appear in Table VIII along with the experimental data. For all values of K'_0 as previously determined which are

(23) G. Schwarzenbach, *Z. physik. Chem.*, **A176**, 133 (1936).

(24) Except for N,N-pentamethylenesuccinanilate.

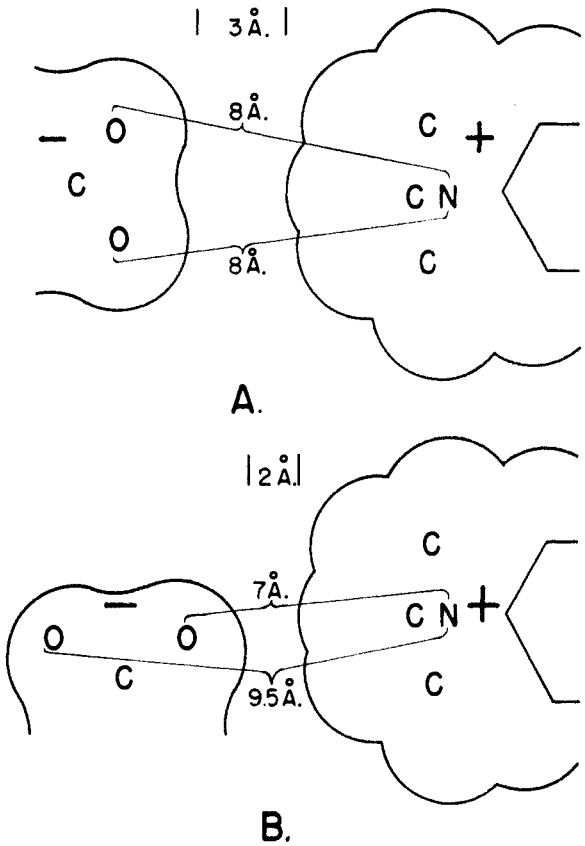


Fig. 1.—Apparent distance of approach of trimethylammonium group of hapten to carboxylate of antibody: A, head-on; B, sideways.

lower than 0.15, the correction is low due to the high hapten concentration required. There is a constant factor due to the binding of the reference ion, but this leaves unchanged the previously discussed structural aspects of the combination of hapten with antibody.

TABLE VII

BINDING TO RABBIT SERUM OF SUCCINANILATE ION: EXTENT OF BINDING AT 1/3 ORIGINAL SERUM CONCN.

	Free concn., molar × 10^5	Total hapten concn., molar × 10^5	Bound, %
p-(*p*'-Hydroxyphenylazo)-succinanilate	2.5	12.5	80
	46	138	67
Succinanilate	108	140	23
	470	500	6

Although some of the differences in values of K'_0 (Table VIII) as determined here using the globulin fraction and as reported previously using whole serum may be due to differences in the pools of serum, it is significant that the differences are precisely those which would be expected for serum binding effects. Substances which have high values of K'_0 in the presence of whole serum have still higher values of K'_0 in the absence of serum. This is especially true in those cases where the substance is known to bind more strongly than the reference substance to serum, *i.e.*, *p*-(*p*'-hydroxyphenylazo)-succinanilate, or where substituents are present which might be expected to increase the binding to serum proteins. On the other hand, those with low values of K'_0 are lower in the absence of albumin due to the greater effect of serum binding on the reference ion. The only change in the conclusions drawn previously concerning the effect of structure on the combination of hapten with antibody in this system is that there seems to be a greater effect of substituents in the para position than was noticed previously. The relative values of K'_0 for the *d*- and *l*-(α-methylbenzyl)-succinamate depend on the pool of serum used; since here and in reference 8 the value is greater for the *l*-isomer, and in reference 11 the value is greater for the *d*-isomer.

The 4-Azophthalate Ion Systems (Anti-X_{mp} System.)[12]—A preliminary experiment with the *p*-(*p*'-hydroxyphenylazo)-*o*-phthalate showed that it was appreciably bound to serum proteins.

All the haptens which showed any appreciable combination with antibody in the presence of whole serum were rerun with the globulin fraction of anti-X_{mp} serum to eliminate the albumin-binding effect. The results are shown in Table IX and the values of K'_0 are generally quite similar to those reported previously for determinations with whole serum. In the new data the 3-nitrophthalate combines less strongly than the 4. Also the tetrachlorophthalate now has a value of less than 1 for K'_0 showing a steric effect of the chloro groups interfering with the hapten antibody combination. The value of K'_0 for bromo is greater than that for chloro, but still less than one. Apparently the greater van der Waals attraction of the bromo groups more than overcomes the greater steric effect of these groups.

All of the other correlations which have been drawn are still valid for the rest of the haptens.

The Effect of Structure on Binding to Serum Albumins.—On the basis that the binding to serum proteins is primarily due to the serum albumins, binding constants were calculated for the interaction of the arsonates with albumin. The constants listed in Table III are for the binding of the first ion to the protein molecule.[25] Those that show bindings of appreciable magnitude are those which would be expected to show the greatest van der Waals attraction due to the substituents present. Thus, *p*-iodobenzoate shows a greater binding than the other *p*-halobenzenearsonates, and substances containing more than one benzene ring are in general more strongly bound than those containing a single ring. The strength of binding is also a function of steric configuration as is shown by the differences in binding strengths of α- and β-naphthylarsonates and of the *o*-, *m*- and *p*-(*p*'-hydroxyphenylazo)-phenylarsonates.

The low binding of the positive ions studies in the anti-A_p system, is especially interesting in view of the fact that the serum proteins carry a net negative charge at *p*H 8.0 and might be expected to

(25) Binding constants were calculated from the binding data at low concentrations of arsonate ion. At these concentrations most of the albumin molecules will be unbound and only an insignificant number of these molecules will bind more than one arsonate ion. The binding constant, k, equals $\frac{(\text{albumin–anion complex})}{(\text{free album in})(\text{free anion})}$. The total albumin concentration of the serum was taken as 6×10^{-4} *M*.

TABLE VIII

EFFECT OF HAPTENS ON THE PRECIPITATION OF ANTI-S_p ANTIBODY WITH S_p -OVALBUMIN

Antigen solution in buffer 0.70 ml. (88 μg.); hapten solution in saline, 0.70 ml.; regenerated globulin fraction of antiserum in saline, 0.70 ml. (23 mg.); three days at 5°

	K'_0	K'_0,[a] uncor.	σ	Hapten concn., molar × 10^5: 0.65	1.3	2.6	5.2	10.4	20.7	41.5	83	166
				Amount of precipitate[b]								
Succinanilate	1.00	1.00	2.5			790		520		260		
Maleanilate	0.11	0.25	2.5				930		820		540	
p-(*p'*-Hydroxyphenylazo)-succinanilate	3.9	1.38	1.5	880		550		200				
p-Nitrosuccinanilate	4.1	1.65	2	840		500		160				
p-Aminosuccinanilate	0.95	1.03	1.5			900		540		240		
p-Bromosuccinanilate	2.2	1.31	1.5		820		470		180			
m-Bromosuccinanilate	1.8	0.72	2		820		560		210			
o-Bromosuccinanilate	0.40	.50	2				840		580		260	
N-α-Naphthylsuccinamate	0.32	.45	2				850		630		330	
N-β-Naphthylsuccinamate	2.2	1.09	2		820		500		150			
N-Cyclohexylsuccinamate	0.10	0.15	2					910		750		450
N-Benzylsuccinamate	.18	.25	2					830		560		310
d-N-(α-Methylbenzyl)-succinamate	.10	.19	2					850		760		420
l-N-(α-Methylbenzyl)-succinamate	.16	.17	2					870		630		290
β-Benzoylpropionate	.70	.59	2				750		460		150	

[a] K'_0 uncorrected values are those reported previously[11] and were determined in presence of albumin. [b] The amounts of precipitate are in parts per mille of the amount in absence of hapten, 391 μg. All values are averages of triplicate analyses with mean deviation 4%.

TABLE IX

EFFECT OF HAPTENS ON THE PRECIPITATION OF ANTI-X_{mp} ANTIBODY WITH X_{mp}-OVALBUMIN

Antigen solution in buffer, 1.00 ml. (700 μg.); hapten solution in saline, 1.00 ml.; regenerated globulin fraction of antiserum in saline, 1.00 ml. (26.7 mg.); three days at 5°

	K'_0	K'_0[a] uncor.	σ	Hapten concn., molar × 10^5: 20	80	330
				amount of precipitate[b]		
Phthalate	1.00	1.00	2	800	510	230
4-Nitrophthalate	1.03	1.15	2.5	770	550	220
3-Nitrophthalate	0.65	1.04	2.5	790	650	310
Tetrachloro-phthalate	.62	1.51	3.5	720	630	380
Tetrabromo-phthalate	.81	0.97	3	770	590	320
Tetraiodophthalate	.35	.29	4	710	680	490
Quinolinate	.20	.36	3	930	800	560
Pyrazine-2,3-dicar-boxylate	(.05)[c]	.05	(3)[c]	1010	950	790
o-Sulfobenzoate	.26	.69	2.5	970	750	500

[a] K'_0 uncorrected values are those reported previously and were determined in the presence of albumin. [b] The amounts of precipitate are in parts per mille of the amounts in absence of hapten: 387 μg. All values are averages of triplicate analyses with mean deviation 3%. [c] Values for the pyrazine-2,3-dicarboxylate were determined from the two higher hapten concentrations.

bind positive ions more strongly than negative or uncharged ions. Klotz has reported that even at a higher *p*H the binding of a similar positive ion, *p*-azophenyltrimethylammonium ion to bovine serum albumin is negligible.[26] The presence of the positive charge even decreases the binding of a strongly bound negative ion. Thus, the hapten "H-acid"—N=N—$C_6H_4C(CH_3)_3$ which contains two negative charges, is very strongly bound to serum proteins. However, replacement of the *t*-butyl group by the isosteric trimethylammonium group which carries a single positive charge essentially destroys the binding power. This large difference in binding powers is probably due to the presence of a positive charge on the serum protein in the region where the *t*-butyl or trimethylammonium ions would be located when these substances are bound. The extent of binding of the substituted "H-acid" compounds is very sensitive as to the nature of the hydrocarbon residue attached. The order of effectiveness is *t*-butylphenyl > naphthyl > phenyl.[27]

Acknowledgment.—We wish to thank Richard Clarke and Rubén Medina for assistance in this work.

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(26) I. M. Klotz, E. W. Gelewitz and J. M. Urquhart, THIS JOURNAL, **74**, 209 (1952).

(27) On the basis of van der Waals interaction the α-naphthyl derivative would be expected to exhibit the highest binding. The higher value for the *t*-butylphenyl indicates the importance of steric factors in binding.