Thianthrene (II) and Nitric Acid. Run I.—To 25 ml. of nitric acid (sp. gr. 1.5) was added at −10°, 4.32 g. (0.02 mole) of powdered II. The temperature was maintained at −15° to −10° during the addition of II. The solution was immediately poured over ice and the white product which separated was filtered, washed with water and dried. Recrystallization from glacial acetic acid gave 2.23 g. (45%) of the α-form[14] of thianthrene-5,10-dioxide (mixed m.p.). The mother liquor was colorless indicating that no nitration had taken place.

Run II.—To 100 ml. of nitric acid (sp. gr. 1.5) was added at −30°, 10.8 g. (0.05 mole) of powdered II. The mixture was vigorously stirred during the addition of II and the temperature was maintained at −30° for a total period of 1 hour. Upon the addition of each portion of II to the nitric acid an intense red coloration was produced which immediately disappeared to give a greenish solution. A brown slurry was formed toward the end of the addition-period of 30 minutes. The mixture was poured over ice and the white product which separated was filtered, washed and dried to yield 4.7 g. (38%) of the α-form of thianthrene-5,10-dioxide (mixed m.p.).

Acknowledgment.—The authors wish to thank the Institute for Atomic Research, Iowa State College, for making available to us the Baird double beam infrared spectrophotometer used in the determination of the spectra of the compounds reported in this paper. We are grateful to Mr. Robert McCord for the actual determination of the spectra.

AMES, IOWA

The Hydration of the Annular Nitrogen Group as a Factor in the Combination of Hapten with Antibody[2,3]

BY DAVID PRESSMAN AND MALCOLM SIEGEL

The relative extent of combination of each of the various pyridine carboxylate and quinoline carboxylate ions with antibody specific to the o-, m- and p-azobenzoate ions was determined. In each case the ion behaved like either a benzoate or napthoate with a large steric substituent in the position occupied by the ring nitrogen. This was interpreted as being due to water of hydration which was attached to the nitrogen affecting its steric configuration, thereby altering its ability to react with a site formed against a substance of known configuration. The results obtained here indicate that water of hydration may be a structural feature of significance in biological systems in general.

Most biological reactions take place in aqueous media and the hydration of the molecules involved is an important structural feature having a role in determining binding strength and specificity. Immuno-chemical systems are very useful in demonstrating that hydration is a structural feature of considerable importance.

Evidence that the hydration of the annular nitrogen group of the pyridine or pyrazine nucleus appears to be an important factor in the combination of hapten with antibody was first observed in a study of the extent of combination of various substances with antibody homologous to the 4-azophthalate ion.[4,5] The combining power of this antibody with simple substances varied markedly in the sequence phthalate ion > pyridine-2,3-dicarboxylate ion > pyrazine-2,3-dicarboxylate ion. This order of decrease was attributed to the hydration of the annular nitrogen atom. The water of hydration attached to the hapten acts sterically to interfere with the combination of these haptens with the antibody to the 4-azophthalate ion. The interference was less with the pyridine compound, which has a single nitrogen, than with the pyrazine derivative which has two nitrogen atoms.

The role of hydration has now been studied further through an investigation of the extent of combination of antiserum specific to the o-, m- and p-azobenzoate ion groups (anti-X_o, anti-X_m, anti-X_p antibodies, respectively) with the various pyridine monocarboxylate ions, picolinate, nicotinate and isonicotinate, and with the various quinoline monocarboxylate ions. The results are reported here.

Landsteiner and van der Scheer[6] have already reported a study of combination of nicotinate and picolinate with the three azobenzoate sera, but their studies were too qualitative and restricted to permit conclusions to be drawn such as those made here.

Experimental Methods

Materials.—The quinoline–carboxylic acids, which were kindly furnished by Prof. Robert C. Elderfield, were pure samples, recrystallized to constant melting point and the correct neutral equivalent. Heterocyclic acids and other haptens were commercial preparations recrystallized to the correct melting point and neutral equivalent.

Protein Antigens.—Immunizing antigen was prepared by diazotizing 500 mg. of o-, m- and p-aminobenzoic acid and coupling each with 100 ml. of regenerated lyophilized bovine serum. (The coupling mixtures were allowed to stand overnight at 3–5° and at pH 10.5.) The azoproteins were dialyzed against many portions of saline solution containing sodium borate until the dialyzate was free of color. Phenol was added to a concentration of 0.2%, and the solutions were stored in the cold.

Test antigens were prepared by diazotizing and coupling 26 mg. of the o-, m- and p-aminobenzoic acids with 250 mg. of ovalbumin at pH 9. After standing overnight in the cold, the coupling mixtures were dialyzed against borate solution until no further passage of color through the membrane was detected. The azoproteins were precipitated twice with acid at pH 3.5 and redissolved in alkaline saline with a final adjustment to pH 7 to 8.

(1) Contribution from the Gates and Crellin Laboratories No. 2133.

(2) Presented in part before the Division of Biological Chemistry at the 115th National Meeting of the American Chemical Society, San Francisco, 1949.

(3) This research was supported at various times by a grant from the Rockefeller Foundation, the American Cancer Society (during tenure of D. P. as Senior Fellow), and the Atomic Energy Commission, Contract No. AT(30-1)-910.

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

(5) D. Pressman and M. Siegel, ibid., 75, 686 (1953).

(6) K. Landsteiner and J. van der Scheer, J. Exper. Med., 54, 295 (1931).

Table I

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. (100 μg. protein); hapten in saline, 1.00 ml. One hour at room temperature and three days at 5°.

| Hapten | K'_0 | ΔF_{rel} | σ | Hapten concn., molar × 10⁵ |
| | | | | 13 | 26 | 53 | 104 | 208 | 417 | 833 | 1670 | 3330 |
				Amount of precipitate[a]
Benzoate	1.00	0	3	860		660		450				
α-Naphthoate	0.22	840	3.5				720		580		350	
β-Naphthoate	3.1	−620	4			480		310		170		
Picolinate	0.09	1320	1.5					1030		700		
Nicotinate	.43	460	2.5			770		630		280		
Isonicotinate	1.71	−300	3.5					370		180		
Quinoline-2-carboxylate	0.28	700	4				680		510			
Quinoline-3-carboxylate	.48	400	3			750		570		320		
Quinoline-4-carboxylate	.14	1080	3				810		680		410	
Quinoline-5-carboxylate	.26	740	3				700		560		290	
Quinoline-6-carboxylate	4.2	−800	3	640		420		170				
Quinoline-7-carboxylate	3.4	−670	3.5	640		460		250				
Quinoline-8-carboxylate	0.03	1900	4						870		730	580

[a] The amount of precipitate is reported as the parts per mille of the amount of precipitate in the absence of hapten, 427 μg. All values are the averages of triplicate analyses with mean deviation of 3%.

Table II

Effect of Haptens on the Precipitation of Anti-X_m-Globulin with X_m-Ovalbumin

Anti-X_m-globulin, 0.50 ml. (11.7 mg. protein); X_m-ovalbumin in borate buffer, 0.50 ml. (111 μg. protein); hapten in saline solution, 0.50 ml. One hour at room temperature and three days at 5°

| Hapten | K'_0 | ΔF_{rel} | σ | Hapten concn., molar × 10⁵ |
| | | | | 26 | 53 | 104 | 208 | 417 | 833 | 1670 | 3330 |
				Amount of precipitate[a]
Benzoate	1.0	0	4		630		430		240		
α-Naphthoate	1.5	220	4		570		360		220		
β-Naphthoate	1.7	290	4			430		300		120	
Picolinate	0.05	650	0.5				1120		800		430
Nicotinate	.58	300	2.5		730		470		270		
Isonicotinate	.24	780	3.5				630		430		230
Quinoline-2-carboxylate	.28	700	4			640		450		390	
Quinoline-3-carboxylate	.20	880	3.5			710		590		360	
Quinoline-4-carboxylate	.31	650	3			710		480		290	
Quinoline-5-carboxylate	1.9	−350	3.5	630		400		250			
Quinoline-6-carboxylate	0.45	440	3.5		730		530		360		
Quinoline-7-carboxylate	2.8	−570	3.5	550		390		200			
Quinoline-8-carboxylate	0.55	330	3.5			580		410		230	

[a] The amount of precipitate is reported in parts per mille of the amount in the absence of hapten; 521 μg. All values are averages of triplicate analyses with mean deviation of 4%.

Table III

Effect of Haptens on the Precipitation of Anti-X_0-Globulin with X_0-Ovalbumin

Anti-X_0-globulin, 1.00 ml. (19.0 mg. protein); X_0-ovalbumin in borate buffer, 1.00 ml. (163 μg. protein); hapten in saline solution, 1.00 ml. One hour at room temperature and 3 days at 5°.

| Hapten | K'_0 | ΔF_{rel} | σ | Hapten concn., molar × 10⁵ |
| | | | | 7 | 13 | 26 | 53 | 104 | 208 | 417 | 833 | 1670 | 3330 |
				Amount of precipitate[a]
Benzoate	1.00	0	1.5				850		550		200		
α-Naphthoate	11.5	−1360	2	750		460		180					
β-Naphthoate	0.27	720	1						830		590	110	
Picolinate	.065	1510	0.5						1170		1080	630	
Nicotinate	.11	1220	2					970		810		560	
Isonicotinate	.08	1390	1.5						990		830		500
Quinoline-2-carboxylate	.17	980	4					810		630		540	
Quinoline-3-carboxylate	.10	1270	2						1070		830	570	
Quinoline-4-carboxylate	.77	140	2.5				810		600		310		
Quinoline-5-carboxylate	1.4	−180	2				720		510		160		
Quinoline-6-carboxylate	0.04	1770	4					1010	870	750			
Quinoline-7-carboxylate	.31	650	2						850	650	330		
Quinoline-8-carboxylate	9.4	−1250	4			580	430	210					

[a] The amount of precipitate is reported in parts per mille of the amount in the absence of hapten; 384 μg. All values are averages of triplicate analyses with mean deviation of 3%.

Antisera.—The antisera used were prepared and pooled by the methods described previously.[7] Each pool was fractionated with $(NH_4)_2SO_4$ (final concn. 1.75 M) and the inhibition studies carried out on the globulin fraction.

Reaction of Antiserum with Antigen and Hapten.—Equal volumes of antigen, antisera and hapten solution were mixed, incubated for 1 hr. at room temperature, then allowed to stand for 3 days at 3–5°. A concentration of antigen diluted with borate buffer[8] was used which yielded a maximum amount of precipitate. The precipitates were washed 3 times with 8 ml. of saline, dissolved in 1 M NaOH and the amount of precipitate determined by a modified Folin procedure.[9]

Hapten stock solutions were prepared by dissolving a weighed quantity of hapten in the calculated quantity of NaOH and adjusting the pH to between 7 and 9. The ionic strength was adjusted to 0.16 with NaCl. Dilutions were made with 0.16 M NaCl.

Results

Extent of Combination of Hapten with Antibody.

—The extent of combination of hapten with antibody was determined as described previously[10] by the ability of the hapten to inhibit the precipitation of the antibody by the hapten homologous ovalbumin antigen, X_o–ovalbumin, X_m–ovalbumin and X_p–ovalbumin, respectively. The amount of antigen used was that which gave the greatest amount of precipitate. The mixtures were buffered at pH 8 since the carboxylic acids were 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 the p-azobenzoate ion (anti-X_p–globulin) with the given concentration of X_p–ovalbumin are given in Table I; data for the X_m system (anti-X_m–globulin and X_m–ovalbumin) in Table II; data for the X_o system (anti-X_o–globulin and X_o–ovalbumin) in Table III. Values for the relative hapten combination constant, K'_0, and the heterogeneity index, σ, obtained on the application of the theory of heterogeneous antisera are listed.[10] There are also listed values for the relative free energy of combination of antibody with hapten, ΔF_{rel}. (*i.e.*, the value relative to that for 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.
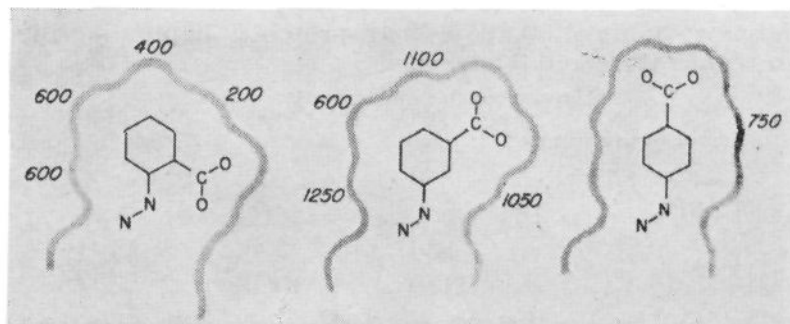

Fig. 1.—Van der Waals outline of the haptens used in the preparation of antibodies. The numbers represent the effect on ΔF_{rel} of replacing a hydrogen with a chlorine in the corresponding position.[11]

Discussion

Closeness of Fit of Antibody about Homologous Hapten Group.

—The van der Waals outline of the three haptens used for the preparation of antisera are shown in Fig. 1. The closeness of fit of antibody about the homologous hapten has been determined previously[11] for such systems by determining the extent of combination of the various chloroazobenzoate ions with these antisera. Values were obtained for the change in free energy of combination when a hydrogen was replaced by chlorine in the various positions on the hapten, and these values are shown around the van der Waals outlines of the hapten in Fig. 1. The values are positive since the chlorine interferes with the combination.

The Effect of Replacing the –CH–Group of the Ring of the Benzoate Ion with a Nitrogen Atom.

—Since the antibody appears to be formed relatively closely about the injected hapten, a large steric contribution to the van der Waals outline contributed by the presence of a single water molecule on the nitrogen atom would affect the combination of hapten with antibody. This is shown in Fig. 2 where the van der Waals outlines of the singly hydrated form of the various pyridine carboxylate ions are superimposed upon the outline of the hapten used for the preparation of the antibody.[12] The relative combining constants, K'_0, are also shown.

Where the water of hydration interferes sterically with the combination of hapten with antibody, one would expect increased free energy of combination and a decreased combining constant; and this is what is observed.

The values of K'_0 for the combination of isonicotinate, nicotinate and picolinate with anti-X_p antibody are 1.7, 0.43 and 0.09, respectively. The largest value is attained with the ring nitrogen in the *para* position; the next largest with the ring nitrogen in the *meta* position; and the lowest, with the nitrogen in the *ortho* position. This is the order previously observed in this system[5,11,13,14] (and related p-azo systems)[5] for the combination of substituted benzoates with substituents in the o-, m- and p-positions. Thus, the pyridine compounds act as though they were benzoates with a large substituent attached to the carbon in the position occupied by the annular nitrogen atom. This is in accord with the fact that the nitrogen is hydrated in aqueous solution and with the hypothesis that this water of hydration acts as a large substituent in the ring.

The values for the combination of isonicotinate, nicotinate and picolinate with anti-X_m antibody are 0.24, 0.58 and 0.05, respectively. The combining constant is greatest when the nitrogen is in the

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

(8) The borate buffer of pH 8.0 was prepared by adding 0.16 M NaOH solution to 0.2 M boric acid in 0.9% sodium chloride.

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

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

(11) D. Pressman, M. Siegel and L. A. R. Hall, *ibid.*, **76**, 6336 (1954).

(12) In the figures showing van der Waals outlines, the outlines have been drawn in accordance with van der Waals radii. Where a hydrated form of a nitrogen compound is drawn, the position of the water is shown as coplanar with the pyridine ring and is entirely schematic since one or more waters may be present and they may be oriented in directions other than that indicated.

(13) D. Pressman, S. M. Swingle, A. L. Grossberg and L. Pauling, THIS JOURNAL, **66**, 1731 (1944).

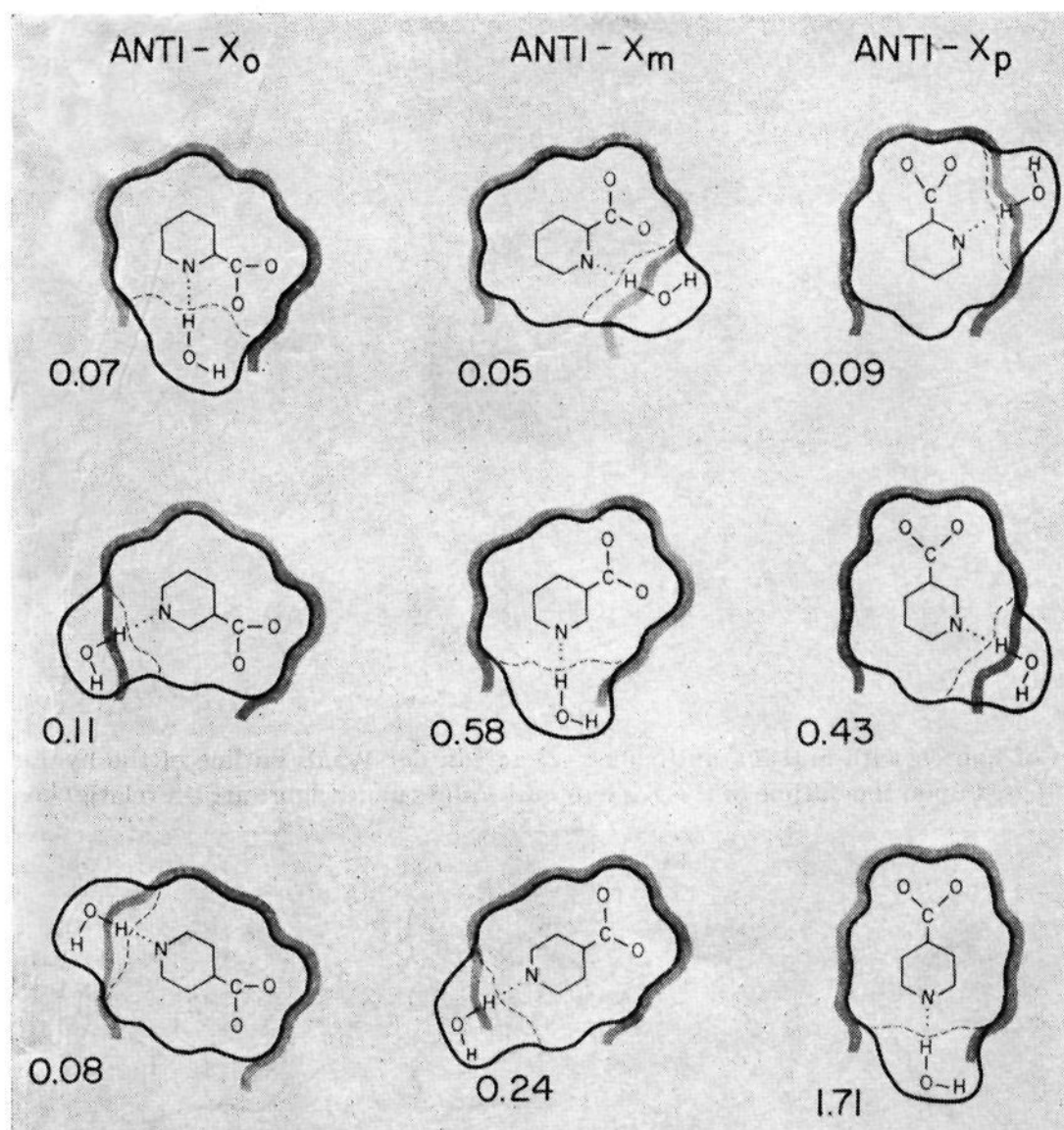(14) D. Pressman and M. Siegel, *Archives Biochem. Biophys.*, **45**, 41 (1953).

Fig. 2.—Van der Waals outline of the hydrated form of pyridine carboxylate ion superimposed upon the outline of the hapten. The number under each figure is the relative combining constant, K_0'.
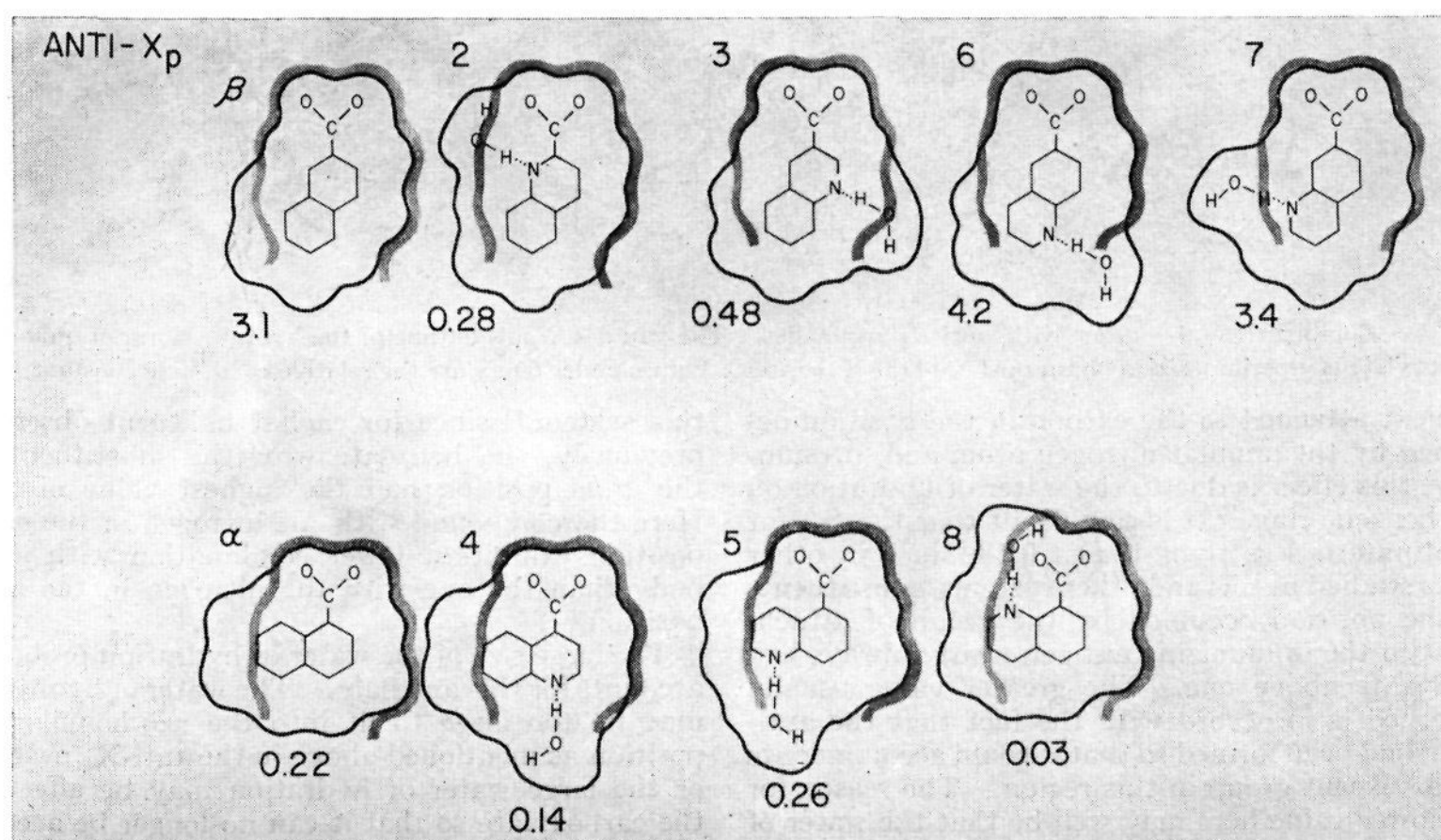

Fig. 3.—Combination of hapten with anti-X_p antibodies. The van der Waals outline of the hydrated form of quinoline carboxylate is superimposed upon the outline of the X_p hapten. Values under figure are the relative combining constant, K_0'.

meta position corresponding to the position of attachment of the benzoate in the injected antigen and is less when the nitrogen is in the *ortho* and *para* positions. This same order has been observed for several substituted haptens in this and related systems.[5,11]

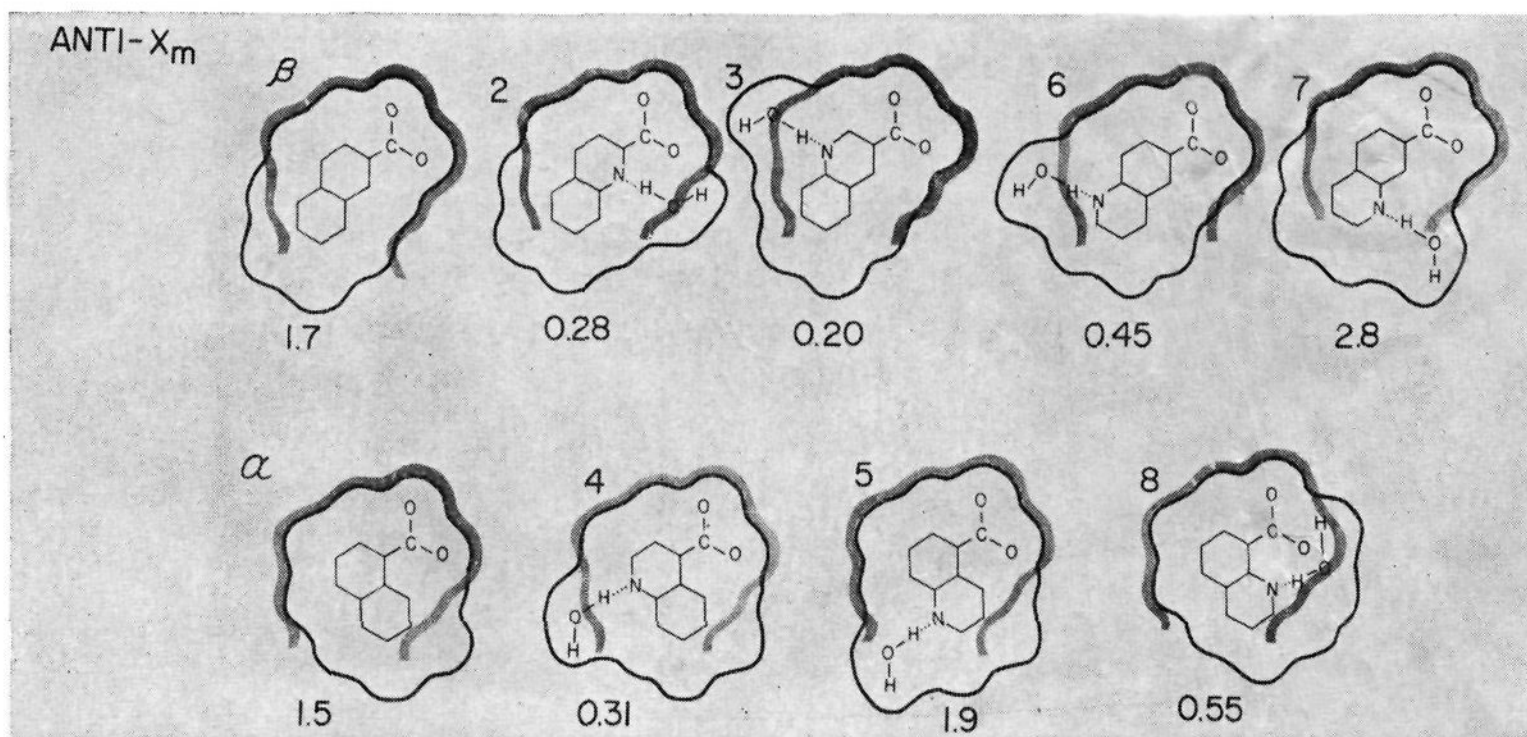In this system, again the pyridine compounds act as though they were benzoates with a large sub-

Fig. 4.—Combination of hapten with anti-X_m antibodies. The van der Waals outline of the hydrated form of quinoline carboxylate is superimposed upon the outline of the X_m hapten. Values under figure are the relative combining constant, K_0'.
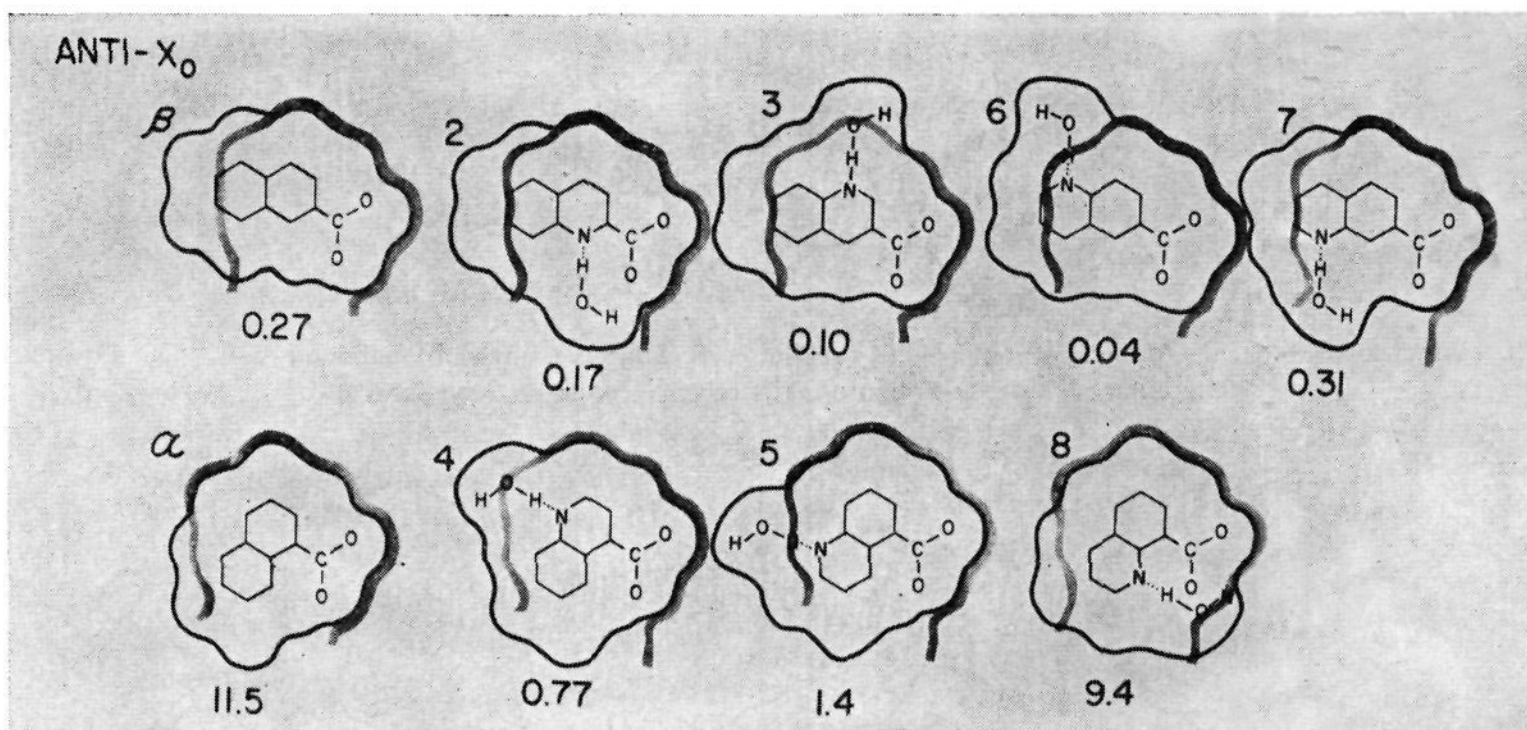

Fig. 5.—Combination of hapten with anti-X_o antibodies. The van der Waals outline of the hydrated form of quinoline carboxylate is superimposed upon the outline of the X_o hapten. Values under figure are the relative combining constant, K_0'.

stituent attached to the carbon in the position occupied by the annular nitrogen atom and, presumably, this effect is due to the water of hydration on the benzene ring. It is significant that the K'_0 for nicotinate is less than 1, i.e., 0.58, since in other cases studied in this and other systems, substituents in the position occupied by the group of attachment in the immunizing antigen nearly always had constants above one. The greater value usually observed is in accord with the fact that the antibody had been formed so that it could accommodate a substituent group in this region. The reason for the lower value here may well be that the water of hydration may be so large that it cannot even fit into the region of the antibody formed against the azo group.

In the anti-X_o system, the K'_0 values for isonicotinate, nicotinate and picolinate are, respectively, 0.08, 0.11 and 0.07. This system does not follow what was observed with substituted benzoates in

this system,[11] since for each substituent observed previously, the benzoate with the substituent in the *ortho* position had the highest value of K'_0. Here the compound with the nitrogen in the *ortho* position exhibits a lower combination with antibody than the one with the nitrogen in the *meta* position.

The large size of the water of hydration probably accounts for this anomaly. The water of hydration may be too large to fit into the azo-homologous position as mentioned above in the anti-X_m system, or the large water of hydration may be affecting the carboxylate so that it can no longer be accommodated by the X_o antibody.

The Effect of Replacing an α-CH-group of Naphthoate Ion with a Nitrogen Atom.—The extent of combination of the quinoline carboxylate ions is best discussed relative to the extent of combination of the related α- or β-naphthoate ions. Thus, the quinoline 2-, 3-, 6- and 7-carboxylate

ions may be compared with β-naphthoate ion and the quinoline 4-, 5- and 8-carboxylates may be compared with the α-naphthoate ion. α-Naphthoate itself, acts as a 2,3-disubstituted benzoate and β-naphthoate acts as a 3,4-disubstituted benzoate. The quinolines act as though there were also a large substituent (presumably water) at the position occupied by the nitrogen as already described for the pyridine acids.

As an aid in discussing combination of antibody with hapten, there are shown in Figs. 3, 4, 5, the van der Waals outline of the α-naphthoate structure, the β-naphthoate structure and the quinoline carboxylates superimposed on the van der Waals outline of the p-, m- and o-azobenzoates. The number on the upper left corner of each figure indicates the position of the nitrogen atom relative to the carboxylate as well as the position of the carboxylate group relative to the nitrogen. The pertinent data are listed along the side of each figure.

In the X_p system, K'_0 for the combination of anti-X_p antibody with the β-naphthoate ion is 3.1. When the nitrogen is in the indicated 2- or 3-position, there is a six to tenfold decrease in combining power which is in accord with the presence of large steric substituents in these positions on the benzene ring. Moreover, picolinate is known to have a very low combining constant in the X_p system, and this is reflected in the 2-quinolinate constant. 3-Quinolinate is a 3,4,5-substituted benzoate, and it is known that 3,5-disubstituted benzoates combine very poorly with anti-X_p antibody.[11]

Nitrogen in the 6- or 7-position does not hinder combination but actually increases combining power slightly, which is to be expected, especially in position 6, in view of the ability of anti-X_p antibody to accommodate large substituents in general in the *para* position and some (iodine and bromine) even in the *meta* position corresponding to position 7.

The second group of haptens consists of those related to α-naphthoate ion. α-Naphthoate itself has a K'_0 value of 0.22, attributable to the size and position of the second ring. Insertion of the nitrogen atom in the 8-position causes a very large decrease in K'_0 as would be expected from insertion of a large substituent in a position around which the antibody would fit closely. Insertion of the nitrogen in the 4-position decreases K'_0 while insertion into the 5-position increases the constant slightly. Although one might expect that the hydrated nitrogen in the 4-position could be accommodated best, the experiment shows that the 5-nitrogen is accommodated best. A reasonable explanation is the following. When the α-naphthoate ion combines with the X_p antibody, the naphthoate is tilted somewhat in order for the second ring to be accommodated (Fig. 6). This tilt and resultant rotation would place the 5-nitrogen in a less hindered position.

In the combination with anti-X_m antibodies, β-naphthoate has a K'_0 value of 1.7 and replacement of the –CH– in the 7-position by hydrated nitrogen increases the constant to 2.8. From Fig. 4, it can be seen that a large substituent in this position can be accommodated. The increase is probably due

to increased van der Waals interaction of the water with the antibody. Placement of the nitrogen in the other positions, 2, 3 and 6, causes a decrease in combining constant as would be expected on the basis of the results obtained with the chlorosubstituted benzoates.[11] Again, with the α-naphthoate structures, it can be seen that a large substituent can be accommodated in position 5 most readily and the quinoline-5-carboxylate does have the highest constant in this group of quinoline carboxylates. The presence of a large substituent in the 4 or 8-positions caused decreased combination as would be expected on the basis of close fit in these positions and the assumption of hydration.

In the combination with anti-X_o antibodies, the β-naphthoate structure itself is not well accommodated and has a K'_0 value of 0.27. Replacement of the –CH– by the hydrated nitrogen atom in the 2-, 3- and 6-position, lowers the combining constant by a factor of 1.5 or more. The low value of the quinoline-2-carboxylate is in line with the anomalously low constant of the related picolinate ion.

Presence of the nitrogen in the 7-position, $K'_0 = 0.31$, increased the constant somewhat over that of the parent β-naphthoate. This is what would be expected for a large substituent here since the β-naphthoate is probably tilted somewhat already (Fig. 6) in order to be accommodated by the anti-X_o
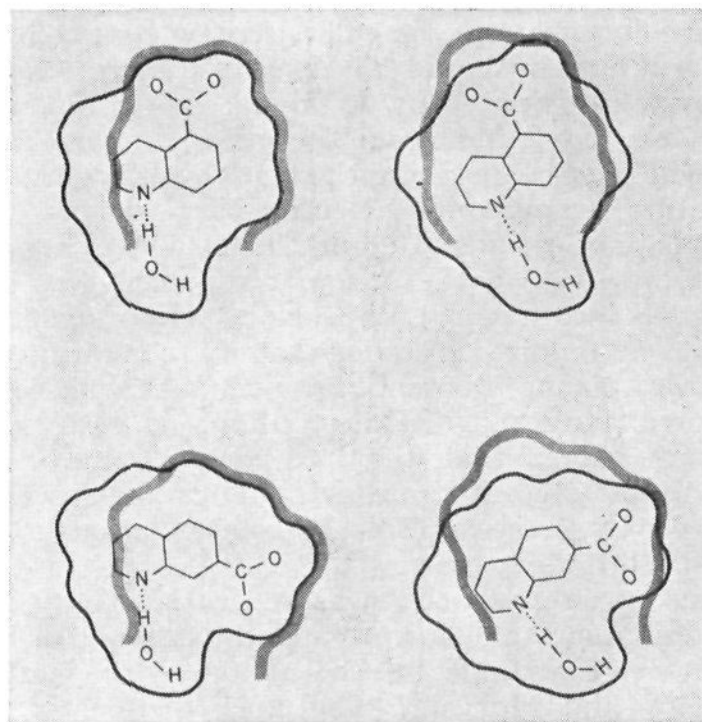

Fig. 6.—Diagrams indicating schematically the better accommodation of the 5-carboxyquinoline by the anti-X_p antibody and of the 7-carboxyquinoline by the anti-X_o antibody when the haptens are tilted.

site. This tilt would bring a large substituent into the region where it can be accommodated by the antibody and the increased van der Waals attraction for the hydrated nitrogen could come into play.

In the combination of the α-naphthoate group of haptens with anti-X_o antibody, α-naphthoate itself shows a high constant 11.5. The nitrogen in the 8-position is easily accommodated, $K'_0 = 9.4$, and as seen from the drawing, a large substituent in this position could be accommodated. However, hydrated nitrogen atoms in the 4- and 5-

position would not be accommodated easily, and that is actually what is observed.

The Role of Water of Hydration of the Annular Nitrogen Group.—In the work reported here and previously, it is apparent that the ring nitrogen acts as though it were a large substituent on an aromatic ring. This effect is attributed to the presence of water of hydration which modifies the steric configuration of the hapten and affects its combination with antibodies.

That pyridine is hydrated in aqueous solution is apparent from the fact that it is miscible with water, whereas benzene is only slightly soluble. Moreover, the heat of hydration of pyridine has been measured by Briegleb[15] and has been found to be 12 kcal. per mole.

(15) G. Briegleb, *Z. Elektrochem.*, **53**, 350 (1949).

Carsten and Eisen[16] reported the heat of reaction of a hapten (ε-DNP-lysine) with its specific antibody to be about 1 kcal. Since the heat of hydration of pyridine is much greater than this value, further evidence points to the fact that combination of the pyridine and quinoline carboxylates with antibody, in the systems studied here, takes place with the water of hydration still attached to the nitrogen atom. Otherwise, the combination would be very weak if energy were required to break the water away first.

For the same reasons, biological reactions in general involving similar annular nitrogen compounds may specifically require the hydrated form for combination to take place.

(16) M. Carsten and H. Eisen, This Journal, **77**, 1273 (1955).

BUFFALO 3, NEW YORK

COMMUNICATIONS TO THE EDITOR

ORGANIC FREE RADICALS IN THE SOLID STATE[1,2]

Sir:

Solid solutions of triphenylmethyl in fluorene, phenanthrene, and triphenylamine, respectively, of unknown composition have been prepared. The solid solutions were the characteristic corn color of the solutions in liquid benzene, in all three cases. They were prepared by dissolving chosen amounts of hexaphenylethane and the other component in benzene and evaporating the benzene at room temperature in a pure nitrogen atmosphere in a dry box.

It is to be assumed that all the hexaphenylethane that dissolved in the second component was dissociated into free radicals since a planar structure would be required in order that the crystal lattice of the planar fluorene or phenanthrene could accommodate it. Triphenylamine is also sufficiently planar that the hexaphenylethane could hardly dissolve in it as such. Thus the susceptibility gave a measure of the concentration in the solid solution.

Susceptibilities were measured with a Gouy balance at room temperature and the amount of free radical in the sample tube calculated by the method of Roy and Marvel.[3] The mole fractions of triphenylmethyl in solid solution in fluorene, triphenylamine, and phenanthrene were found to be approximately 0.24, 0.22, and 0.17, respectively, assuming complete dissociation in the solid solution. If all the hexaphenylethane were in solid solution, the dissociation in each case would be 17, 14, and 10%, respectively.

In all cases the resultant diamagnetic susceptibility was somewhat less than half that calculated if there were no dissociation to free radicals. If all samples obeyed the Curie–Weiss law, the same

(1) Publication No. 97 of the Cryogenic Laboratory of the College of Chemistry and Physics, The Pennsylvania State University, University Park, State College, Pennsylvania.

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(3) M. L. Roy and C. S. Marvel, *J. Am. Chem. Soc.*, **69**, 2622 (1937).

amount of free radical would correspond to a considerable paramagnetism at helium temperatures.

The susceptibility was therefore studied by an inductance method down to 1.6°K.; the method was sufficiently sensitive to detect one per cent. of the hexaphenylethane present if it were in the form of free radical active as a perfect paramagnetic substance (due to spin only). No paramagnetism was detected.

However, it was found that under similar conditions hexabiphenylethane, which is completely dissociated into free radicals in the solid at room temperature also gave no paramagnetism. Hexabiphenylethane roughly follows the Curie–Weiss law down to liquid nitrogen temperatures.[4] We found that the susceptibility measured by the inductance method was also essentially zero at 20°K. The "paramagnetism" measured in the Gouy balance in this laboratory at room temperature corresponded to complete dissociation. The solid solutions are being investigated spectroscopically and the transition below 70°K. responsible for loss of paramagnetism of tribiphenylmethyl is being investigated.

We wish to thank Dr. C. Haas for the opportunity to use his Gouy balance.

(4) E. Müller, I. Müller and W. Bunge, *Ann.*, **520**, 235 (1935).

(5) On leave from the University of Osaka, Japan.

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THE BIOSYNTHETIC PRECURSOR OF THE EXTRA CARBON ATOM IN THE SIDE-CHAIN OF STEROIDS[1]

Sir:

The concept of the biosynthesis of steroids from acetate through squalene accounts for the source of all the carbon atoms of C_{27} and C_{30} compounds

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