INHIBITION OF THE PRECIPITIN REACTION BY OPTICALLY ACTIVE HAPTENS AND BY DERIVATIVES OF THE HOMOLOGOUS HAPTEN

Thesis of
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INTRODUCTION

The work described in this thesis is part of a program of investigation of immunological reactions being carried out at the California Institute of Technology by Drs. Linus Pauling, David Pressman, Dan H. Campbell, and others. Part I consists largely of the results reported in Paper X of the series "The Serological Properties of Simple Substances", published in the Journal of the American Chemical Society.

The phenomomen of hapten inhibition was discovered by Landsteiner, when, to explain the fact that no precipitation occurred with an excess of test azoprotein, he postulated that a reaction did occur between the antibodies and the specific groups of the test antigen, but the products were soluble. A similar effect should be excpected if molecules of the simple hapten were present in the solution; i.e., the amount of precipitate formed with optimum amounts of antiserum and antigen should be much less with hapten present than with antiserum and antigen alone. This effect was observed by Landsteiner.[1]

It was also found that simple compounds having a structure closely similar to that of the homologous hapten would inhibit the precipitation, though to a lesser degree. This fact has been utilized in this investigation as described in Part I, using d- and l-N-(α-methylbenzyl)succinamic acid, a derivative of N-benzylsuccinamic acid which closely resembles the homologous hapten, succinanilic acid. The affect of various substituents in various positions on the molecule of the homologous hapten is described in Part II.

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(1) K. Landsteiner, Biochem. Zeitshft., 104, 280 (1920).

PART I

INHIBITION OF THE PRECIPITIN REACTION BY OPTICALLY ACTIVE HAPTENS

It was shown by Landsteiner and van der Scheer[2,3] that by innoculating animals with suitable antigens antisera can be produced which can distinguish between optical isomers. Their first experiments were carried out with antisera prepared with use of immunizing antigens made by coupling proteins with diazotized d- and l-p-aminobenzoylphenylaminoacetic acid; each of the two antisera precipitated preferentially the test azoprotein containing the corresponding hapten. Similar results were also obtained with azoproteins containing azo derivatives of the stereoisomeric tartranilic acids as haptenic groups.

These experimental results show the significance of spatical configuratio in serological reactions. They do not, however, depend in any way on the fact that antibodies themselves have inherent optical activity. Because of the optical activity (l-configuration) of the amino acid residues of proteins, the possibility exists that an antiserum made by use of an antigen prepared from an optically inactive haptenic substance may combine preferentially with one of a pair of optically isomeric substances. Such an antiserum (anti-S_p serum) was prepared by injecting rabbits with an azoprotein made from the inactive substance succinanilic acid. It was found by hapten-inhibition experiments that the antibodies in the serum combine more strongly with l-N-(α-methylbenzyl)succinamic acid than with the d isomer.

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(2) K. Landsteiner and J. van der Scheer, J. Exptl. Med., 48, 315(1928).

(3) K. Landsteiner and J. van der Scheer, ibid., 50, 407(1929).

PREPARATION OF COMPOUNDS

Succinanilic acid was prepared by the method of Auwers[4] by slowly adding 0.16 mole of aniline to a boiling solution of 0.15 mole of succinic anhydride in 150 ml. of chloroform. The reaction took p lace immediately with precipitation of succinanilic acid. The product was recrystallized from water, with a yield of 68%. M. p., 147.2-147.7°; reported, 148.5°.[4] Acidic equivalent weight: calcd. for $C_{10}H_{11}O_3N$, 193.1; found, 191.4, 191.7.

p-Nitrosuccinanilic acid was prepared by Dr. W. B. Renfrow, Jr., by mixing hot solutions of 0.2 mole of succinic anhydride and 0.2 mole of p-nitraniline in 50 ml. portions of dioxane and heating in a boiling water bath for five minutes. The p-nitrosuccinanilic acid crystallized on cooling. M. p., 194-195°; reported, 194-195°.[5]

p-Aminosuccinanilic acid was prepared by Dr. W. B. Renfrow, Jr., by reducing 0.08 mole of p-nitrosuccinanilic acid in 175 ml. of 90% methnaol with hydrogen at 3 atms. over a catalyst of paddadium on calcium carbonate. The aminosuccinanilic acid precipitated on cooling. It was dissolved in the theoretical amount of sodium hydroxide solution, filtered, and reprecipitated with the theoretical amount of hydrochloric acid. M. p., 184-185°; reported, 183-184°.[5]

d- and l-α-Methylbenzylamines were prepared by resolution of Eastman dl-α-methylbenzylamine using the method of Lovén.[6] The dl-amine was redistilled

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(4) K. Auwers, Ann., 309, 326(1899).

(5) K. Landsteiner and J. van der Scheer, J. Exptl. Med., 56, 399(1932).

(6) J. M. Lovén, J. prak. Chem., 72, 310(1905).

through 5 cm. of glass rings, the fraction boiling from 186.0 to 186.6° (uncorr.) being taken. 0.43 mole of the amine was added to a solution of 0.44 mole of ℓ-malic acid in 235 ml. of water. Crystallization took place over night. Yield of dried d-α-methylbenzylammonium-ℓ-malate was 52.0 gms., 94% of the theoretical quantity.

The filtrate from the crystals of d-α-methylbenzylammonium-ℓ-malate was made basic with sodium hydroxide and the released amine extracted with ether. The ether was removed on a water bath, and a solution of 0.22 mole of d-tartaric acid in 130 ml. of water added to the amine. It was allowed to crystallize over night. The yield of ℓ-α-methylbenzylammonium-d-tartrate was 36.0 gms., 61% of the theoretical quantity.

The d-α-methylbenzylammonium-ℓ-malate and ℓ-α-methylbenzylammonium-d-tartrate crystals were recrystallized twice from water.

The free amines were prepared by adding sodium hydroxide solution to the salts, extracting with ether, removing the ether on the water bath, and distilling. The specific gravities and optical rotations were taken:

d-α-Methylbenzylamine: b. p., 185.5-186.0° (uncorr.); $d_4^{26.5} = 0.9475$; $[\alpha]_D^{26.5} = +39.2°$ ($\alpha = +37.16°$, $\ell = 1$ dm.); yield, 11.4 gms., 44%.

ℓ-α-Methylbenzylamine: b. p., 186.0-186.5° (uncorr.); $d_4^{26.5} = 0.9466$; $[\alpha]_D^{26.5} = -39.4°$ ($\alpha = -37.32°$, $\ell = 1$ dm.); yield, 9.6 gms., 38%.

d-(α-Methylbenzyl)succinamic aicd was prepared by adding 0.091 mole of d-α-methylbenzylamine in 25 ml. of chloroform to a chloroform solution of 0.091 mole of succinic anhydride. When all the succinic anhydride had dissolved the solution was extracted with a solution of 0.091 mole of sodium hydroxide. This aqueous solution was extracted with ether and the acid precipitated with hydrochlorie acid. It was recrystallized from water.

Yield, 12.6 gms., 63%. M. p., 100.9-101.1°(corr.); $[\alpha]_D^{25}$ = +129.2° (α = +2.586°, ℓ = 2 dm., c = 10.01 gms./liter). Acidic equivalent weight: calcd. for $C_{12}H_{15}O_3N$, 221.1; found, 220.9, 221.5.

ℓ-(α-Methylbenzyl)succinamic acid was prepared in the same manner form 0.075 mole of the amine and 0.075 mole of succinic anhydride. Yield, 10.4 gms., 63%. M. p., 100.9-101.2° (corr.); $[\alpha]_D^{25}$ = -127.0° (α = -2.540°, ℓ = 2 dm., c = 10.00 gms./liter). Acidic equivalent weight: calcd. for $C_{12}H_{15}O_3N$, 221.1; found, 221.6, 221.9.

Preparation of Protein Anitgens.--The immunizing antigen was prepared by diazotizing 0.006 mole of p-aminosuccinanilic acid and coupling with 100 ml. of sheep serum at pH 9. The antigen was purified according to the directions of Landsteiner and van der Scheer.[3]

The test antigen (S_p-ovalbumin) was prepared by diazotizing 0.0002 4 mole (0.050 gm.) of p-aminosuccinanilic aicd and coupling with 0.50 gm. of ovalbumin at about pH 9. The azoportein was dialyzed overnight against tap water, precipitated twice at pH 3.5 from 50 ml. of solution, and finally dissolved in saline solution at pH 7.

Antisera.--The method of preparing and pooling antisera has been described elsewhere by Pauling and his coworkers.[7]

Reaction of Antiserum with Antigen and Hapten.--The reactants were mixed and permitted to stand for one hour at room temperature and over two nights at 5° C. The precipitates were centrifuged and washed three times with 10-ml. portions of 0.9% sodium chloride solution and were analyzed by the standard method.[8]

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(7) L. Pauling, D. Pressman, D. H. Campbell, C. Ikeda, and M. Ikawa, J. Am. Chem. Soc., 64, 2994 (1942).

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

DISCUSSION

The results of the hapten inhibition experiments are given in Table I and Figure 1. The values of 0.8 to 3.5 of the heterogeneity index σ obtained on application of the theory of heterogeneous antisera[9] to the data are similar in magnitude and in trend to those found for other systems. The values of the hapten inhibition constant K_o' (aside from the difference for the d and l haptens) are reasonable; succinanilic acid, which is very closely related in structure to the haptenic group of the immunizing antigen (the N-(p-azophenyl)-succinamic acid group), combines five to ten times more strongly with the antibody than the optically active haptens, and about fifty times more strongly than the simple hapten succinic acid.

Data were obtained for three different amounts of the precipitating antigen, S_p-ovalbumin, extending into the regions of antibody excess and antigen excess. It is interesting that in these experiments a given amount of hapten was found to be more effective in inhibiting precipitation the greater the amount of antigen; this corresponds to the simple theory of homogeneous antibody,[10] but is contrary to earlier work with another type of antiserum.[9]

The striking result of the experiments is the difference in inhibiting power shown by the d and l isomers of N-(α-methylbenzyl)succinamic acid, amounting in the region of antibody excess to a factor in K_o' greater than 2, and to a difference of about 500 cal. per mole in the standard free energy of combination of hapten and antibody. This difference is presumably to be attributed to the presence of optically active amino acid residues in the antibody γ-globulin, causing the stable configurations complementary to the symmetric haptenic group of the immunizing antigen to be asymmetric. An

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(9) L. Pauling, D. Pressman, and A. L. Grossberg, J. Am. Chem. Soc., 66, 784(1944).

(10) L. Pauling, D. Pressman, D. H. Campbell, and C. Ikeda, ibid., 64, 3003 (1942).

alternative but less likely explanation could be based on the presence of optically active amino acid residues in the protein part of the immunizing antigen; this exp lanation is rendered improbable by the fact that the part of the haptenic group in the immunizing antigen which corresponds to the asymmetric carbon atom of the d and l haptens is separated from the asymmetric carbon atom of the amino acid residue by a considerable distance (the length of a benzene ring, azo group, and the amino acid side chain.).

Our present knowledge of the structure of antibodies is not sufficiently detailed to permit an interpretation to be made of the observation that it is the l isomer which combines the more strongly with the antibody.

A reasonable explanation can be given of the observed dependence of the optical isomer effect on the amount of precipitating antigen and on the amount of hapten. The antiserum is heterogeneous--it contains antibodies which in configuration approximate only roughly to the haptenic group of the immunizing azoprotein and the precipitating azoportein, and hence exert only weak attraction for these groups, and antibodies which fit very closely to the haptenic group, and form strong bonds with it. It is to be expected that weak antibodies, fitting the haptenic group only loosely, could be built in a great many different ways, of which some would combine more strongly with the d isomer of a pair of asymmetric haptens than with the l isomer, and others would combine more strongly with the l isomer than with the d isomer, and that these preferential effects would largely cancel each other. A very good antibody molecule, however, would have to assume a very well-defined configuration, bringing it into the closest approximation to the haptenic group and locating and orienting its charged groups and hydrogen-bond-forming groups in the most satisfactory way for attracting and holding the haptenic group; the nature

of this more complementary configuration would be determined by the fact that the building stones are *l*-amino acid residues, and the configuration would accordingly be expected to correspond more closely to one than to the other of an enantiomorphic pair of asymmetric molecules.

When a small amount of antigen is added to a portion of antiserum, it is the antibody molecules with the greatest attraction for the antigen which form the precipitate with it. The argument given above leads to the expectation that the optical isomer effect would be larger in this case than when a large amount of precipitating antigen is used. This expectation is confirmed by the experimental results.

Moreover, in the region of the equivalence zone, with the precipitate containing poor antibody as well as good antibody, the part of the precipitate which is dissolved first on addition of hapten is that containing the poor antibody (with small conbining power for the haptenic group of the immunizing antigen and precipitating antigen); the good antibody tends to remain in the undissolved precipitate. Accordingly, by the argument given above, the difference in effect of *d* and *l* haptens would be small at low hapten concentrations and larger at high hapten concentrations, at which a considerable fraction of the precipitate is dissolved. This effect, shown clearly by the middle pair of curves in Figure 1, leads to a difference in apparent heterogeneity of the antiserum with respect to the isomers.

The small difference shown by the isomeric haptens in the region of slight antigen excess may be the result of the action of the excess of antigen in favoring the formation of soluble complexes involving good antibody molecules.

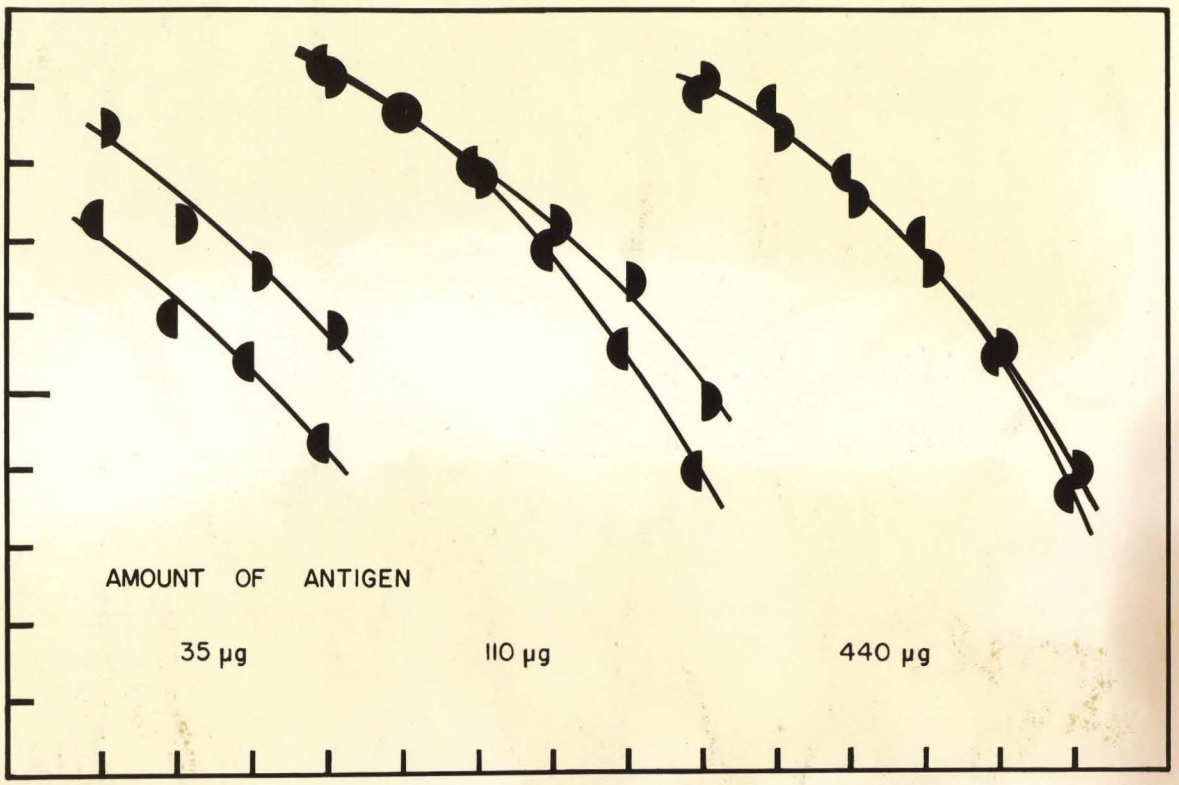

Figure 1.--Effect of d- and l-N-(α-methylbenzyl)succinamic acid in inhibiting the precipitation of the anti-S_p serum and S_p-ovalbumin; points for the d and l haptens respectively are represented by the right and left halves of circles.

Table I

Effect of Optically Active Haptens on the Precipitation of Anti-S_p Serum with S_p-Ovalbumin

Antigen solution in borate buffer at pH 8, 1 ml.; antiserum, 1 ml.; hapten solution in saline, 1 ml.; pH of supernates 8.2 [a]

Amount of Antigen, μg.	Hapten [b]	K'_0	σ	Moles of hapten added, x 10^8						
				15.6	31.3	62.5	125	250	500	1000
				Amount of precipitate [c]						
35	d-I	0.070	3.5				845	720	657	577
	l-I	.167	3.5				724	594	540	435
110	d-I	.092	3.5		909	864	777	717	642	485
	l-I	.165	2.5		927	864	(791)	685	553	397
	II	1.00	1.5	883	785	652	439			
	III								864	799
440	d-I	0.17	3.0		903	(834)	(749)	656	552	394
	l-I	.19	2.0		887	(871)	786	702	543	363
	II	1.00	0.8	966	(877)	741	503			
	III								927	882

For notes see next page.

Notes to Table I

[a] Optimum precipitation 850 μg. at 220 μg. of antigen.

[b] I = N-(α-Methylbenzyl)succinamic acid; II = succinanilic acid; III = succinic acid.

[c] The amounts of precipitate are in parts per mille of the amounts in the absence of hapten: 680,660, and 239 μg. Blanks of serum and buffer 8.8, and 16 μg., respectively. Values are averages of triplicate analyses, with mean deviation ±2%, except for duplicate analyses in parentheses.

PART II

INHIBITION OF THE P RECIPITIN REACTION BY DERIVATIVES OF THE HOMOLOGOUS HAPTEN

A number of hapten inhibition experiments were performed by Landsteiner and van der Scheer[11] with anti-S_p serum, employing a number of substances related to but not derivatives of the homologous hapten with the exception of p-nitro- and p-aminosuccinanilic acids. The experiments described below, which use a number of such derivatives, have been carried out in a manner previously employed with anti-benzoic acid serum.[12]

PREPARATION OF COMPOUNDS

Succinanilic acid was prepared by the method of Auwers[2] by adding 0.15 mole of aniline to a boiling chloroform solution of 0.15 mole of succinic anhydride. The crude yield was nearly quantitative. It was purified by dissolving in the calculated amount of sodium hydroxide solution, extracting twice with ether, reprecipitating with hydrochloric acid, and recrystallizing from water.

M. p., 145.1-146.9° (corr.); reported, 148.5°.[4] Acidic neutral equivalent: calcd. for $C_{10}H_{11}O_3N$, 193.1; found, 191.1, 192.2.

p-Nitrosuccinanilic acid was prepared by the method of H. E. Fierz-David, W. Jadassohn, and W. F. Zürcher[13] by dissolving 0.10 mole of succinic anhydride and 0.10 mole of p-nitraniline separately in 125 ml. of chlorobenzene and pouring the two solutions together. Crystallization soon occurred throughout the solution. The crystals were filtered (yield, 81%), and purified by dissolving in sodium hydroxide solution, extracting with ether, reprecipitating

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(11) K. Landsteiner and J. van der Scheer, J. Exptl. Med., 59, 751 (1934).

(12) D. Pressman, S. M. Swingle, A. L. Grossberg, and L. Pauling, J. Am. Chem. Soc., 66, 1731 (1944).

(13) H. E. Fierz-David, W. Jadassohn, and W. F. Zürcher, Helv. Chim. Acta, 20, 21 (1937).

with hydrochloric acid, and recrystallizing from alcohol.

M. p., 194.4° (corr.); reported, 194-195°.[5] Acidic equivalent weight: calcd. for $C_{10}H_{11}O_5N_2$, 238.1; found, 240.8, 241.6.

p-Aminosuccinanilic acid was prepared by Dr. W. B. Renfrow, Jr., as described previously. M. p., 184-185°; reported, 183-184°.[5] Acidic equivalent weight: calcd. for $C_{10}H_{12}O_3N_2$, 208.1; found, 207.2, 208.3.

o-Bromosuccinanilic acid was prepared by the method of Auwers[4] from 0.06 mole of o-bromoaniline and 0.075 mole of succinic anhydride in chloroform solution. Yield, 9.0 gms., 57%. It was purified by dissolving in sodium hydroxide solution, extracting with ether, precipitating with hydrochloric acid, and recrystallizing from water. M. p., 154.1-156.1° (corr.). Acidic equivalent weight: calcd. for $C_{10}H_{10}O_3NBr$, 272.1; found, 270.1, 270.4

m-Bromosuccinanilic acid was prepared and purified in the same manner from 0.06 mole of m-bromoaniline and 0.075 mole of succinic anhydride. Yield, 9.0 gms., 57%.

M. p., 150.9-151.9° (corr.). Acidic equivalent weight: calcd. for $C_{10}H_{10}O_3NBr$, 272.1; found, 266.7, 266.7.

p-Bromosuccinanilic acid was prepared and purified in the same manner from 0.17 mole of p-bromoaniline and 0.17 mole of succinic anhydride. It was recrystallized from alcohol. Crude yield, 44.7 gms., 97%; purified yield, 27.9 gms., 65%.

M. p., 187.2-188.2° (corr.); reported, 186-187°.[14] Acidic equivalent weight: calcd. for $C_{10}H_{10}O_3NBr$, 272.1; found, 272.7, 270.8.

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(14) Beilstein, Vol. XII, p. 644.

N-(α-Naphthyl)succinamic acid was prepared by the method of Auwers[4] from 0.14 mole of α-naphthylamine and 0.14 mole of succinic anhydride. It was purified by dissolving in sodium hydroxide solution, extracting with ether, reprecipitating with hydrochloric acid, and recrystallizing from alcohol. Yield of purified material, 13.0 gms., 38%.

M. p., 171.1-171.6° (corr.). Acidic equivalent weight: calcd. for $C_{14}H_{13}O_3N$, 243.1; found, 241.4, 241.2.

N-(β-Naphthyl)succinamic acid was prepared and purified in the same manner described above from 0.14 mole of β-naphthylamine and 0.14 mole of succinic anhydride. Yield of purified material, 23.3. gms., 68%.

M. p., 189.4-190.0° (corr.); reported, 184-185°.[15] Acidic equivalent weight: calcd. for $C_{14}H_{13}O_3N$, 243.1; found, 240.5, 240.6.

p-(p-Hydroxyphenylazo)succinanilic acid was prepared by diazotizing 0.01 mole of p-aminosuccinanilic acid, making alkaline, and adding the solution to an alkaline solution of 0.10 mole of phenol. Coupling wa s complete in less than 30 minutes. The pH of the solution was adjusted to about 7, and it was extracted twice with ether. The acid was precipitated with hydrochloric acid, filtered, and recrystallized twice from alcohol and water using decolorizing charcoal the second time. Yield, 1.33 gms., 42%.

M. p., 231.5° (corr.) with decomposition. Acidic equivalent weight: calcd. for $C_{16}H_{15}O_4N_3$, 313.2; found, 312.7.

Preparation of Protein Antigens.--The immunizing antigen was prepared by the method of Landsteiner and van der Scheer[3] by diazotizing 0.006 mole

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(15) K. Auwers, Ann., 292, 190 (1896).

of p-aminosuccinanilic acid, and adding it to 100 ml. of cold alkaline sheep serum. Coupling was allowed to take place over night. The azoprotein was precipitated with hydrochloric acid, centrifuged, dissolved in a small amount of sodium carbonate solution, and again precipitated by adding ethyl alcohol. After centrifugin the azoprotein was ground with a little water and the volume made up to 200 ml. with 0.9% saline.

The test antigen was prepared by diazotizing 0.001 mole of p-aminosuccinanilic acid, making the solution alkaline, and adding it to a basic solution of 2.00 gms. of crystalline ovalbumin. Coupling was complete after 45 minutes. The solution was dialyzed over nightin sausage casing against tap water. The pH of the solution was adjusted to 3.5, the precipitated azoprotein centrifuged, and dissolved in a small amount of sodium hydroxide solution. It was again precipitated, centrifuged, and dissolved. The volume of the solution was made up to about 150 ml. with saline and the pH adjusted to about 7.

Reaction of Antiserum with Antigen and Hapten.--The reactants were mixed and permitted to stand for one hour at room temperature and over two nights at 5° C. The precipitates were centrifuged and washed three times with 10-ml. portions of 0.9% sodium chloride solution and were analyzed by the standard method.[8]

DISCUSSION

The data obtained in the hapten inhibition experiments were interpreted with the aid of the extended quantitative theory of the phenomomen.[9] Application of this theory leads to the evaluation of two constants for each antibody-antigen-hapten system: one of these constants, K'_o, is an average hapten inhibition constant, representing the average bond strength of antibody and hapten relative to that of antibody and antigen, and the other constant, σ ,

is an index of the effective heterogeneity of the antiserum. Values for K_0' and σ for the haptens investigated are given in Table II. The reference point for K_o' is taken as $K_0' = 1.00$ for the hapten succinanilic acid.

The results are similar to what has been obtained previously in this type of experiment,[12] with one notable exception: the p-(p-hydroxyphenylazo)succinanilic acid did not inhibit as well as the p-nitro- and p-bromosuccinanilic acids. This result would not be expected, as the N-(p-phenylazo)succinamic acid group is present in the immunizing antigen, and accordingly a compound containing such a group should inhibit better than any of the other related compounds tested. The reasons for the observed results are not clear.

For the bromine substituent, the dependence of the inhibition constant on position in the ring is $p > m > o$. This is observed with the benzoic acid derivatives, as shown in Table III. The change of the bromine from the para to the meta position changes the inhibition constant by a factor of 3, and the change from meta to ortho changes the constant by a factor of about 1.3. For benzoic acid these factors are 4 and 17 respectively.

The order of activity of groups in the para position of succinanilic acid is $NO_2 > Br > HOC_6H_4NN > NH_2$. The order of activity for benzoic acid is $HOC_6H_4NN > NO_2 > Br \gg NH_2$. The reduced activity of the HOC_6H_4NN group in the succinanilic acid series constitutes the only major difference between these two series. The benzoic acid series, however, shows greater extremes of values for K_0' (varying from 0.074 to 21.9) than the succinanilic acid derivatives (values ranging from 0.55 to 4.22).

The greater inhibiting effect of compounds with groups in the para position is due to the interaction of these groups with the antibody,

which is complementary to a para-azo group. The effect of substitution in the meta and ortho positions seems to be steric, for the antibody would be unable to fit closely around the hapten molecule. The decrease in effectiveness is greater for the ortho than for the meta position. The greatly decreased effectiveness of N-(α-naphthyl)succinamic acid, which can be considered as a 2,3-substituted succinanilic acid, is likewise due to the steric effect. However, the increased effect of N-(β-naphthyl)succinamic acid (a 3,4-substituted succinanilic acid) is again due to interaction of the para-substituent with the antibody.

Table II

Effect of Substituted Haptens on the Precipitation of Anti-S_p Serum with S_p-Ovalbumin

Antigen solution in borate buffer at pH 8, 1 ml.; antiserum (original antiserum diluted with equal volume of buffer), 1 ml.; hapten solution in saline, 1 ml.

			Moles of hapten added, x 10^8						
	K'_0	σ [b]	15.6	31.3	62.5	125	250	500	1000
			Amount of precipitate [a]						
p-(p-Hydroxyphenylazo)-succinanilic acid	2.00	0.8		395	221	61			
Succinanilic acid	1.00	1.3			373	228	86		
o-Bromosuccinanilic acid	0.71	(1)					139	9	4
m-Bromosuccinanilic acid	.915	0.8				246	76	7	
p-Bromosuccinanilic acid	2.72	1.0	471	338	151	18			
p-Nitrosuccinanilic acid	4.22	1.0	379	206	77	2			
p-Aminosuccinanilic acid	1.41	(1.5)			304	167	(29)		
N-(α-Naphthyl)-succinamic acid	0.55	(1)					199	72	5
N-(β-Naphthyl)-succinamic acid	1.55	(1)			(284)	94	6		

For notes see next page.

Notes to Table II

[a] The amounts of precipitate are in parts per mille of the amounts in the absence of hapten: 657 μg. with S_p-ovalbumin. Blank of serum and buffer, 14 μg. Values are averages of triplicate analyses, with mean deviation $\pm$2.3%, except for duplicate analyses in parentheses.

[b] Values of σ in parentheses are assumed, there being no more than two points to determine the heterogeneity curve.

Table III

Comparison of Inhibition Constants and Heterogenity Indexes for Substituted Benzoic and Succinanilic Acids

Substituent	Benzoic Acid		Succinanilic Acid	
	K'_0	σ	K'_0	σ
----------	1.00	2.5	1.00	1.3
p-(p-Hydroxyphenyl-azo)-	21.9	2.0	2.00	0.8
o-Bromo-	0.074	2.8	0.71	(1)
m-Bromo-	1.25	2.0	.915	0.8
p-Bromo-	5.0	2.5	2.72	1.0
p-Nitro-	11.5	3.0	4.22	1.0
p-Amino-	0.88	3.0	1.41	(1.5)
α-Naphthyl-derivative	0.18	4.0	0.55	(1)
β-Naphthyl-derivative	10.7	3.0	1.55	(1)