tion of thioketones and nitroso compounds is correlated with the energy of the triplet state.

Other substances of short lived phosphorescence, phenazine and dibenzalacetone, showed S–T absorption bands, but superposed upon the large bands of normal absorption. In looking for the extremely weak S–T bands of substances of long phosphorescence life, it was necessary to make an incidental study of some of the pure rotation–vibration bands that extend into the visible (Fig. 11). The whole absorption spectrum of liquid (and dissolved) benzene was determined between 12,500 Å. and 2200 Å., including four weak S–T bands. Similar S–T bands were found for *p*-dichlorobenzene.

The phenomenon of phosphorescence in non-rigid solvents is discussed, and in the last section it is shown that the luminescence of the vapors of biacetyl and acetyl propionyl is true phosphorescence. It is almost identical with the phosphorescence in a rigid medium at very low temperature. The absorption of light by these vapors was redetermined and is shown to be due to the superposition of narrow S–T bands upon the broad band of normal absorption.

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[CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, No. 997]

The Serological Properties of Simple Substances. IX. Hapten Inhibition of Precipitation of Antisera Homologous to the *o*-, *m*-, and *p*-Azophenylarsonic Acid Groups

BY LINUS PAULING AND DAVID PRESSMAN

During their extensive studies of the properties of antisera prepared by injecting animals with artificial conjugated antigens containing groups of known chemical structure, Landsteiner and his collaborators investigated the effect of position of substituents in the benzene ring of haptens and haptenic groups on the precipitation reactions of antisera and azoproteins and the inhibition of precipitation by haptens.[1] They found that combination of antiserum and antigen or hapten is decreased by the presence of substituent groups in the precipitating antigen or the hapten in positions other than those occupied in the immunizing antigen. Similar results have been obtained in our studies.[2,3,4,5] In order to learn more about the nature of the intermolecular forces operative in these serological systems, we have extended our quantitative studies of hapten inhibition to include the reactions of antisera homologous to the *o*-, *m*-, and *p*-azophenylarsonic acid groups (hereafter called anti-R_o serum, anti-R_m serum, and anti-R_p serum, respectively) with the corresponding azo-ovalbumins R_o-ovalbumin, R_m-ovalbumin, and R_p-ovalbumin, and have obtained the results reported in this paper. The hapten-inhibition data have been interpreted by use of the heterogeneity theory[4] to provide values of the heterogeneity index σ and the hapten inhibition constant K_0' for each hapten with each system of antiserum and precipitating antigen, and it has been found possible to formulate a detailed discussion of the effective intermolecular forces which accounts in the main for the observed relative values of K_0'.

(1) K. Landsteiner, "The Specificity of Serological Reactions," Charles C. Thomas, Springfield, Ill. 1936.

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

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

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

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

Experimental Methods

Haptens.—The *o*-chlorophenylarsonic, *m*-chlorophenylarsonic, 2,4-dinitrophenylarsonic, and 2,4-dichlorophenylarsonic acids were prepared by the Bart synthesis by Mr. George Cleland. The *o*- and *m*-(*p*-hydroxyphenylazo)-phenylarsonic acids were prepared by coupling diazotized arsanilic acid with a ten-mole excess of phenol at *p*H 9.5–10. The acids were finally purified by crystallization from 50% alcohol and washing the crystals with water acidified with hydrochloric acid. The rest of the haptens used have been reported previously.[6]

Protein Antigens.—The immunizing antigens used for inoculations except for diazotized *p*-arsanilic acid coupled with sheep serum were prepared by Mr. Allan Grossberg by coupling diazotized *o*-arsanilic acid or *m*-arsanilic acid with whole sheep serum. For each antigen 0.1, 0.2, and 0.3-g. portions of the amine were diazotized and coupled with three 70-ml. portions of sheep serum at *p*H 8.5. After the coupling was complete, the three products containing different amounts of hapten were mixed and purified by the method of Landsteiner and van der Scheer.[7] This was done to increase the possibility of using an azoprotein of optimum antigenicity. The antigen made by coupling diazotized *p*-arsanilic acid with sheep serum has been described previously.[8]

The azoprotein test antigens were made by diazotizing 0.2 g., 0.2 g., and 0.15 g. of *o*-, *m*-, and *p*-arsanilic acid, respectively, and coupling individually with 0.1-g. portions of crystallized hen ovalbumin at *p*H 10.0. The solutions were dialyzed overnight against running tap water and then precipitated at *p*H 3.0–3.5, redissolved at *p*H 10, reprecipitated at *p*H 3.0–3.5, and dissolved in 50 ml. saline to *p*H 7. The antigens thus prepared when analyzed were found to contain approximately 2.5% arsenic.

Antisera.—The preparation of anti-R_p serum has been described previously.[3] The anti-R_o and anti-R_m sera were prepared by a similar method. A single pool of each antiserum was used.

(6) D. Pressman and D. H. Brown, *ibid.*, **65**, 540 (1943).

(7) K. Landsteiner and J. van der Scheer, *J. Exptl. Med.*, **55**, 781 (1932).

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

TABLE I

EFFECT OF HAPTENS ON THE PRECIPITATION OF ANTI-R_o SERUM WITH R_o-OVALBUMIN

Antigen added, 33 μg.; *p*H of supernates, 8.1–8.2.

Hapten (arsonic acid)	K_0'	σ	Moles of hapten added × 10^9: 4.9	9.8	19.5	39	78	156	313	625	1250	2500
			Amount of precipitate[a]									
Phenyl	1.00	3.0			762		(550)		328			
o-(*p*-Hydroxyphenylazo)-phenyl	7.3	3.0	700		465		253					
m-	0.158	3.5					[783]		(642)		414	
p-	0.0040	7						828		721		664
o-Nitrophenyl	5.8	3.0	721		455		284					
m-	0.54	5				642		471		404		
p-	0.064	5					775		686		516	
o-Methylphenyl	3.5	4			544		397		178			
m-	0.87	4				650		471		263		
p-	0.169	4				827		653		516		
o-Chlorophenyl	8.2	3.5		560		328		171				
m-	1.60	3.5			684		471		297			
p-	0.77	5				625		492		318		
o-Aminophenyl	1.38	3.5				595		414		219		
m-	0.417	3.0					(721)		428		276	
p-	.268	3.0				835		670		420		
α-Naphthyl	1.66	4		739		556		403				
β-	0.104	4					803		652		499	
2,4-Dinitrophenyl	.17	10					574		516		492	
2,4-Dimethylphenyl	.33	4.5					663		499		345	
2,4-Dichlorophenyl	.91	4				(612)		499		276		

[a] The amounts of precipitate are in parts per mille of the amount in the absence of hapten, 293 μg., and are corrected for blanks of serum and buffer, 9 μg. Values are averages of triplicate analyses, with mean deviation ±2%; duplicate analyses are given in parentheses and single analyses in brackets.

Reaction of Antiserum with Antigen and Hapten.—One-milliliter portions of antigen solution, antiserum, and hapten solution were mixed and permitted to stand one hour at room temperature and over two nights at 5°. The amount of antigen which gave optimum precipitation in the absence of hapten was used. The precipitates were centrifuged and washed three times with 10-ml. portions of 0.9% sodium chloride solution. All dilutions of antigen and hapten were made with a buffer solution of *p*H 8.0, prepared by adding 0.16 *N* sodium hydroxide solution to 0.2 *M* boric acid in 0.9% sodium chloride solution.

Method of Analysis.—The amount of protein in the precipitates was determined with the Folin–Ciocalteu protein reagent by the modification discussed elsewhere.[9]

The Precipitation Reaction.—The pools of antiserum used were chosen to give the same optimum amount of precipitate with the homohaptenic antigens in order to keep the experiments comparable with respect to the amount of serum protein present. With R_o-, R_m-, and R_p-ovalbumins the anti-R_o serum gave amounts of precipitate in the optimum in the ratios 1:1:0.5, anti-R_m serum gave amounts in the ratio 0.2:1:0.3, and anti-R_p serum gave amounts in the ratios 0.0:1:1. (Landsteiner and Lampl[10] have reported the cross reaction of an R_o-antigen with an anti-R_p serum, and we have observed this also with stronger anti-R_p sera.)

We do not believe that much weight can be placed on the relative amounts of precipitate obtained with each antiserum and the different antigens, since the protein antigens may have contained some free hapten even after our purification procedure. Any free hapten present would have caused inhibition and the inhibition would have been greatest in the precipitation of an antiserum with the homologous antigen, since the homologous hapten is a much stronger inhibitor than a heterologous hapten which would be present in a heterologous antigen. This effect is probably the reason for the observation that R_m-ovalbumin is as effective as R_o-ovalbumin in the precipitation of anti-R_o serum and that R_m-ovalbumin is as effective as R_p-ovalbumin in the precipitation of anti-R_p serum.

In general the amounts of the different antigens required for optimum precipitation were found to be roughly the same for any one antiserum. It is interesting that six to ten times as much R_m-ovalbumin was required to give optimum precipitation with the homologous antiserum as with the heterologous antisera.

Hapten Inhibition of Precipitation

Data on hapten inhibition of precipitation for the five antibody–antigen systems are given in Tables I to V. The values of the heterogeneity constant σ and the hapten inhibition constant K_0' obtained by application of the heterogeneity theory[4] are also given in the tables.

For four of the five systems the values found for σ lie for the most part in the range 1.5 to 3.0 found in the earlier studies[4,5] of anti-R_p, anti-R′, and anti-X_p sera. Some indication is seen of the rough correlation previously noted[4] between σ and K_0', an increase in σ with decrease in K_0'.

The values of σ for the deviating system, anti-R_o serum and R_o-ovalbumin, range from 3.0 to 5 or more. A possible explanation of these large values is that they result from an abnormally great variability of strength of attraction of antibody and azoprotein antigen because of the close approximation of the protein part of the antigen to the antibody in this system. The arsonic acid group, which is the strongest antigenic part of the haptenic group, is closer to the protein part of the immunizing antigen when it is in the ortho position than when it is in another position,

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

(10) K. Landsteiner and H. Lampl, *Biochem. Z.*, **86**, 343 (1918).

TABLE II

EFFECT OF HAPTENS ON THE PRECIPITATION OF ANTI-R_o SERUM WITH R_m-OVALBUMIN

Antigen added, 38 μg.; *p*H of supernates, 8.1–8.2.

Hapten (arsonic acid)	K'_1	σ	Moles of hapten added × 10^9 → Amount of precipitate[a]										
			2.5	4.9	9.8	19.5	39	78	156	313	625	1250	2500
o-(*p*-Hydroxyphenylazo)-phenyl	6.65	2.5		695		515		(187)					
m-	0.43	2.0					885		609		140		
p-	.045	5							735		651		494
o-Nitrophenyl	15.5	2.0	720		432		151						
m-	1.30	2.0				792		519		212			
p-	0.23	2.0						851		548		281	
o-Chlorophenyl	5.92	2.0			626		335		90				
m-	2.24	2.5					540		295		68		
p-	1.05	2.5						(555)		(259)		(68)	
o-Aminophenyl	2.46	3.0				626		418		187			
m-	1.70	3.0						(461)		248		43	
p-	0.92	2.5					695		446		187		

[a] The amounts of precipitate are in parts per mille of the amount in the absence of hapten, 278 μg., and are corrected for blanks of serum and buffer, 14 μg. Values are averages of triplicate analyses, with mean deviation ±2%; duplicate analyses are given in parentheses.

TABLE III

EFFECT OF HAPTENS ON THE PRECIPITATION OF ANTI-R_m SERUM WITH R_m-OVALBUMIN

Antigen added, 380 μg.; *p*H of supernates, 8.1–8.2.

Hapten (arsonic acid)	K'_1	σ	Moles of hapten added × 10^9 → Amount of precipitate[a]						
			156	313	625	1200	2500	5000	10000
Phenyl	1.00	2.0		729		378		119	
o-(*p*-Hydroxyphenylazo)-phenyl	0.37	2.0			782		490		167
m-	2.35	1.5	736		337		30		
p-	0.47	2.5	874		720		424		
o-Nitrophenyl	0.64	2.0		794		479		202	
m-	5.5	2.0	498		195		28		
p-	0.35	3.5		786		578		(407)	
o-Methylphenyl	.51	2.0		782		598		231	
m-	.98	2.0		729		416		123	
p-	.25	3.0		812		709		(420)	
o-Chlorophenyl	.89	2.0		[798]		350		(163)	
m-	1.29	2.0		694		301		82	
p-	0.40	2.0		850		666		276	
o-Aminophenyl	.55	2.0		811		538		231	
m-	1.05	2.0		730		362		131	
p-	0.44	1.5		873		636		(248)	
α-Naphthyl	.37	1.5		947		635		267	
β-	.49	2.0		835		574		256	
2,4-Dinitrophenyl	.32	2.5			721		474		256
2,4-Dimethylphenyl	.18	2.0			876		663		(299)
2,4-Dichlorophenyl	.78	2.0		771		487		127	

[a] The amounts of precipitate are in parts per mille of the amount in the absence of hapten, 528 μg., and are corrected for blanks of serum and buffer, 14 μg. Values are averages of triplicate analyses, with mean deviation ±2%; duplicate analyses are given in parentheses and single analyses in brackets.

and hence it may be expected that the combining regions of anti-R_o antibodies will conform to the adjacent protein structures more closely for anti-R_o serum than for anti-R_m serum or anti-R_p serum. Moreover, the protein part of R_o-ovalbumin will be brought into closer juxtaposition with the protein-complementary regions of the anti-R_o antibodies than would the protein parts of R_m-ovalbumin or R_p-ovalbumin with their hapten-homologous antibodies, and accordingly a larger protein–antiprotein interaction would be expected for the system anti-R_o serum: R_o-ovalbumin than for the other systems. Since the protein parts in the neighborhood of the haptenic group would be of various kinds both in the immunizing azoprotein and in the precipitating azoprotein, the protein–antiprotein interaction for different pairs of molecules would make widely different contributions to the bond energy, and in this way would increase the apparent heterogeneity of the antiserum in its competitive interaction with hapten and precipitating antigen.

The values of K'_0 in Tables I to V are referred

TABLE IV

EFFECT OF HAPTENS ON THE PRECIPITATION OF ANTI-R_p SERUM WITH R_m-OVALBUMIN

Antigen added, 63 μg.; *p*H of supernates, 8.1–8.2.

Hapten (arsonic acid)	K_0'	σ	Moles of hapten added × 10^9: 39	78	156	313	625	1250	2500	5000	10000
			Amount of precipitate[a]								
Phenyl	1.59	2.0				668		372		46	
o-(*p*-Hydroxyphenylazo)-phenyl	0.32	2.5					758		585		278
m-	4.00	2.0		775		477		97			
p-	17.3	1.5	645		268		23				
o-Nitrophenyl	1.21	2.0				725		444		143	
m-	4.00	2.0			638		306		41		
p-	15.4	2.5		472		228		23			
o-Methylphenyl	0.64	2.0					146		415		700
m-	2.20	2.0				610		291		49	
p-	5.14	2.0			570		255		23		
o-Chlorophenyl	1.05	2.0				753		467		143	
m-	3.00	2.0			705		(383)		77		
p-	6.4	2.0		660		368		84			
o-Aminophenyl	0.73	2.0				790		528		224	
m-	1.62	2.0				710		344		77	
p-	2.07	2.0			765		465		156		
α-Naphthyl	1.07	2.0				754		450		(161)	
β-	6.2	2.0		689		358		54			
2,4-Dinitrophenyl	4.2	2.0			580		296		71		
2,4-Dimethylphenyl	1.67	2.5				(574)		383		110	
2,4-Dichlorophenyl	3.3	2.5			643		372		105		

[a] The amounts of precipitate are in parts per mille of the amount in the absence of hapten, 392 μg., and are corrected for blanks of serum and buffer, 5 μg. Values are averages of triplicate analyses, with mean deviation ±2%; duplicate analyses are given in parentheses.

TABLE V

EFFECT OF HAPTENS ON THE PRECIPITATION OF ANTI-R_p SERUM WITH R_p-OVALBUMIN

Antigen added, 76 μg.; *p*H of supernates 8.1–8.2.

Hapten (arsonic acid)	K_0'	σ	Moles of hapten added × 10^9: 39	78	156	313	625	1250	2500	5000	10000
			Amount of precipitate[a]								
Phenyl	1.00	2.0				750		506		144	
o-(*p*-Hydroxyphenylazo)-phenyl	0.123	3.0					854		724		450
m-	1.66	2.0			759		540		162		
p-	5.8	1.5		685		367		27			
o-Nitrophenyl	0.60	2.5				795		576		300	
m-	1.48	2.0			800		570		162		
p-	5.3	2.5	768		565		257				
o-Methylphenyl	0.21	2.5					854		660		345
m-	0.78	2.0				786		536		187	
p-	1.93	1.5			813		482		95		
o-Chlorophenyl	0.30	2.0				867		633		(349)	
m-	1.26	2.5				666		437		45	
p-	2.21	1.5			777		424		101		
o-Aminophenyl	0.26	3.0				835		723		453	
m-	0.56	2.0				854		599		266	
p-	1.23	2.0				(741)		408		119	
α-Naphthyl	0.44	2.5				854		604		333	
β-	3.3	2.0			699		340		38		
2,4-Dinitrophenyl	1.88	2.0			755		450		(212)		
2,4-Dimethylphenyl	0.53	2.0				845		621		290	
2,4-Dichlorophenyl	0.83	3.0				698		516		83	

[a] The amounts of precipitate are in parts per mille of the amount in the absence of hapten, 444 μg., and are corrected for blanks of serum and buffer, 9 μg. Values are averages of triplicate analyses, with mean deviation ±2%; duplicate analyses are given in parentheses.

for the several antisera to the value $K_0' = 1$ for phenylarsonic acid in the systems anti-R_o serum: R_o-ovalbumin, anti-R_m serum:R_m-ovalbumin, and anti-R_p serum:R_p-ovalbumin. The K_0' values

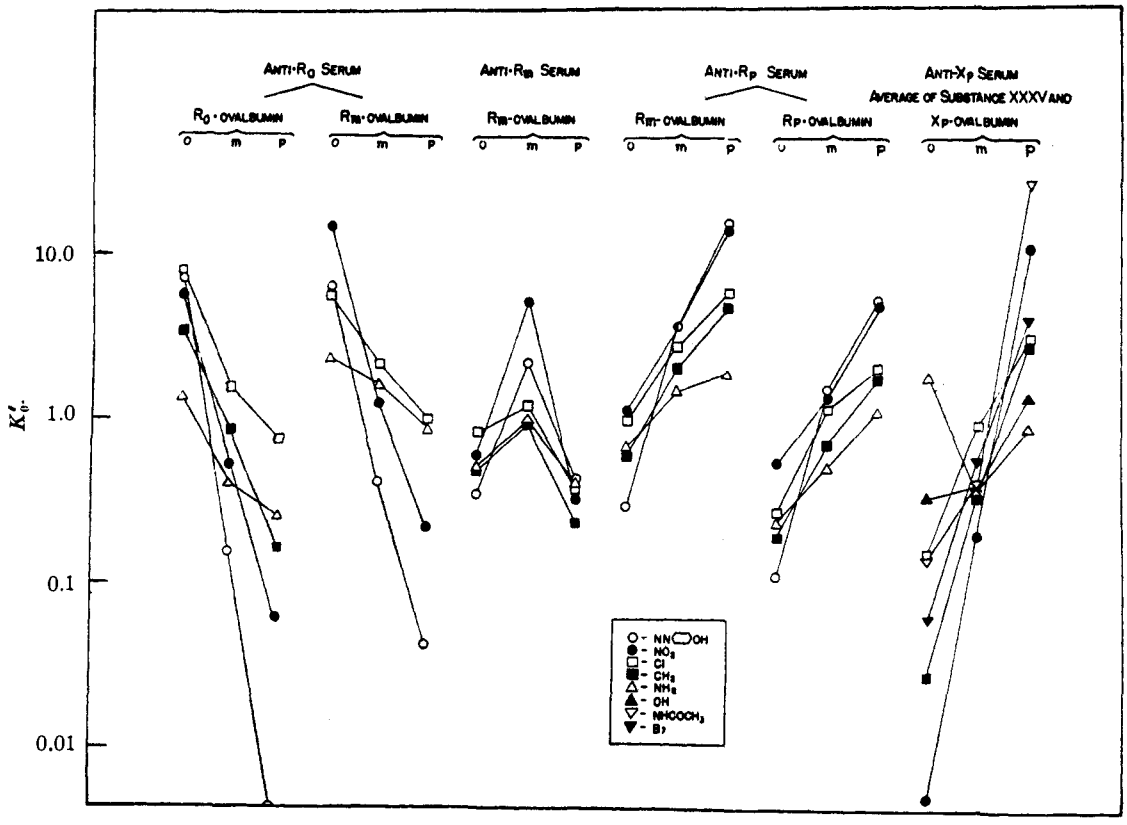


Fig. 1.—The effect of substituents on the K_0' values of haptens.

for anti-R_o serum with R_m-ovalbumin and for anti-R_p serum with R_m-ovalbumin[11] are greater than those for precipitation of the antisera with the homohaptenic azo-ovalbumins because the haptens can displace the heterologous haptenic groups more easily than the homologous haptenic groups. The magnitude of this effect is to be seen from Fig. 1, in which there are plotted the values of log K_0' for the singly substituted phenylarsonic acid haptens. The logarithmically averaged factor of K_0' is 2.3 for change in haptenic group of the precipitating antigen from R_o to R_m for anti-R_o serum and 2.6 for change from R_p to R_m for anti-R_p serum. This factor is roughly the same as that resulting from the corresponding (reverse) change in position of the substituent group in a substituted phenylarsonic acid serving as inhibiting hapten.

The values of K_0' for the various haptens are discussed in the following section.

The Intermolecular Forces Operating in Hapten Inhibition

It is to be expected that electrostatic attraction of charged groups, hydrogen-bond forces, electronic van der Waals attraction (London dispersion forces), steric repulsion determined by molecular sizes and shapes, and other forces recognized in the classification of intermolecular interactions may all enter into the phenomenon of hapten inhibition. The attraction of the azophenylarsonic acid haptenic group (mainly $NNC_6H_4AsO_3H^-$ at physiological pH) to the antibody would contain contributions of the attraction of the negative charge to a complementary positive charge in the antibody, the hydrogen bond formed with the antibody by the undissociated hydrogen atom of the arsonic acid, the electronic van der Waals attraction of the haptenic group and the adjacent parts of the antibody, and possibly a hydrogen bond formed by an azo nitrogen atom with a proton-donating group of the antibody. The forces of steric repulsion would operate to preserve suitable distances of contact between the atoms of the haptenic group and the atoms of the antibody. Similar forces of attraction and repulsion would be operative between the antibody and a substituted phenylarsonic acid serving as hapten.

Electronic van der Waals Attraction.—Let us discuss first the expected effect of electronic van der Waals interaction of a substituent group in the position of the azo group in the immunizing antigen on the value of K_0', and compare the results of a simple theoretical treatment with the experimental data obtained for substituted benzoic acids and substituted phenylarsonic acids as

(11) The results of a study reported in ref. 4 of the effect of change in the amount of precipitating antigen on the values of K_0' suggest that the relative values of K_0' for the systems compared are reliable to within a few per cent.

haptens. The principal force of attraction between neutral molecules was recognized by London[12] in 1930 as being the result of the polarization of each molecule in the rapidly changing electrical field arising from the instantaneous configuration of the electrons and nuclei of the other molecule. London developed for the interaction energy of two atoms, groups, or molecules A and B the approximate expression

$$W = -\frac{3}{2}\frac{\alpha_A\alpha_B}{r_{AB}^6}\frac{I_AI_B}{I_A+I_B} \qquad (1)$$

in which α_A and α_B are the electronic polarizabilities of the two groups, I_A and I_B are average energy differences for normal and excited states (approximately equal to the energies of ionization), and r_{AB} is the distance between the groups. This expression may be rewritten, with introduction of the value $I_A = I_B = 14$ electron volts (which is an average of the rather closely agreeing values for several molecules), as

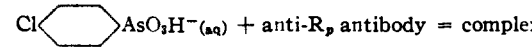
$$W = -\frac{38000}{r_{AB}^6}R_AR_B \text{ cal. mole}^{-1} \qquad (2)$$

in which R_A and R_B are the mole refractions of the groups in cm.[3] (related to α_A and α_B by the factor $4\pi N/3$), and r_{AB} is in Ångström units.

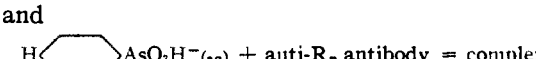
The methyl group, hydroxyl group, azo group, amino group, and other groups have van der Waals radii close to 2 Å., and the half-thickness of aromatic rings is the same; as an approximation we may take 2.0 Å. as the effective radius of groups in the antibody molecule adjacent to the hapten. The antibody may well have some elasticity, permitting these groups to come into contact with parts of the hapten differing slightly in size from the azo group of the immunizing antigen; and accordingly r_{AB} may be taken as the sum of the radius 2.0 Å. of the groups in the antibody and the radius of the group in the hapten. This radius is 1.35 Å. for F, 1.8 for Cl, 1.95 for Br, 2.15 for I, 1.6 for OH, 1.8 for NH_2, and 2.0 for the other groups.[13]

It is also necessary to take into consideration the contribution of van der Waals repulsion to the interaction energy. The effect of the repulsive potential is to decrease the energy of reaction somewhat, leaving the values for different substituent groups in essentially the same order. The amount of this reduction varies from thirty to fifty per cent. for repulsive potential functions indicated by the properties of gases and crystals (inverse twelfth to fifteenth power of r_{AB}, or corresponding exponential expressions). (The energy of deformation of the antibody to adjust itself to the antigen might be appreciable in some cases; its consideration, however, must await more detailed information about the structure of antibodies, and we accordingly neglect it.) As an average, we shall accept the value 40% as the correction for the repulsive potential, and introduce in Equation 2 the factor 0.60.

We shall apply Equation 2 with the factor 0.60 for the calculation of the difference in the free energy changes of a reaction such as



and



in order to predict the effect of replacement of hydrogen by chlorine (or other substituent) on the hapten inhibition constant K_0'. Four energy quantities are to be considered: the electronic van der Waals interaction of the substituent A (such as chlorine) with water, that of A with the antibody, that of the hydrogen atom of the unsubstituted acid with water, and that of the hydrogen atom with antibody. The resultant difference in energy of reaction is

$$\Delta W_A = -\frac{23000}{r_{AB}^6}(R_A - R_H)(R'_{\text{antibody}} - R'_{\text{water}}) \qquad (3)$$

Here R'_{antibody} and R'_{water} represent the mole refraction values integrated over the region of material in contact with the group A. This may be estimated as the volume of eight water molecules (twelve water molecules would completely surround a group of this size; the change to eight makes room for the benzene ring and for the similar residue attached on the other side of the azo group in the immunizing antigen); and hence R'_{water} may be taken as $8R_{H_2O} = 29.6$ cm.^{3}. (For convenience we use values of R for the sodium D lines instead of the slightly different values of R_∞ obtained by extrapolation.) For antibody the value 47.2 cm.^{3} may be taken, calculated from the index of refraction, 1.55, of a protein, squash seed globulin, with correction for its 8% water content.[14] These values lead to

$$\Delta W_A = -\frac{400000}{r_{AB}^6}(R_A - R_H) \qquad (4)$$

which may be compared with the difference in free energy change $RT \ln K_0'$ for the reactions.

There are plotted at the left of Fig. 2 values of $-\Delta W_A/2.303\,RT$ calculated by Equation 4 with use of the following values of $R_A - R_H$, which are the differences in mole refraction values[15] of the substituted benzenes and benzene itself: CH_3CONH, 13.0; NO_2, 6.58; I, 12.98; Br, 7.79; Cl, 5.00; F, −0.14; OCH_3, 6.86; CH_3, 4.92; OH, 1.81; and NH_2 4.44. Values are also shown of log K_0' for the *p*-substituted benzoic acids and anti-X_p serum (averages of values for antigen

(12) F. London, *Z. Physik*, **63**, 245 (1930).

(13) These values, indicated by interatomic distances in crystals, are generally accepted: L. Pauling, "The Nature of the Chemical Bond," Cornell University Press, Ithaca, New York, Second Edition, 1940, p. 189. The results of the calculation are not essentially changed by taking 2.0 Å. as the radius for all of the groups.

(14) A. N. Winchell, "The Optical Properties of Organic Compounds," University of Wisconsin Press, Madison, 1943.

(15) The values are calculated from data given in Landolt–Börnstein, except for the value for the acetamino group, which was calculated from data for acetanilide given by Winchell, *loc. cit.*

XXXV and X_p-ovalbumin, Table IV of ref. 5), for the *p*-substituted arsanilic acids with anti-R_p serum (averages of values for antigen VI, antigen XXX, and R′-ovalbumin, Table VII of ref. 4), and for the arsanilic acids with anti-R′ serum (averages of three sets of values, Table VII of ref. 4). It is seen that for the iodo, bromo, chloro, methyl, and hydroxy groups the correlation between calculated and observed values is good. The change in scale from anti-R_p serum to anti-R′ serum indicates, as mentioned before,[3,4] a poorer fit of the anti-R′ antibodies than of the anti-R_p antibodies; an increase in r_{AB} for antibody and hapten by only a few tenths of an Ångström unit would account for the observed effect—because of the inverse sixth power of r_{AB} in the expression for the electronic van der Waals energy a large effect is caused by a small change in the degree of approximation of the antibody to the hapten, and it is accordingly not surprising that the values of K_0' vary widely from antiserum to antiserum. The effect might instead result from the inability of the anti-R′ antibody to bring as much protein into contact with the hapten; that is, from the poorer fit of the anti-R′ antibody than the anti-R_p antibody to the hapten.

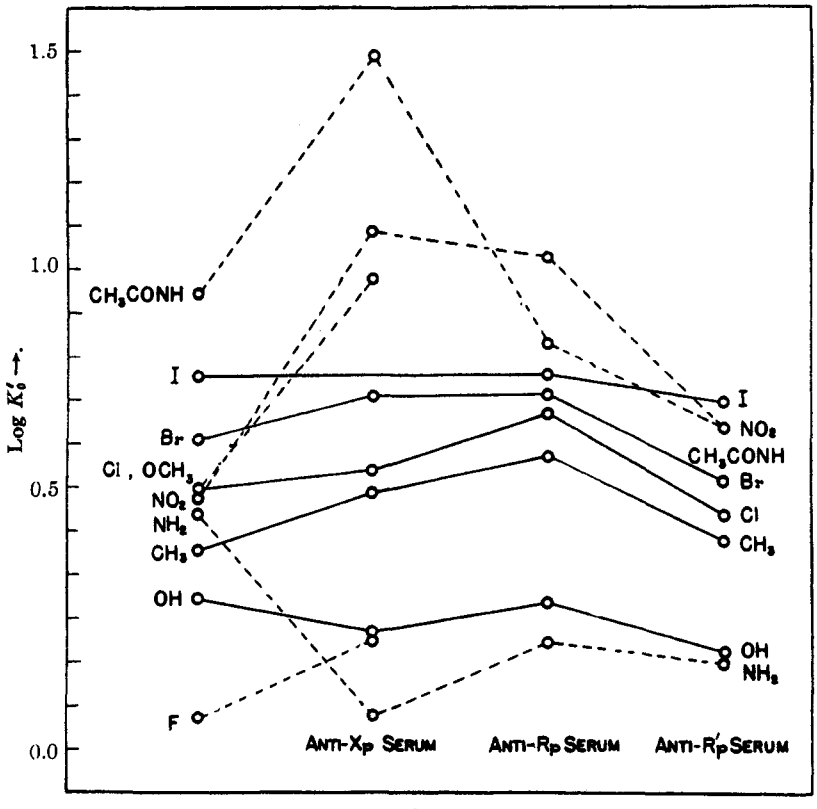


Fig. 2.—Comparison of values of log K_0' for para substituted haptens with calculated van der Waals attraction energies.

The Effect of Hydrogen Bonds.—It seems likely that the increase over the calculated values of the values of K_0' shown by haptens containing the acetamino, nitro, methoxy, and fluoro groups is the result of the formation of hydrogen bonds between these groups and the antibody, with the antibody providing the proton for the bond. These groups are all characterized by the ability to form bonds of this type, and this ability is not shown to any significant extent by any of the other groups represented in the figure except the hydroxyl group, which is discussed in greater detail below. Moreover, it would be anticipated that the antibody in building up a structure complementary to that of the immunizing antigen would provide an amino group or similar group which could form a hydrogen bond with an azo nitrogen atom of the azoprotein. The energy effect indicated for anti-X_p serum in Fig. 2, 400 to 700 cal. mole^{-1}, represents of course the difference in energy of the hydrogen bond formed by the hapten with antibody and with water.

The discrepancy shown by the amino group has a different origin. The aromatic amino group cannot form hydrogen bonds with proton-donating groups, because its unshared electron pair is used in conjugation with the ring. It can, however, form hydrogen bonds with use of its own hydrogen atoms. Bonds of this sort would be formed by the hapten with water molecules, but not in general with antibody homologous to azoprotein, because the azo group does not contain any hydrogen atom to attract a suitable group to this portion of the antibody molecule during the formation of the antibody; accordingly this effect would result in low values for K_0' for amino haptens, as observed.

The phenolic hydroxyl group and the acetamino group can form hydrogen bonds of both kinds, and the two resultant opposed effects on the value of K_0' appear largely to cancel each other in the case of the hydroxyl group; for the acetamino group the first effect is the more important.

The large phenolazo and benzoylamino groups give rise to large values of K_0', which, however, are not so large as would result from van der Waals interaction with antibody contiguous to the entire group. This fact indicates that the combining groups of antibody molecules made with use of azoproteins are directed mainly against the azobenzoic acid or azophenylarsonic acid group,

and only to a smaller extent against the aromatic ring of the tyrosine or histidine residue to which it is attached.

The Effect of Steric Factors.—The order of K_0' values of substituted phenylarsonic acids represented in Fig. 1 is essentially the same as in the systems just discussed when the substituent group occupies the same position in the benzene ring as was occupied by the azo group in the immunizing antigen. The only pronounced change in order is that the *o*-nitro and *o*-(*p*-hydroxyphenylazo) groups with anti-R_o serum are weaker than the *o*-chloro group. The reason for this change is not obvious.

Many changes in the order of inhibiting power occur when the substituents occupy positions in the ring different from that of the azo group in the immunizing antigen. These changes are the result in the main of variation in the amount of steric hindrance for the different groups. A quantitative discussion of steric hindrance in these systems is made impossible by the lack of knowledge of the detailed structure of antibodies. However, a simple treatment may be carried out on the basis of the assumption that a combining region of the antibody surrounds a cavity into which an azophenylarsonic group (*o*, *m*, or *p*) fits more or less closely. Drawings were made of these groups with use of the accepted bond distances and van der Waals radii, and circumferential contours, representing the cavity in the plane of the benzene ring, were drawn (with re-entrant angles rounded off with radius of curvature 2.0 Å.) to fit the groups closely (Fig. 3), or with radial dilatation of 0.2 Å., 0.4 Å., ... Scale drawings of the haptens were then superimposed, with the arsonic acid group in the same position and with approximately the same orientation as for the immunizing group, and the extent of dilatation needed for each hapten with each antiserum was estimated. The results are given in Table VI.

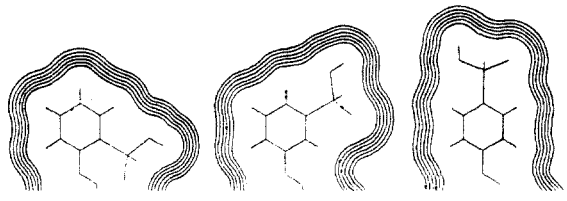


Fig. 3.—Scale drawings of the antibody cavity specific to the *o*-, *m*-, and *p*-azophenylarsonic acid haptenic groups, with circumferential contours corresponding to close fit and to radial dilatation in increments of 0.2 Å.

It is seen that a radial dilatation of about 1 Å. is needed whenever the substituent group is in a position other than that of the azo group in the immunizing azoprotein, and that the amount of dilatation needed increases as the group moves farther away, being greater for the para than for the meta position with anti-R_o serum and greater for the ortho than for the meta position with anti-R_p serum. These facts, with the assumption that increase in the dilatation needed infers increase in steric repulsion and hence decrease in K_0', explain the main features of Fig. 1. In addition some details are explained. There is complete correlation between the K_0' values and the fact that with anti-R_m serum there is greater steric hindrance for nitro, methyl, chloro, and amino groups in the para position than in the ortho position, whereas the greater hindrance for the phenolazo group is in the ortho position; this reversal of steric effects causes the value of K_0' to be greater for the *p*-phenolazo group than for the other *p*-groups, and less for the *o*-phenolazo group than for the other *o*-groups. The other observed changes in relative values of K_0' for the phenolazo and nitro groups and the other groups are also in the main to be accounted for by the steric relations indicated in Table VI. The only pronounced discrepancy is that between the large value of K_0' for *m*-(*p*-hydroxyphenylazo)-phenylarsonic acid with anti-R_p serum and the large steric effect expected from the calculated required dilatation of 1.5 Å.

TABLE VI

RADIAL DILATATION OF ANTIBODY MOLECULES NEEDED FOR COMBINATION WITH SUBSTITUTED PHENYLARSONIC ACID HAPTENS

Values are for the radial dilatation in Ångström units; no dilatation is needed for ortho substituents with anti-R_o serum, etc. The ortho substituted haptens fit into anti-R_m antibodies in two ways, with dilatations different by 0.1 Å. or less, and the meta substituted haptens fit into anti-R_o antibodies in two ways, with dilatations differing by about 0.2 Å., except for phenolazo haptens.

Antiserum	Anti-R_o		Anti-R_m		Anti-R_p	
Position of substituent in hapten	*m*	*p*	*o*	*p*	*o*	*m*
Substituent group						
Phenolazo	>4	>2	2	1.0	>3	1.5
Nitro	0.9	1.6	0.8	1.0	1.0	0.8
Methyl	.7	1.2	.6	0.9	0.8	.7
Chloro	.8	1.2	.7	.9	.8	.7
Amino	.8	1.2	.6	.9	.8	.6

The extent to which the interpretation of the values of K_0' can be carried may be further illustrated by the data for anti-X_p serum[5] shown at the right of Fig. 1. The steric effects are on the average somewhat larger than for anti-R_p serum, for which they are larger than for anti-R′; this fact has been remarked before[5] as showing that the closeness of fit of antibody to haptenic group falls off in the order anti-X_p, anti-R_p, anti-R′. The changes in order of values of K_0' as the substituents are changed in position are the result of differences in steric repulsion; the effect of the greater mole refraction, giving the bromo group in the para position a larger value of K_0' than the chloro group, is overcome in the meta position by the greater size, which requires 0.15 Å. more dilatation of the antibody, and to a still greater extent in the ortho position, where 0.3 Å. more radial dilatation is required.

The surprisingly large value of K'_0 for *o*-aminobenzoic acid has been mentioned before[5] as probably related to the formation of an intramolecular hydrogen bond with the carboxyl group. The effect of this hydrogen bond would be two-fold: the intramolecular bond would largely interfere with hydrogen-bond formation by the free hapten with water, which decreases the values of K'_0 for the meta and para haptens, and it would hold the carboxyl ion group coplanar with the ring, whereas other ortho substituents push it into a non-coplanar position,[16] which is unfavorable to attraction of the antibody. The *o*-hydroxyl group shows a similar but smaller effect.

The accruing evidence thus provides an increasingly detailed conception of the nature of the combining regions of antibodies homologous to haptenic groups of the sort which we have been studying. The structure of the antibody is such that the molecule can approximate itself closely not only to the phenylarsonic acid or benzoic acid portion of the haptenic group or hapten but also to the azo group or other substituent group; and this approximation is close enough to correspond to intermolecular "contact," as determined by the outer electron shells of the atoms. On the other hand, the antibody has sufficient elasticity of configuration to permit the insertion of a much larger hapten than that to which it was molded, and yet it retains the impress of the immunizing antigen sufficiently to cause combination to occur more readily with haptens similar to the immunizing antigen than with others. It is likely that some of the combining regions of antibodies may have the form of two protein layers of suitable configuration, between which the haptenic group fits—the interfering steric action of a larger coplanar substituent group such as the azophenol group would be much smaller for such an antibody than for an antibody which rigidly circumscribed a cavity. Some antibody molecules might provide merely a flat depression in which the hapten or haptenic group lies on its side. We hope to learn more about these details by further experimental work.

The Effect of Symmetry.—The *p*-azophenylarsonic acid group has (except for the jog in the azo group) a vertical plane of symmetry, and it might be expected that the combining regions of anti-R_p antibodies would approximate this symmetry, whereas those of anti-R_o and anti-R_m antibodies would not. Similarly para-substituted haptens have a vertical plane of symmetry, and the other haptens do not. It would hence be expected that a symmetry-number term $-R \ln 2$ would occur in the entropy of reaction of *p*-substituted haptens with anti-R_o and anti-R_m serum, increasing K'_0 by the factor 2. There is, however, no indication of this factor in the data represented in Fig. 1, which show a reasonable dependence on position of substituent without correction by this factor.

The reason for this situation is provided by the steric considerations summarized in Table VI. The ortho-substituted haptens (other than that containing the phenolazo group) can fit nearly equally easily in two ways into the anti-R_m antibodies, giving two different complexes with nearly equal stability; and this duplexity of reaction has the effect of nearly doubling the equilibrium constant, and thus balancing the symmetry-number factor 2 for the para-substituted haptens. A meta-substituted hapten may react in two ways with anti-R_o antibodies to give two different complexes differing somewhat in stability, corresponding to a difference in dilatation required of about 0.2 Å.; accordingly the equilibrium constant would be increased by a factor intermediate in value between 1 and 2.

The Effect of Two or More Substituents.—It was pointed out in the discussion of data for anti-benzoic acid serum[5] that the free-energy effects of two or more substituents are roughly additive; the values of K'_0 calculated as the products of corresponding values for singly-substituted haptens agree moderately well with experimental values. Similar rough agreement is also found between the calculated and observed values for the disubstituted phenylarsonic acids given in Tables I, III, IV, and V. The observed values for α- and β-naphthylarsonic acid agree approximately with those calculated for the corresponding dimethylarsonic acids.[17]

The Interpretation of Hapten Inhibition Data.—In this paper and preceding papers in this series data on the inhibition by haptens of the precipitation of antibodies and antigens have been discussed in terms of the framework theory of serological precipitation and of a quantitative theory of heterogeneous antisera.[4] It is worthy of mention that the evaluation and discussion of differences in the standard free energy of combination of different haptens with antibody is not dependent on the assumption of any particular theories of the precipitation of antibody and antigen. We may consider that the precipitation with antigen is used as a method of fixing a standard concentration of free antibody (for example, that which corresponds under the conditions of the experiment to the formation of one-half as much precipitate as is formed in the absence of hapten); the concentration of hapten necessary to reduce, by combination with part of the antibody, the concentration of free antibody to the standard value is determined experimentally, and the ratio of concentrations for two haptens may then be introduced in the well-known equation relating standard free energy change and equilib-

(16) V. Meyer and J. J. Sudborough, *Ber.*, **27**, 1580 (1894); V. Meyer, *ibid.*, **28**, 1254 (1895); R. H. Birtles and G. C. Hampson, *J. Chem. Soc.*, 10 (1937); C. E. Ingham and G. C. Hampson, *ibid.*, 981 (1939); J. F. J. Dippy and R. H. Lewis, *ibid.*, 1426 (1937); J. F. J. Dippy, D. P. Evans, J. J. Gordon, R. H. Lewis, and H. B. Watson, *ibid.*, 1421 (1937).

(17) A pronounced and unexplained discrepancy is shown by 2,4-dichlorophenylarsonic acid with anti-R_o serum and R_o-ovalbumin; its calculated value of K'_0 is 6.3, and the observed value is 0.91.

rium constant to give a value of the difference in the standard free energy change of combination of the two haptens with antibody. The only assumption underlying our treatment of hapten inhibition data is that inhibition of precipitation is the result of combination of the antibody with molecules of the hapten.

This investigation was carried out with the aid of a grant from The Rockefeller Foundation. Mr. Allan L. Grossberg, Mr. George Cleland, and Mr. Dan Rice aided in the preparation of antigens and haptens and in the analytical work. We are indebted to Professor Dan H. Campbell and Dr. Verner Schomaker for their interest and advice.

Summary

Experiments have been made on the precipitation of antisera homologous to the *o*-, *m*-, and *p*-azophenylarsonic acid groups, prepared by injecting rabbits with sheep serum coupled with diazotized *o*-, *m*-, and *p*-arsanilic acids, with azo-ovalbumins containing these groups, and on the effect of various haptens, mainly substituted phenylarsonic acids, in inhibiting this precipitation.

It has been found that the relative values of the hapten-inhibition constants of the substituted phenylarsonic acids can be in large part accounted for by consideration of the operative intermolecular forces, including electronic van der Waals attraction of the substituent group and the antibody, the formation of hydrogen bonds, and steric hindrance.

PASADENA, CALIFORNIA RECEIVED DECEMBER 26, 1944

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

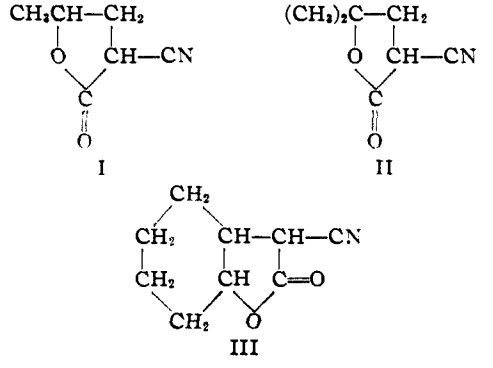
Condensation of Ethyl Cyanoacetate with Alkene Oxides

BY SAMUEL A. GLICKMAN AND ARTHUR C. COPE

The reaction of alkene oxides with the sodium derivatives of ethyl malonate and ethyl acetoacetate has been used as a method for the preparation of α-carbethoxy and α-acetyl-γ-lactones.[1-5] In these condensations, the base (sodium ethoxide) and alkene oxides were used in equivalent amounts. Two instances have been reported in which improved yields were obtained by using two molar equivalents of ethyl malonate.[6,7]

This paper describes the condensation of ethyl cyanoacetate with propylene oxide, isobutylene oxide and cyclohexene oxide, in the presence of one equivalent and one-tenth equivalent of sodium ethoxide, and an investigation of the structures of the reaction products.

Condensation of ethyl cyanoacetate with the three alkene oxides in the presence of one molar equivalent of alcoholic sodium ethoxide proceeded simply to the α-cyano-γ-lactones I, II and III.



The structures of the α-cyanolactones were established by hydrolysis to α-carboxylactones, which were decarboxylated by distillation to known γ-lactones. These γ-lactones were converted to known solid derivatives of the corresponding γ-hydroxy acids by reaction with phenylhydrazine or ammonia. Cleavage of the unsymmetrical alkene oxides by attachment of the enolate anion to the least substituted carbon atom is in agreement with the mode of cleavage observed by Rothstein in reactions with ethyl malonate.[8]

The course of the reaction [7,9-11] is presumed to involve attachment of the enolate anion to the least substituted carbon atom with rupture of the oxide link, lactonization of the resulting anion and elimination of ethoxide ion.

Surprisingly, the expected α-cyanolactone II was not isolated from the condensation of isobutylene oxide with ethyl cyanoacetate in the presence of one-tenth equivalent of sodium ethoxide. Instead, a product IV with the molecular formula $C_9H_{13}NO_3$ (m. p. 64.5–65.5°) was isolated. IV was soluble in dilute hydrochloric acid at room temperature, and hydrolyzed without heating to give α-carbethoxy-γ-isocaprolactone (V). V was identified by hydrolysis to α-

(1) Traube and Lehmann, *Ber.*, **34**, 1971 (1901).
(2) Haller and Blanc, *Compt. rend.*, **142**, 1471 (1906).
(3) Coffey, *Rec. trav. chim.*, **42**, 387 (1923).
(4) Kötz and Hoffman, *J. prakt. Chem.*, **110**, 101 (1925).
(5) Rothstein, *Bull. soc. chim.*, [5] **2**, 80 (1935).
(6) Leuchs, *Ber.*, **44**, 1507 (1911).
(7) Grigsby, Hind, Chanley and Westheimer, THIS JOURNAL, **64**, 2606 (1942).
(8) Rothstein, *Bull. soc. chim.*, [5] **2**, 1936 (1935).
(9) Hudson and Hauser, THIS JOURNAL, **63**, 3162 (1941).
(10) Dubinen and Chelentsev, *J. Gen. Chem.* (U.S.S.R.) **7**, 2368 (1937); *C. A.*, **32**, 2123 (1938).
(11) Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1940, pp. 143, 303.