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The Reactions of Antiserum Homologous to the 4-Azophthalate Ion[1,2]

BY DAVID PRESSMAN[3] AND LINUS PAULING

In connection with our studies[4] on the nature of the forces operating in the combination of haptens with hapten-homologous antisera it became apparent that a study should be made of an antiserum prepared against a haptenic group containing two or more discrete negative charges, in order to determine the difference in combining power with this antibody of substances containing a single negative charge and substances containing two charges. Great specificity for the charged group has been observed for singly charged haptenic groups[5,6,7,8]: thus benzenesulfonate ion and benzenearsonate ion were found not to combine with antibodies homologous to the benzoate ion group, and benzenesulfonate ion and benzoate ion were found not to combine with antibodies to the benzenearsonate ion group; but when the ions are very closely related, as are benzenephosphonate ion and benzenearsonate ion, there is appreciable cross reaction.[5,9] We were interested to discover whether antibodies homologous to haptenic groups containing two charges would show greater or less specificity than was shown by these antibodies.

Some work was done by Landsteiner and van der Scheer[10] on antibodies against complex haptenic groups containing two negative charges widely separated on the molecule. They found that individual antibody molecules seemed to be formed against the charged groups separately.

For our present study we have chosen antibodies homologous to the 4-azophthalate ion (anti-X_{mp} serum), prepared by inoculating rabbits with sheep serum coupled with diazotized 4-aminophthalic acid (X_{mp} sheep serum). Quantitative studies were made of the effect of phthalate ion containing various substituents and of other ions containing one and two negatively charged groups on the precipitation of X_{mp}-ovalbumin by anti-X_{mp} serum, and inhibition constants for the haptens were calculated from the data. It was found that two negatively charged groups (not necessarily both carboxylate ion groups) must be present in positions ortho to each other on the benzene ring in order to produce significant combination of the hapten with anti-X_{mp} antibody. The antibody was found to combine only very weakly, under the conditions of this experiment, with any hapten carrying a single negative charge. The specificity of the antiserum was found to be less than that of anti-benzoic acid serum.

Experimental Methods

Protein Antigens.—The immunizing antigen used for inoculating the rabbits was prepared by diazotizing 1.5 g. of 4-aminophthalic acid and coupling it with 150 ml. of sheep serum at *p*H about 9. After coupling was complete the mixture was dialyzed overnight against running tap water, and then the azoprotein was precipitated at *p*H 3.5 by the addition of hydrochloric acid. The mixture was centrifuged and the precipitated protein was redissolved by the addition of 1 *M* sodium hydroxide solution to a final *p*H of 7. The test antigen was prepared by diazotizing 0.2 g. of 4-aminophthalic acid by the inverted method using excess sodium nitrite. The excess sodium nitrite was decomposed by sulfamic acid and the mixture was added to 1 g. of crystallized ovalbumin with sufficient sodium carbonate to keep the *p*H about 9. The mixture was allowed to stand overnight to permit the completion of the reaction (ovalbumin does not couple so rapidly with the reagent as do the serum proteins). The solution was then dialyzed against tap water, and the product was purified by precipitating from 50 ml. of solution at *p*H 3, followed by solution of the precipitated protein in saline at *p*H 7 by the addition of 1 *M* sodium hydroxide solution, and repetition of the precipitation and re-solution. The protein concentration of the antigen was determined by digesting with sulfuric acid and determining the ammonia formed with Nessler reagent.

Preparation of Antisera.—Antisera were obtained and pooled in a manner similar to that described for the preparation of anti-R_p-sera.[11] One pool of antiserum was used throughout the experiment.

Simple Haptens.—The haptens used were the commercially available products which had been recrystallized to the correct melting point and acidic equivalent weight, except for 4-aminophthalic acid, which was prepared by Dr. William B. Renfrow, Jr., and Dr. A. Recsei by the reduction of 4-nitrophthalic acid with hydrogen in the presence of a catalyst; both palladium and platinum were satisfactory catalysts.

The Reaction of Antiserum with Antigen and Hapten.—The reactants were mixed and permitted to stand for one hour at room temperature and forty hours at 5°. The precipitates were centrifuged, washed 3 times with 10-ml. portions of 0.16 *M* sodium chloride solution,[12] and

(1) The Serological Properties of Simple Substances. XV. For Nos. XIV and XIII of this series see (a) A. B. Pardee and L. Pauling, THIS JOURNAL, **71**, 143 (1949), and (b) D. Pressman, J. H. Bryden and L. Pauling, *ibid.*, **70**, 1352 (1948).

(2) Presented before the Division of Biological Chemistry of the American Chemical Society at the Atlantic City Meeting, April, 1947.

(3) Present address: Sloan-Kettering Institute for Cancer Research, New York, N. Y.

(4) See ref. 1 and earlier papers.

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

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

(7) L. Pauling, D. Pressman, and D. H. Campbell, *ibid.*, **66**, 330 (1944).

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

(9) H. Erlenmeyer and E. Berger, *Biochem. Z.*, **255**, 429 (1932).

(10) K. Landsteiner and J. van der Scheer, *J. Exptl. Med.*, **67**, 709 (1938).

(11) L. Pauling, D. Pressman, D. H. Campbell, C. Ikeda, and M. Ikawa, THIS JOURNAL, **64**, 2294 (1942).

(12) In earlier work (L. Pauling, D. Pressman, D. H. Campbell, and C. Ikeda, *ibid.*, **64**, 3003 (1942)) we found with a similar system that eight or ten extra washings with 10-ml. portions of solution decreased the amount of precipitate by only 5 to 15%. Similar results have been obtained also in a recent investigation, to be described in a separate note.

analyzed by our standard method.[13] The antiserum and solutions of antigen and hapten were brought to the desired *p*H values by the addition of hydrochloric acid or sodium hydroxide, and dilutions of the antigen were made with borate buffer with the same *p*H. Dilutions of the haptens were made with 0.16 *M* sodium chloride solution. The borate buffers were made by adding 0.16 *M* sodium hydroxide solution to a solution 0.2 *M* in boric acid and 0.16 *M* in sodium chloride.

Discussion

The Specificity of the Reaction.—The properties of the system X_{mp}-ovalbumin:anti-X_{mp} serum were found to be similar to those of the other azoprotein:hapten-homologous serum systems that we have studied. As usual, a maximum in the amount of precipitate was found at a particular concentration of the azoprotein antigen, the decrease with further increase in the amount of antigen being attributed to the formation of soluble complexes. The antigen X_{mp}-ovalbumin was found not to form a precipitate with normal rabbit serum; cross-reactivity with other antisera was not tested. The specificity of the antiserum to the homologous haptenic group is indicated by its strong combination with various haptens (such as the tetrachlorophthalate ion) which have been found to have practically no combining power with other antisera.[6]

The Effect of Hydrogen-ion Concentration on the Precipitation Reaction.—The effect of hydrogen-ion concentration on the precipitation of anti-X_{mp} serum with X_{mp}-ovalbumin is shown in Table I. Optimum precipitation takes place in the same *p*H range, between 7.4 and 8.1, that has been observed for azoprotein systems involving other negatively charged haptenic groups.[1,6] There was observed a shift with *p*H in the amount of antigen producing optimum precipitation, as was observed previously with other systems.[1,6] The shift is such that less antigen is required at higher *p*H's. At *p*H 8 the optimum amount of antigen was about 230 micrograms; this amount was used in the hapten-inhibition experiments reported in Table II.

TABLE I

EFFECT OF HYDROGEN-ION CONCENTRATION ON THE PRECIPITATION OF ANTI-X_{mp} SERUM AND X_{mp}-OVALBUMIN

Antigen solution, 1.0 ml.; antiserum, 1 ml.; buffer, 1 ml. Antiserum and antigen solution were adjusted to the *p*H indicated before mixing.

		Amount of antigen added, μg.			
Initial *p*H	*p*H of supernate	26	77	230	700
		Amount of protein precipitated, μg.[a]			
6.0	6.5	69	309	427	461
7.0	7.4	73	600	866	622
8.0	8.1	95	597	896	464
9.0	8.8	(10)	401	627	(244)

[a] Averages of triplicate analyses, with mean deviation ±4%; averages of duplicate analyses in parentheses. Blank of serum and buffer, 17 μg. at *p*H 6, 11 μg. at *p*H 7, 12 μg. at *p*H 8, and 18 μg. at *p*H 9.

Inhibition of Precipitation by Haptens.—Data on hapten inhibition are given in Table II. Values of the hapten inhibition constant K_0' and the heterogeneity index σ obtained on application of the theory of heterogeneous antisera[14] are also listed. The haptens were run in three different groups, labelled Series A, B, and C in Table II. These haptens were found to have no effect on the amount of precipitate formed when 1 ml. of anti-ovalbumin serum, 1 ml. of ovalbumin at the optimum concentration, and 1 ml. of 0.01 *M* hapten solution were mixed.

The Effect of Substituents in the Benzene Ring on the Interaction of Phthalate Ion with Antibody.—In Fig. 1 there are reproduced scale drawings showing the van der Waals outline of the 4-azophthalate group, the phthalate ion, several substituted phthalate ions, and other ions used in these experiments. The drawings have been made with use of the usual covalent radii and van der Waals radii.

The introduction of a nitro group into the phthalate ion in the 4 position increases the combining constant by only 15%. This effect (factor 1.15) is very small, smaller even than the effect (factor 1.65) of introducing a nitro group in the para position of succinanilate ion on the combination with anti-S_p serum[1] (serum homologous to the *p*-azosuccinanilate ion group), and very much less than the effects (factors 4, 11, and 12, respectively) observed with anti-R_p' serum[14] (serum homologous to the *p*-(*p*-azobenzeneazo)-benzenearsonate ion group), anti-R_p serum[14] (serum homologous to the *p*-azobenzenearsonate ion group), and anti-X_p serum[6] (serum homologous to the *p*-azobenzoate ion group). The small effect with anti-X_{mp} serum shows that there is a very pronounced looseness of fit of the antibody around the region on the opposite side of the phthalate ion from the charged groups.

Even the insertion of a nitro group in the 3 position of the phthalate ion causes a slight increase over the combining constant of the unsubstituted ion rather than the decrease that might be expected to result from steric hindrance. This indicates also some looseness of fit of antibody around the position adjacent to at least one of the carboxylate groups of the phthalate ion. The existence of this looseness of fit is further borne out by the fact that the replacement of the four hydrogen atoms of the ring by four chlorine atoms, to give the tetrachlorophthalate ion, increases the constant by 50%; it might be expected that the steric effect of forcing the four large chlorine atoms into the region of the antibody fitted to the ring hydrogen atoms would appreciably decrease the combining power of the hapten and antibody, but it is apparent that instead the effect of the greater van der Waals attraction of the chlorine atoms than of the replaced hydrogen atoms is more important than the steric effect. In the case of the tetrabromo-

(13) D. Pressman, *Ind. Eng. Chem., Anal. Ed.*, **15**, 357 (1943).

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

TABLE II

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

Antigen solution in buffer of *p*H 8, 1.0 ml. (230 μg.); antiserum, 1.0 ml.; hapten solution, 1.0 ml. Series A, B, and C were run on consecutive days.

Hapten (ion)	K_0'	σ	Moles of hapten added × 10^8: 31.3	62.5	125	250	500	1000
			Amount of precipitate[a]					
			Series A					
Phalate	1.00	2	820		530		250	
4-Nitrophthalate	1.15	1.5	850		530		160	
3-Nitrophthalate	1.04	3.0	740		530		300	
Tetrachlorophthalate	1.51	1.5	750		(440)		(180)	
Tetrabromophthalate	0.97	2	923		(530)		210	
Tetraiodophthalate	.29	2		940		640		380
Pyridine-2,3-dicarboxylate	.36	2				610	470	310
Pyrazine-2,3-dicarboxylate	.05	2					(850)	780
o-Sulfobenzoate	.69	2.5	850		590		360	
			Series B					
Phthalate	1.00	2.5	750		490		210	
1,8-Naphthalate	0.22	2.5	1000		790		530	
Benzoate	.01						970	880
o-Chlorobenzoate	.01						940	920
o-Bromobenzoate	.01						920	910
o-Iodobenzoate	.02						920	860
o-Aminobenzoate	.00						980	990
o-Acetaminobenzoate	.005						950	940
o-Methoxybenzoate	.01						970	880
o-Hydroxybenzoate	.005						960	950
o-Nitrobenzoate	.01						960	880
o-Benzoylbenzoate	.01						960	870
			Series C					
Phthalate	1.00	2.5			490		220	
Benzoate							970	980
o-Nitrobenzoate							950	920
m-Nitrobenzoate							970	970
p-Nitrobenzoate							1050	960
2,4-Dinitrobenzoate							1020	960
2,5-Dinitrobenzoate							1010	980
Maleate								970
Fumarate								1000
Succinate								1000

[a] The amounts of precipitate are in parts per mille of the amounts in absence of hapten, 849, 869, and 832 for series A, B, and C, respectively. Blanks of serum and buffer 15 μg., 13 μg., and 18 μg., respectively. *p*H of supernates, 8.0. Values are averages of triplicate analyses, with mean deviation ±4%; averages of duplicate analyses in parentheses.

phthalate ion the increased van der Waals attractive force is just counterbalanced by the steric effect of the still larger bromine atoms, whereas the very large size of the iodine atoms in the tetraiodophthalate ion causes the steric effect to overcome the still greater van der Waals attraction of these more polarizable groups, the inhibition constant being only 29% of that for the unsubstituted phthalate ion. The relative sizes of the tetrachloro-, tetrabromo-, and tetraiodophthalate ions can be seen from Fig. 1. An increase in diameter of the hapten by 2.0 Å. (from the phthalate ion to the tetraiodophthalate ion) causes the hapten inhibition constant in this system to decrease to one-third its value, whereas the same decrease in the *p*-azobenzoate system is caused by an increase in diameter of only 0.8 Å. (the introduction of a single bromine atom in the 3 position of benzoic acid). These numbers may be taken as representing the average closeness of fit of the antibody to the haptenic group in the two systems.

The Effect of Charge on the Combination of Antibody and Hapten.—The antibody to the phthalate ion does not appear to combine with the singly charged benzoate ion, nor does there appear to be any combination with the *o*-nitrobenzoate ion, which has essentially the same steric configuration and size (Fig. 1) as the phthalate ion but has only one charge. In Table II there are also listed several other ortho-substituted benzoate ions, none of which combined strongly with anti-X_{mp} serum. In the 2,4-dinitrobenzoate and 2,5-dinitrobenzoate ions the nitro

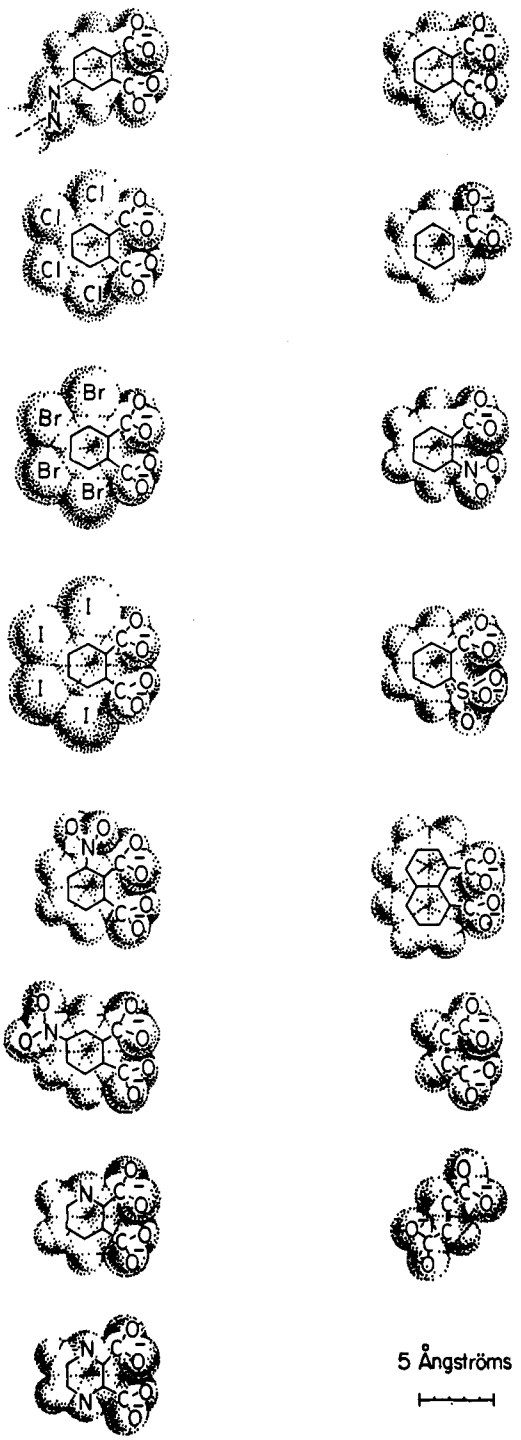

Fig. 1.—Van der Waals scale drawings of (first column) the 4-azophthalate ion group, tetrachlorophthalate ion, tetrabromophthalate ion, tetraiodophthalate ion, 3-nitrophthalate ion, 4-nitrophthalate ion, pyridine-2,3-dicarboxylate ion, and pyrazine-2,3-dicarboxylate ion, and (second column) the phthalate ion, benzoate ion, *o*-nitrobenzoate ion, *o*-sulfobenzoate ion, 1,8-naphthalate ion, maleate ion, and fumarate ion.

group in the 4 or 5 position presents the possibility of fitting into the homologous azo position on the antibody and directing the carboxylate ion into the position para to the azo position or meta to the azo position, letting the other nitro group occupy the remaining position specific to the second carboxyl group of the phthalate ion. However, the *o*-sulfobenzoate ion combines almost as strongly with anti-X_{mp} serum as does the homologous phthalate ion. This observation shows that although two negative charges are essential for the effective combination of a hapten with anti-X_{mp} serum there is very little specificity for the structure of the second charged group, a sulfonate ion group being almost as effective as a carboxylate ion group. The 1,8-naphthalate ion also combines appreciably with anti-X_{mp} serum, giving $K_0' = 0.22$. The fit of this hapten into the antibody cavity cannot be very good, for naphthalene is much larger than benzene and also the carboxylate ions do not occupy the same relative positions in the napthalate ion as when they are ortho to each on the benzene ring (Fig. 1). These results also indicate a considerable looseness of fit between antibody and hapten, the effective diameter of the combining-region cavity of the antibody being at least 1.5 Å. greater than the diameter of the homologous haptenic group (the diameter of 1,8-naphthalate ion parallel to a line connecting the two carboxyl groups is 8.8 Å., and that of the phthalate ion is 7.3 Å.).

Maleate, fumarate, and succinate ions were also studied and were found not to combine with anti-X_{mp} serum. These ions are similar to the phthalate ion in that they are composed of two carboxylate ion groups separated by two carbon atoms. Fumarate ion was not expected to combine with the antibody, since in it the carboxylate ion groups are in the *trans* configuration, nor was succinate ion expected to combine, since it also appears to have mainly a *trans* configuration.[1b] However, the maleate ion has a configuration similar to that of the phthalate ion. It would be expected to combine less strongly with the antibody than the phthalate ion because of its smaller power of van der Waals attraction. Application of Equation 2 of the following section, with the difference in mole refraction of phthalate ion and maleate ion taken as 15.5, leads to a predicted value 0.06 for K_0' for the maleate ion. The experimental data indicate a considerably smaller value than this, which suggests that there is a larger difference in structure of the maleate ion and the phthalate ion in aqueous solution than has been suspected.

A Quantitative Discussion.—It is of interest to attempt to develop a rough quantitative treatment of the various intermolecular interactions involved in combination of the antibody with the haptenic group.

The energy of van der Waals electronic attraction between a group in the para position of a

hapten and the closely surrounding portion of the antibody has been represented by the equation[15]

$$\Delta W_A = -\frac{400{,}000}{r_{AB}^6} R_A \qquad (1)$$

This equation is derived from the equation of London[16] by use of 14 e. v. for the average excitation energy, −40% as the correction for the repulsive potential, and 17.6 cm.^{3} as the difference in mole refraction of the atoms of antibody interacting with the group on the hapten and the molecules of water replaced by the antibody (the assumption being made that eight molecules of water are in contact with the group in aqueous solution, and the same volume of the antibody molecule when the hapten is combined with it). To get an idea of the order of magnitude of the electronic van der Waals energy we may replace r_{AB} by an average value,[15] 4 Å., and assume that any hapten or portion of hapten for which it is desired to make the calculations is interacting with the amount of antibody material described above. The equation then becomes

$$\Delta W_A = -100 R_A \qquad (2)$$

Here ΔW_A is given in cal. mole^{-1} when R_A is the mole refraction of the hapten or haptenic group in cm.^{3}.

Application of this equation may be illustrated by the comparison of the phenyltrimethylammonium ion and the tetramethylammonium ion, in combining with antiserum homologous to the *p*-azobenzenetrimethylammonium ion group.[17] There should not be any steric effect in this case, and the change in hapten inhibition constant should be due entirely to the difference in mole refraction. This difference amounts to 19.0 cm.^{3} (the value of R for benzene and methane being 25.8 and 6.8, respectively), and the corresponding energy difference of 1900 cal. mole^{-1} leads to a ratio of hapten inhibition constants of 1:0.033. This ratio agrees satisfactorily with the observed values, which are 1:0.061, 0.052, 0.043, and 0.023, in separate experiments. A similar example is provided by succinanilate ion and N-methylsuccinamate ion, in combining with anti-*p*-azosuccinanilate serum, their ratio of values of K_0' being 1:0.053.

It seems likely that the poorness of the fit of the anti-X_{mp} antibodies would cause the van der Waals attraction to be less than indicated by this equation. Thus the difference in mole refraction of a nitro group and a hydrogen atom, 6.6, corresponds from Equation 2 to an increase in hapten inhibition constant by the factor 3.3. An increase of this magnitude is indeed observed with other systems, as mentioned above, but the factor is only 1.15 for the present system (4-nitrophthalate ion, Table II). Application of Equation 1 with R_A replaced by 6.6 cm.^{3} and W_A equated to the standard free-energy change RT ln K_0' leads to r_{AB} = 3.5 Å. for the *p*-azobenzoate system, with K_0' for *p*-nitrobenzoate ion equal to 12 (relative to 1 for benzoate ion), 3.6 Å. for the *p*-azobenzenearsonate system (K_0' = 11), 3.9 Å. for the *p*-(*p*-azobenzeneazo)-benzenearsonate system (K_0' = 4), 4.6 Å. for the *p*-azosuccinanilate system (K_0' = 1.65), and 5.9 Å. for the 4-azophthalate system (K_0' = 1.15). It has been previously suggested that the average effective radius of the surface atoms of the combining region of the antibody can be taken as about 2.0 Å. If we add to this the van der Waals radius 1.6 Å. of the atoms in the nitro group we obtain the predicted value 3.6 Å. for r_{AB} in case that the antibody fits tightly around the group. It is seen that this case is apparently realized in the *p*-azobenzoate and *p*-azobenzenearsonate systems, and we conclude that in these systems there is complete complementariness in structure (to within the uncertainty of the foregoing calculations, about 0.3 Å.) between the antibody and the haptenic group in the region of the *para* position of the benzene ring. The radial dilatation indicated for this region of the other types of antibody is 0.3 Å. for the *p*-(*p*-azobenzeneazo)-benzenearsonate system, 1.0 Å. for the *p*-azosuccinanilate system, and 2.3 Å. for the 4-azophthalate system.

Evidence of Hydration of Heterocyclic Rings.—For the pyridine-2,3-dicarboxylate ion, which differs from the phthalate ion in having a nitrogen atom in place of one of the C–H groups in the benzene ring adjacent to a carboxylate ion, the hapten inhibition constant drops to the value 0.36, and a further drop, to 0.05, is shown by pyrazine-2,3-dicarboxylate ion, in which there is a similar second nitrogen atom. These successive decreases correspond to a change in free energy of combination of about 750 cal. mole^{-1} per nitrogen atom. The mole refractions of benzene, pyridine, and pyrazine are 25.8, 24.0, and 22.6 cm.^{3}, respectively, the differences corresponding to a van der Waals effect of only 150 cal. mole^{-1} per nitrogen atom, if the fit is close enough to permit Equation 2 to apply, or to a smaller energy value for a somewhat looser fit. We thus must attribute the major effect of the nitrogen atoms to another cause, which is without doubt their hydration in aqueous solution[15] through the formation of a hydrogen bond with use of the unshared electron pair of each ring nitrogen atom. In order for one of these heterocyclic haptens to combine with the antibody it is necessary for the water molecule attached to each nitrogen atom by a hydrogen bond to be removed, and the energy of the hydrogen bond is indicated by the numbers given above to be about 600 cal. mole^{-1} (the difference between the total effect, 750 cal. mole^{-1}, and the calculated van der Waals effect, 150 cal. mole^{-1}), or slightly larger, if the van der Waals effect is smaller than that calculated. This

(15) L. Pauling and D. Pressman, THIS JOURNAL, **67**, 1003 (1945).
(16) F. London, *Z. Physik*, **63**, 245 (1930).
(17) D. Pressman, A. L. Grossberg, L. H. Pence, and L. Pauling, THIS JOURNAL, **68**, 250 (1946).

value for the hydrogen bond energy is similar to the value, 400 to 700 cal. mole^{-1}, found for the hydrated amino group.[15]

The Effect of the Charged Groups in the Haptens.—The large difference in combining power of the haptens with two negatively charged groups and those with one negatively charged group, estimated from the data in Table II to correspond to about a factor of 100 in K_0', shows that the energy of electrostatic attraction of the negative charges of the hapten and corresponding positive charges in the antibody is very great. In the case of the positively charged hapten phenyltrimethylammonium ion and its homologous antiserum the conclusion was reached[17] that the complementary negative charge in the antibody lies about 7 Å. from the positive charge of the hapten, this distance being 2 Å. greater than the minimum possible distance of approach of these two charges. The effect of the electrostatic attraction in this case corresponds to a ratio of 15.5 of the inhibition constants for charged and uncharged haptens. A factor as great as 100 resulting from the removal of one of the two negative charges of the phthalate ion (replacement of the carboxyl group by a nitro group) corresponds to an energy of interaction of the electric charge of the replaced carboxylate ion and the positive charge of the antibody amounting to 2600 cal. mole^{-1}. This is found with use of the Schwarzenbach function[18] for the effective dielectric constant to be equal to the coulomb energy of a positive charge and a negative charge 5.6 Å. apart. It is interesting to note that this distance is roughly the same amount greater than the minimum distance of approach (3.0 Å., for a carboxyl oxygen and an ammonium ion, carrying the positive charge in the antibody) as was found for the system in which the positive charge was carried by the hapten.

The results obtained in this investigation do not answer the question as to whether a single positive charge is induced in the combining region of the antibody by the two carboxylate ion groups, or two positive charges are induced. It might well be expected that an ammonium ion group, such as the ε-amino group of a lysine residue, would carry the positive charge for the antibody. Such a group might be in a position within (on the average) 2.6 Å. of the minimum possible distance of approach of 3.0 Å. from each of the two carboxyl groups. However, there might instead be present two positively charged groups, on opposite sides of the haptenic group, each positively charged group being approximately 5.6 A. from one of the carboxyl groups.

It is possible to understand the poorness of fit of anti-X_{mp} antibody to the homologous haptenic group, as compared with other serological systems, by consideration of the postulated mechanism of antibody production.[19] In this postulated process the combining region of the antibody is built up on a portion of the surface of the antigen, which serves as a template, and thermal agitation leads to the dissociation of the antigen-antibody bond, producing free antibody in the circulating blood stream. In order for this dissociation to occur when the antigen contains a strong group, capable of forming very strong bonds with the antibody, the degree of complementariness of antibody and antigen must not be too great; the better antibody molecules are not able to dissociate away from their antigen template, but remain attached to it. This argument was applied in making the deduction that antibodies to antigens containing strong groups should show low specificity, and those to antigens containing weak groups should show high specificity. In the present case, the two negatively charged groups and their complementary positive charges in the antibody make a contribution of 5,000 cal. mole^{-1} to the bond energy purely through their electrostatic interaction. If the combining region of the antibody were to be closely complementary in structure to the rest of the haptenic group, the total energy of interaction would be so great as to constitute a bond that would not dissociate appreciably, and accordingly the antibody could not readily escape from the antigen template on which it was formed. The antibody molecules that do separate from the template, and escape into the blood stream, are those in which the fit to the haptenic group is poor enough that the total energy of interaction is not much greater than the part due to coulomb interaction of charges alone. The anti-X_{mp} serum extends the sequence anti-X_p, anti-R_p, anti-R_p', in which increasing strength of the haptenic group has been found to be accompanied by a decreasing degree of complementariness in structure of the homologous antibody.

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Summary

A study has been made of the reaction of antiserum prepared by injecting rabbits with sheep serum coupled with diazotized 4-aminophthalate ion.

This serum combines strongly with phthalate ion itself and with the *o*-sulfobenzoate ion, which contains two negatively charged groups in adjacent positions on the benzene ring. However, it combines only very weakly with the *o*-nitrobenzoate ion, which is closely similar to the phthalate ion except that one of the two groups does not carry a negative charge. The large difference in combining power of these two haptens is interpreted as showing that there is present in

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

the combining region of the antibody a positive charge, or two positive charges, close to the negative charges of the two carboxylate ions of the hapten. The calculated distance between the negative charge of the carboxylate ion and the positive charge (assumed to be a single charge) in the antibody is 5.6 Å., which is 2.6 Å. greater than the minimum possible distance of approach of complementary charges in the two molecules.

A large decrease in combining power with the antibody is observed in the sequence phthalate ion, pyridine-2,3-dicarboxylate ion, pyrazine-2,3-dicarboxylate ion. This decrease is interpreted as resulting from hydration of the heterocyclic ions in solution. The energy required to break the hydrogen bonds between a heterocyclic nitrogen atom and an attached water molecule is calculated from the hapten-inhibition data to lie between 700 and 850 cal. mole^{-1}.

Unusually small steric effects of groups substituted in the phthalate ion have been observed. These observations are interpreted as showing that the antibodies fit only rather loosely about the haptenic group, the diametral dilatation being about 2 Å. in the region near the carboxyl ion groups and somewhat larger on the opposite side of the benzene ring. An explanation of the greater looseness of fit of these antibodies than of those in the systems previously studied is presented.

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[CONTRIBUTION FROM THE VENABLE CHEMICAL LABORATORY OF THE UNIVERSITY OF NORTH CAROLINA]

Reaction of Acetylglycine with Ethyl Orthoformate in Acetic Anhydride

BY RICHARD H. WILEY, OLIN H. BORUM AND LEONARD L. BENNETT, JR.[1]

The reaction of acetylglycine and ethyl orthoformate in acetic anhydride has been reported to be anomalous in that no 4-ethoxymethylene-5-oxazolone is obtained.[2] We wish to report the isolation of two new compounds from this reaction. One is diacetylglycine ethyl ester; the other a solid for which the formula $CH_3C(OC_2H_5)=NCH_2CO—O—COCH_3$ is proposed. The same diacetyl ester has also been obtained in up to 88% yield from acetylglycine ethyl ester and acetic anhydride with or without pyridine. This ester reacts slowly with water and with butylamine to give acetylglycine N-*n*-butylamide, previously undescribed and N-*n*-butylacetamide. The ester is reduced with lithium aluminum hydride to diethylethanolamine.

$$(CH_3CO)_2NCH_2CO_2C_2H_5 \longrightarrow (C_2H_5)_2NHCH_2CH_2OH$$

The solid, m. p. 103–104°, obtained in the ethyl orthoformate reaction reacts readily with water to form acetylglycine quantitatively along with identifiable amounts of ethyl acetate; reacts with butylamine to form N-butylamides of acetylglycine and acetic acid; and is readily oxidized by permanganate. Analytical data show the addition of one acetyl and one ethyl group to acetylglycine. Of several possible structures, the ethoxyethylidene anhydride (A) and the N-ethyl anhydride (B) are considered likely although neither O- nor N- alkylation of amides by ethyl orthoformate has been established. The structure (A) is indicated by the reaction with water.

$$CH_3C(OC_2H_5)=NCH_2CO—O—COCH_3$$
(A)

$$CH_3CON(C_2H_5)CH_2CO_2COCH_3$$
(B)

Hydrolysis of the imido ester to acetylglycine rather than glycine is possible by a reaction similar to the Schotten–Baumann reaction in which the mixed anhydride acetylates the amino group more rapidly than it is hydrolyzed by water.

Infrared absorption spectra[3] show an absorption band at 5.6 microns characteristic of the anhydride carbonyl; absence of the characteristic ester carbonyl absorption band at 5.8 microns and the presence of absorption bands at 6.0, 8.0, and 8.4 microns attributable to C=N, and C—O (C unsaturated) linkages by comparison with N-(ethoxyethylidene)-glycine ester. The absorption band at 5.6 microns, the only band the solid has in common with acetic anhydride, is also observed with phthalimide. This suggests a diacylamino structure for the solid which, however, is not consistent with other data. The spectra are given in Figs. 1 and 2.

Experimental

Reaction of Acetylglycine with Ethyl Orthoformate and Acetic Anhydride.—Acetylglycine, 50 g., and 260 g. of acetic anhydride were heated together with stirring for 20 min. at 90° to dissolve whereupon 63 g. of ethyl orthoformate was added. After heating at 90–100° for one hour and fractionating, 26–34 g. of product b.p. 126–136° at 4 mm. was collected. At 120–130°, 42–43 g. of this fraction was obtained. On standing 13–20 g. of solid separated from this liquid. The solid was separated by filtration, and the liquid purified by washing with potassium permanganate. Thus purified the product had b.p. 112° at 3.5 mm. and $n^{25}D$ 1.4532 and was similar in refractive index and analysis to the diacetylglycine ethyl ester isolated by fractionation from a refluxed mixture of acetylglycine ethyl ester and acetic anhydride with or without pyridine.

Anal. Calcd. for $C_8H_{13}O_4N$: C, 51.33; H, 7.00; N, 7.5; mol. wt., 187. Found: C, 51.27; H, 6.80; N,

(1) Taken in part from the Ph.D. thesis of L. L. B.; address, Birmingham, Alabama.

(2) Barber, *et al.*, British Patent 585,089, January 30, 1947.

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