only 6.6% of the total entropy for $CaO \cdot B_2O_3$ and is lower for the other substances.

The entropies of $3CaO \cdot B_2O_3$, $2CaO \cdot B_2O_3$, and $CaO \cdot B_2O_3$ differ, in order, by 9.2 and 9.6 units, corresponding to successive decreases of one mole of calcium oxide. These figures are to be compared with the measured value for free calcium oxide,[10] $S^0_{298.16} = 9.5 \pm 0.2$. This type of approximate additivity of entropies of some inter-oxidic compounds has been noted previously in work of this Laboratory and is the result of compensation of plus and minus deviations from additivity of heat capacities. In the case of $CaO \cdot 2B_2O_3$ such compensation is quite incomplete and the entropy difference between $CaO \cdot 2B_2O_3$ and $CaO \cdot B_2O_3$ is only 7.1 units, whereas the entropy of crystalline boric oxide[11] is $S^0_{298.16} = 13.0 \pm 0.1$.

Related Thermal Data.—Free energies of formation at 298.16°K. of the four calcium borates from the oxides and from the elements are given in Table III, being obtained from the relationship $\Delta F^0 = \Delta H - T\Delta S$. The heats of formation, $\Delta H_{298.16}$, are from the paper of Torgeson and Shomate.[5] The entropies employed in calculation of the $\Delta S_{298.16}$ values are from publications of Kelley.[10,11]

Precision uncertainties have been assigned to the free energies of formation from the oxides. It is not possible to do this for the values from the elements because the probable error in the heat of formation of crystalline boric oxide, on which the free energies depend, is not known.

TABLE III

FREE ENERGIES OF FORMATION AT 298.16°K., CAL./MOLE

Substance	$\Delta H_{298.16}$	$\Delta S_{298.16}$	$\Delta F^0_{298.16}$
	From oxides		
$3CaO \cdot B_2O_3$	−60,000 ± 40	2.4 ± 0.7	−60,720 ± 210
$2CaO \cdot B_2O_3$	−45,760 ± 30	2.7 ± 0.5	−46,570 ± 150
$CaO \cdot B_2O_3$	−29,420 ± 20	2.6 ± 0.3	−30,200 ± 90
$CaO \cdot 2B_2O_3$	−42,930 ± 20	−3.3 ± 0.5	−41,950 ± 150
	From elements		
$3CaO \cdot B_2O_3$	−858,200	−136.4 ± 0.6	−817,500
$2CaO \cdot B_2O_3$	−692,100	−111.2 ± 0.5	−659,000
$CaO \cdot B_2O_3$	−524,000	− 86.3 ± 0.5	−498,300
$CaO \cdot 2B_2O_3$	−880,200	−156.2 ± 0.9	−833,700

The free energy of formation values from the oxides follow a normal pattern. The formation of $CaO \cdot B_2O_3$ from the oxides gives a decrease in free energy of 30,200 cal. Smaller decreases in free energy accompany each successive step of adding one mole of oxide to $CaO \cdot B_2O_3$ to form the other calcium borates.

Summary

Low temperature heat capacity measurements of $3CaO \cdot B_2O_3$, $2CaO \cdot B_2O_3$, $CaO \cdot B_2O_3$, and $CaO \cdot 2B_2O_3$ were made throughout the temperature range 52° to 298.16°K.

The entropies of the four calcium borates were determined as 43.9 ± 0.3, 34.7 ± 0.2, 25.1 ± 0.2, and 32.2 ± 0.3 cal./deg./mole, respectively.

Free energy of formation values from the oxides and from the elements are included.

BERKELEY, CALIFORNIA RECEIVED OCTOBER 24, 1947

(10) Kelley, *Bur. Mines Bull.*, 434 (1941).

(11) Kelley, THIS JOURNAL, **63**, 1137 (1941).

[CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, No. 1154]

The Reactions of Antiserum Homologous to the *p*-Azosuccinanilate Ion Group[1a]

BY DAVID PRESSMAN,[1b] JOHN H. BRYDEN AND LINUS PAULING

It was discovered by Landsteiner and van der Scheer[2] that the precipitation of azoprotein containing the *p*-azosuccinanilate ion haptenic group by hapten-homologous antiserum (anti-S_p serum) is inhibited just as well by maleate ion as by succinate ion, whereas fumarate ion is practically ineffective, and from this observation the cautious conclusion was drawn[3,4] that "Accordingly, one could suppose that the succinic acid molecule can exist in a form corresponding to the *cis* configuration, or that the antibodies adjust themselves to this." Because of our interest in the use of immunochemical techniques for the determination of the configuration of molecules and haptenic groups,[5] we have extended our quantitative studies of hapten inhibition of serological precipitation to include the S_p system, and have investigated the effect of over fifty haptens on the precipitation of S_p-ovalbumin and anti-S_p serum. The analysis of the data has shown that the normal configuration of the *p*-azosuccinanilate ion group in aqueous solution is a *cis* configuration, presumably stabilized by a hydrogen bond, and has provided information about the configuration of other ions.

Experimental Methods

Haptens.—The following substances used in this work have been described previously[6]: succinanilic acid, *p*-aminosuccinanilic acid, *p*-nitrosuccinanilic acid, and *d*-

(1a) The Serological Properties of Simple Substances. XIII. For No. XII of this series see D. Pressman, A. L. Grossberg, L. H. Pence, and L. Pauling, THIS JOURNAL, **68**, 250 (1946).

(1b) Present address: Sloan-Kettering Institute for Cancer Research, New York.

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

(3) K. Landsteiner, "The Specificity of Serological Reactions," Charles C Thomas, Springfield, Illinois, 1936, p. 129.

(4) K. Landsteiner, "The Specificity of Serological Reactions," Revised Edition, Harvard University Press, Cambridge, Mass., 1945, p. 192.

(5) D. Pressman, *Register of Phi Lambda Upsilon*, **29**, 30 (1944).

(6) D. Pressman, J. H. Bryden, and L. Pauling, THIS JOURNAL, **67**, 1219 (1945).

and *l*-N-(α-methylbenzyl)-succinamic acids. The substances prepared in this investigation are described in the following section. All other substances used were commercial preparations purified to the correct melting point and acidic equivalent weight.

Antiserum and Protein Antigens.—The preparation of antiserum and antigens used in this work has been described previously.[6] Only one pool of anti-S_p serum and one preparation of S_p-ovalbumin were used in these experiments.

Reaction of Antiserum with Antigen and Hapten.—One-milliliter portions of S_p-ovalbumin, anti-S_p serum, and hapten solution were mixed and permitted to stand about one hour at 37° and over two nights at 5°. The amount of antigen used, 320 μg. (by Nessler analysis), was that which gave optimum precipitation in the absence of hapten. The hapten solution was made with 0.9% sodium chloride solution and the antigen solution was made with borate buffer of *p*H 8.0.[7] The precipitates were centrifuged, washed three times with 10-ml. portions of 0.9% sodium chloride solution, and analyzed by our standard method.[8]

Preparation of Substances

Malonanilic acid was prepared by the method of Rügheimer,[9] by heating a mixture of 0.24 mole of malonic acid and 0.24 mole of aniline at 105° for one hour. The resultant mass was dissolved in 2 *N* sodium hydroxide solution and filtered, and the filtrate was acidified with hydrochloric acid. Malonanilic acid separated on partial evaporation and cooling. The product was recrystallized from water; m. p. 132.0–132.5°, reported 132°. *Acidic equivalent weight:* calcd. for $C_9H_9O_3N$, 179.1; found 185.6, 186.4.

Glutaranilic acid was prepared by heating a mixture of 0.06 mole of glutaric acid, 0.06 mole of aniline, and 2 g. of fused zinc chloride in an oil-bath at 160 to 170° for forty-five minutes. After cooling, the mixture was extracted with potassium hydroxide solution and was filtered, and the filtrate was acidified with hydrochloric acid. The crystals which separated were recrystallized from water; m. p. 127.0–128.0°, reported 126–127°.[10] *Acidic equivalent weight:* calcd. for $C_{11}H_3O_3N$, 207.1; found 208.8, 209.0.

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

(8) D. Pressman, *Ind. Eng. Chem., Anal. Ed.*, **51**, 357 (1943). It has been suggested by E. A. Kabat (*Ann. Rev. Biochem.*, **15**, 511 (1946)) that our experimental results are unreliable because the mixtures are allowed to stand only two days, instead of five, before the precipitates are removed and analyzed. We have continued to use the two-day period. for convenience, and we feel that no significant error is introduced thereby. If the tubes containing antiserum and antigen and those also containing hapten were allowed to stand three days longer the amounts of precipitate would increase somewhat, and their ratios might change slightly (by perhaps 5%), leading to correspondingly small changes in the derived values of K_0'. But the values of K_0' obtained with different pools of antiserum differ by as much as two-fold (although usually without changing the order of various haptens), so that the small expected effects of increasing the time of standing are unimportant.

If a true equilibrium were achieved in five days it might be worth while to adopt this longer period. However, on still longer standing the amount of precipitate decreases, presumably as the result of slow degradation of the materials; this suggests that the use of the shorter rather than the longer period may give the more reliable results.

The suggestion has also been made by W. C. Boyd and J. Behnke (*Science*, **100**, 13 (1944)), and repeated by Kabat, that some of the conclusions drawn from our experimental results may be invalidated by the polymerization (aggregation) of some of the haptens or simple precipitating antigens in solution. A detailed discussion of this question will be published shortly; it may be pointed out here that the hapten-inhibition studies reported in the present paper were made with an azoprotein, rather than a polyhaptenic simple substance, as precipitating antigen, and the haptens themselves are so simple as to have little tendency to aggregate, and that for these reasons (as well as others, to be discussed in the later paper) it is unlikely that the arguments presented are to any extent invalidated by the possibility of aggregation of the hapten molecules.

(9) L. Rügheimer, *Ber.*, **17**, 736 (1884).

Adipanilic acid was prepared by the method of Dieckmann,[11] by heating a mixture of 0.1 mole of adipic acid, 0.1 mole of aniline, and 2 g. of fused zinc chloride on an oil-bath at 150–160° for one and one-half hours. Upon cooling, the material was dissolved in sodium hydroxide solution and was filtered, and the adipanilic acid was precipitated with hydrochloric acid. The product was recrystallized from water: m. p. 152.0–153.0°, reported 152–153°. *Acidic equivalent weight:* calcd. for $C_{12}H_{15}O_3N$, 221.1; found, 216.9, 218.7.

Maleanilic acid was prepared by the method of Anschütz,[12] by adding 0.3 mole of aniline dissolved in 75 ml. of anhydrous ether to 0.26 mole of maleic anhydride dissolved in 250 ml. of anhydrous ether. The product precipitated as it was formed, and was purified by dissolving it with sodium hydroxide solution and reprecipitating with hydrochloric acid; m. p. 197–198°, reported 198°. *Acidic equivalent weight:* calcd. for $C_{10}H_9O_3N$, 191.1; found, 185.3, 190.5.

Fumaranilic acid was prepared by slowly adding 0.12 mole of aniline in 100 ml. of chloroform to 0.12 mole of fumaryl chloride in 100 ml. of chloroform. The chloroform was evaporated from the emulsion formed by the addition of 350 ml. of 1 *N* sodium hydroxide solution. The solution was filtered and the filtrate was acidified with hydrochloric acid. The precipitate was dissolved with sodium hydroxide solution and reprecipitated with hydrochloric acid; m. p., 238–238.5°, reported, 233–234.0°. *Acidic equivalent weight:* calcd. for $C_{10}H_9O_3N$, 191.1; found, 195.9, 196.9.

***d*-Tartranilic acid** was prepared by slowly adding 0.4 mole of aniline to 0.4 mole of *d*-α,β-diacetoxysuccinic anhydride in 400 ml. of chloroform at the refluxing temperature. The cooled solution was extracted with about 400 ml. of 1 *N* sodium hydroxide solution. The aqueous phase was treated with 200 ml. of concentrated hydrochloric acid. A colorless oil separated which dissolved on heating. Subsequent cooling produced crystals; m. p. 181.9–182.4°, reported 180°[13] $[\alpha]^{23}D$ in water, +106.2° (α, +2.13°, 1 dm., 20 g./l.); reported[14] $[\alpha]^{15}D$ +105.6°. *Acidic equivalent weight:* calcd. for $C_{10}H_{11}O_5N$, 225.1; found 224.8, 225.3.

α,β-Diacetylsuccinic anhydride was prepared by the method of Lucas and Pressman.[15]

***o*-Bromosuccinanilic acid, *m*-bromosuccinanilic acid, *p*-bromosuccinanilic acid, N-α-naphthylsuccinamic acid,** and **N-β-naphthylsuccinamic acid** were prepared by adding 0.06–0.2 mole of the appropriate amine to a boiling chloroform solution of an equimolar amount of succinic anhydride. The products precipitated as formed and were purified by dissolving in sodium hydroxide solution, extracting with ether, precipitating from the aqueous phase with hydrochloric acid, and finally crystallizing from water or alcohol. The melting points and acidic equivalent weights are as follows:

Substance, acid	Formula	M. p., °C.	Acidic equivalent weight Calcd.	Obs.
o-Bromosuccinanilic	$C_{10}H_{10}O_3NBr$	154.1–156.1	272.2	270.1, 270.1
m-Bromosuccinanilic	$C_{10}H_{10}O_3NBr$	150.9–151.9	272.1	266.7, 266.7
p-Bromosuccinanilic	$C_{10}H_{10}O_3NBr$	187.2–188.2 reported[16] 186–187°	272.1	272.7, 270.8

(10) L. Balbiano and L. Angeloni, *Gazz. chim. ital.*, **35**, I, 150 (1905).

(11) W. Dieckmann, *Ann.*, **317**, 62 (1901).

(12) R. Anschütz, *Ber.*, **20**, 3215 (1887).

(13) A. E. Arppe, *Ann.*, **93**, 352 (1855).

(14) L. Casale, *Gazz. chim. ital.*, **471**, 272 (1917).

(15) H. J. Lucas and D. Pressman, "Theory and Practice in Organic Chemistry Laboratory," to be published.

(16) S. Hoogewerff and W. A. van Dorp, *Rec. trav. chim.*, **9**, 48 (1890).

Substance, acid	Formula	M. p., °C.	Acidic equivalent weight Calcd.	Obs.
N-α-Naphthylsucinamic	$C_{14}H_{13}O_3N$	171.1–171.6	243.1	241.4, 241.2
N-β-Naphthylsuccinamic	$C_{14}H_{13}O_3N$	189.4–190.0 reported[17] 184–185°	243.1	240.5, 240.6

p-(p-Hydroxyphenylazo)-succinanilic acid was made by diazotizing 0.01 mole of p-aminosuccinanilic acid, making the solution neutral, and adding it to 0.10 mole of phenol in the presence of sodium hydroxide solution. Coupling was complete within fifteen minutes. The solution was neutralized and was extracted twice with ether, and the free acid was precipitated with hydrochloric acid from the aqueous phase. The product was crystallized twice from dilute alcohol; m. p. 231.5 dec. *Acidic equivalent weight:* calcd. for $C_{16}H_{15}O_4N_3$, 313.2; found, 312.7.

Succinamic acid was prepared by adding an equimolar amount of 15 *N* ammonium hydroxide to solid succinic anhydride. The solid product was recrystallized from acetone; m. p., 156.3–157.8°; reported, 157°.[18] *Acidic equivalent weight:* calcd. for $C_4H_7O_3N$, 117.1; found, 116.9, 116.7.

N-Methylsuccinamic acid was synthesized by adding slowly 0.56 mole of anhydrous methylamine to a mixture of 0.54 mole of succinic anhydride and 200 ml. of anhydrous ether under a "Dry Ice" reflux condenser. The waxy lumps which resulted were broken up several times during the addition. After two days the ether was decanted and the solid residue was recrystallized from absolute alcohol; m. p. 107.7–108.2°. *Acidic equivalent weight:* calcd. for $C_5H_9O_3N$, 131.1; found, 132.1, 132.3.

N,N-Dimethylsuccinamic acid was prepared similarly from dimethylamine; m. p. 81.6–82.6°. *Acidic equivalent weight:* calcd. for $C_6H_{11}O_3N$, 145.1; found, 145.7, 145.1.

N-Isopropylsuccinamic acid was prepared similarly from isopropylamine but at the boiling point of ether; m. p. 97.9–98.9°. Acidic equivalent weight: calcd. for $C_7H_{13}O_3N$, 159.1; found, 162.0, 163.1.

N,N-Diethylsuccinamic acid was prepared similarly from dimethylamine at the refluxing temperature. The product was recrystallized from isopropyl ether; m. p. 82.1–84.1°. *Acidic equivalent weight:* calcd. for $C_8H_{15}O_3N$, 173.1; found 173.2, 173.8.

N-Methylsuccinanilic acid was prepared by the method of Auwers[11] from 0.20 mole of methylaniline and 0.20 mole of succinic anhydride in chloroform solution. The chloroform solution was extracted with sodium hydroxide solution. The aqueous phase was extracted with ether and then treated with hydrochloric acid to precipitate the product, which was then recrystallized from water; m. p. 89.3–89.8°, reported, 91–92.5°. *Acidic equivalent weight:* calcd. for $C_{11}H_{13}O_3N$, 207.1; found, 205.8, 207.8.

N-Benzylsuccinamic acid was prepared similarly from benzylamine; m. p. 137.7–138.2°; reported, 139°.[19] *Acidic equivalent weight:* calcd. for $C_{11}H_{13}O_3N$, 207.1; found, 207.2, 207.4.

N-Cyclohexylsuccinamic acid was prepared similarly from cyclohexylamine; m. p. 166.5–167.0°. *Acidic equivalent weight:* calcd. for $C_{10}H_{17}O_3N$, 199.1; found, 199.1, 200.0.

N,N-Pentamethylenesuccinamic acid was prepared by adding 0.32 mole of piperidine to 0.32 mole of succinic anhydride in 200 mole of anhydrous ether and refluxing. The ether was decanted from the heavier liquid phase, which crystallized upon the removal of residual ether under vacuum. The solid was recrystallized from ethyl acetate; m. p. 93.8–94.8°. *Acidic equivalent weight:* calcd. for $C_9H_{15}O_3N$, 185.1; found, 186.1, 186.1.

γ-Anilinobutyric acid hydrochloride was prepared by the method of Anschütz and Beavis,[20] by hydrolyzing 1 g. of N-phenyl-α-pyrrolidone with barium hydroxide octahydrate in 10 moles of water in a sealed tube for twenty hours. The solution was diluted and carbon dioxide was added to precipitate excess barium hydroxide. The silver salt of the acid was precipitated from the filtrate by adding silver nitrate solution. The dried silver salt was suspended in absolute ether and saturated with hydrogen sulfide. The silver sulfide was removed by filtration and the γ-anilinobutyric acid hydrochloride was precipitated by saturating the ether solution with dry hydrogen chloride; m. p. 135.5–136.5°, reported 135.5–136.5°.

The N-phenyl-α-pyrrolidone was prepared by the method of Anschütz and Beavis,[20,21] by heating 0.21 mole of succinanil with 0.86 mole of phosphorus pentachloride at about 130–140° until all the solid was dissolved to form dichloromaleanil chloride, which was purified by distilling at reduced pressure; b. p. 218–219° at 35 mm. The dichloromaleanil chloride was reduced by slowly adding a solution of 0.05 mole of the compound in 50 ml. of acetic acid and 100 ml. of anhydrous ether to 800 g. of 3% sodium amalgam with agitation and cooling in an ice-bath. The mixture was allowed to stand two weeks. The ether phase was fractionally distilled and the N-phenyl-α-pyrrolidone was collected at 193–195° at 24 mm. The N-phenyl-α-pyrrolidone was recrystallized from petroleum ether containing a few drops of alcohol; m. p. 59.0–61.0°, reported, 68–69°.

Phenylhydantoic acid was prepared by the method of Paal,[22] by stirring 0.21 mole of phenylisocyanate with a solution of 0.21 mole of glycine in sodium hydroxide. After thirty minutes of stirring the odor of the isocyanate had disappeared. The solution was filtered, the phenylhydantoic acid was precipitated with hydrochloric acid, and the solid was recrystallized from water; m. p. 196.5–197.0°, reported, 195°. *Acidic equivalent weight:* calcd. for $C_9H_{10}O_3N_2$, 194.1; found, 195.0, 196.6.

γ-Benzoylbutyric acid was prepared by the method of Somerville and Allen[23] and was recrystallized from water; m. p. 127.5–128.5°, reported, 125–126°. *Acidic equivalent weight:* calcd. for $C_{11}H_{12}O_3$, 192.1; found, 193.7, 194.9.

δ-Phenyl-n-valeric acid was prepared by heating γ-phenyl-n-propylmalonic acid and a few drops of hydrochloric acid on a water-bath for eight hours. The product was crystallized from water; m. p. 54.4–55.5°, reported, 57°[24]. *Acidic equivalent weight:* calcd. for $C_{11}H_{14}O_2$, 178.1; found, 181.2, 181.4.

The γ-phenyl-n-propylmalonic acid used above was prepared as an oil by reducing 0.05 mole of cinnamalmalonic acid in 100 ml. of ethanol with hydrogen in the presence of platinum oxide, removing the catalyst by filtration, and evaporating the alcohol. The cinnamalmalonic acid was prepared by the method of Stuart,[25] by refluxing 0.20 mole of malonic acid, 0.20 mole of cinnamaldehyde, and 25 g. of glacial acetic acid for nine hours. The solid product was filtered off, washed with chloroform, and recrystallized from absolute alcohol.

Citraconic acid was prepared by the method of Shriner, Ford, and Roll[26]; m. p. 92.8–93.8°, reported 92–93°. *Acidic equivalent weight:* calcd. for $C_5H_6O_4$, 65.1; found, 65.3, 65.4.

Mesaconic acid was prepared by the method of Shriner, Ford, Roll[27]; m. p. 204.6–205.6°; reported, 203–205°. *Acidic equivalent weight:* calcd. for $C_5H_6O_4$, 65.1; found, 65.2, 65.2.

Discussion

The Effect of Hydrogen-ion Concentration on the Precipitation Reaction.—The effect of hydro-

(17) K. Auwers, *Ann.*, **292**, 190 (1896).
(18) L. Wolff, *ibid.*, **260**, 114 (1890).
(19) E. A. Werner, *J. Chem. Soc.*, 630 (1889).
(20) R. Anschütz and C. Beavis, *Ann.*, **295**, 41 (1897).
(21) R. Anschütz and C. Beavis, *ibid.*, **263**, 158 (1891).
(22) C. Paal, *Ber.*, **27**, 975 (1894).
(23) L. F. Somerville and C. F. H. Allen in "Organic Syntheses," Coll. Vol. II, J. Wiley and Sons, Inc., New York, N. Y., 1943, p. 82.
(24) W. Borsche, *Ber.*, **45**, 622 (1912).
(25) C. M. Stuart, *J. Chem. Soc.*, 365 (1886).
(26) R. L. Shriner, S. C. Ford, and L. V. Roll, in "Organic Synthesis," Coll. Vol. II, J. Wiley and Sons, Inc., New York, N. Y., 1943, p. 140.
(27) R. L. Shriner, S. C. Ford, and L. V. Roll, *ibid.*, p. 382.

gen-ion concentration on the precipitation of anti-S_p serum with S_p-ovalbumin is shown in Table I. Optimum precipitation takes place between pH values of 7.4 and 8.1, as has been found previously for other azo-protein antigens with negatively charged haptenic groups.[28,29] The antigen concentration for optimum precipitation was found to be between 240 and 480 μg. of antigen added. In the experiments reported in Table II 320 μg. of antigen was used.

TABLE I

THE EFFECT OF HYDROGEN-ION CONCENTRATION ON THE PRECIPITATION OF ANTI-S_p SERUM WITH S_p-OVALBUMIN

Antigen solution, antiserum, and buffer, 1 ml. each.

Initial pH	pH of supernate	Amount of antigen added, μg., 30	60	120	240	480
		Amount of precipitate, μg.[a]				
6.0	6.5	91	208	333	434	453
7.0	7.4	136	295	543	697	735
8.0	8.1	117	320	519	704	724
9.0	8.9	144	244	394	432	377

[a] Averages of triplicate analyses, with mean deviation ± 2%.

TABLE II

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

Antigen solution in borate buffer at pH 8, 1 ml. (320 μg.); antiserum, 1 ml.; hapten solution in saline, 1 ml.; pH of supernate, 8.1

Hapten	K_0'	σ	Moles of hapten added $\times 10^8$: 15.6	62.5	250	1000
			Amount of precipitate[a]			
Series A						
Malonanilate ion	0.03	(1.5)			920	830
Succinanilate	1.00	1.5		670	300	50
Glutaranilate	0.03	(1.5)				830
Adipanilate	.01					920
d-Tartranilate	.00					1030
Maleanilate	.25	2.5		850	640	330
Fumaranilate	.01					900
p-(p-Hydroxyphenylazo)-succinanilate	1.38	1.0	920	610	200	
p-Nitrosuccinanilate	1.65	1.0	870	570	120	
p-Aminosuccinanilate	1.03	2.0		650	300	60
p-Bromosuccinanilate	1.31	1.5		640	200	0
m-Bromosuccinanilate	0.72	1.0		920	360	60
o-Bromosuccinanilate	.50	1.0		890	490	110
N-α-Naphthylsuccinamate	.45	1.5		840	540	160
N-β-Naphthylsuccinamate	1.09	1.0		680	220	0
Series B						
Succinanilate ion	1.00	1.5		670	280	100
Succinate	0.01				990	870
Succinamate	.035	(2.5)		970	980	760
N-Methylsuccinamate	.053	(2.5)		890	820	650
N-Isopropylsuccinamate	.064	(2.5)		930	840	600
N-Cyclohexylsuccinamate	.150	2		870	760	400
N-Benzylsuccinamate	.255	2		800	650	280
d-N-(α-Methylbenzyl)-succinamate	.194	2.5		860	660	400
l-N-(α-Methylbenzyl)-succinamate	.169	2		870	(700)	400
N,N-Dimethylsuccinamate	.134	2		(920)	720	460
N,N-Diethylsuccinamate	.122	2		960	760	470
N-Phenyl-N-methylsuccinamate	.128	2		920	740	470
N,N-Pentamethylenesuccinamate	.165	2		910	720	392
Series C						
Succinanilate ion	1.00	1.5		640	270	60
Benzoate	<0.01					930
Phenylacetate	< .01				990	960
β-Phenylpropionate	.01				(930)	870
γ-Phenylbutyrate	.01				980	890
δ-Phenylvalerate	.02				920	830
β-Benzoylpropionate	.59	1.5		770	(410)	130
γ-Benzoylbutyrate	.053	(2.5)			810	640
Benzylsuccinate	.134	2.0		980	750	440
Phenylhydantoate	.102	2.0		930	790	490
Maleate	.03			930	(900)	790
Fumarate	.00					980
Citraconate	.02				910	860
Mesaconate	.00					1020
Succinate	.01				970	890
Valerate	< .01					950
Levulinate	.066	(2)		1010	845	620
Series D						
Succinanilate ion	1.00	2		670	300	90
γ-Anilinobutyrate	0.01			1030	1030	880
δ-Phenylvalerate	.01			970	930	910
β-Benzoylpropionate	.63	1.5		810	410	160
Succinate	.01			1010	950	900
Glutarate	.00			1040	1030	1000
Adipate	.01			940	890	910
Pimelate	.00			1060	1060	990
Sebacate	.03	(2)		1060	930	790
Aspartate	< .01			990	990	950
Asparagine	.00			1000	1010	1000
Glutamate ion	.00			1000	970	1000

[a] The amounts of precipitate are in parts per mille of the amounts in the absence of hapten: 665, 664, 699, and 649 μg. for series A, B, C, and D, respectively. Blanks of serum and buffer 27, 27, 27, and 17, respectively. Values are averages of triplicate analyses, with mean deviation ±2%, except for duplicate analyses in parentheses.

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[30] are also listed.

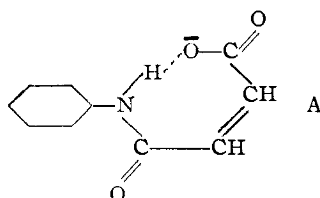
The Structure of the p-Azosuccinanilate Haptenic Group.—Through the consideration of the relative inhibiting powers of haptens of known molecular configuration knowledge can be obtained about the configuration of the combining regions of the antibodies, and hence about the normal configuration of the haptenic groups of the immunizing antigen, if we accept the postulate that these regions are complementary to this antigen. In addition, the data for haptens with uncertain configuration may be interpreted to provide information about either the normal configurations of these haptens in aqueous solution or about configurations which do not differ greatly in energy from the normal ones.

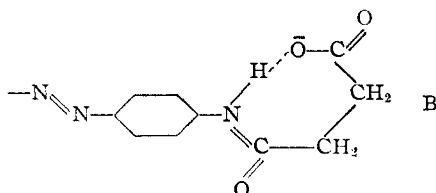
(28) D. Pressman, S. M. Swingle, A. L. Grossberg, and L. Pauling, THIS JOURNAL, 66, 1731 (1944).

(29) Our work showing the same effect of hydrogen-ion concentration on the precipitation with protein antigen of antiserum specific to the p-azophenylarsonate ion and the p-(p-azophenylazo)-phenyl arsonate ion has not been published.

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

It was found by Landsteiner and van der Scheer that the maleate ion combines much more strongly with anti-S_p serum than does the fumarate ion, and that the citraconate (methylmaleate) ion combines much more strongly than does the mesaconate (methylfumarate) ion. We have verified these observations (Table II), and have also found that the hapten inhibition constant for the maleanilate ion (0.25) is much greater than that for the fumaranilate ion (0.01). It may accordingly be concluded that the antibody is complementary to a *cis* configuration similar to that of the maleanilate ion, A; and, since there is no reason to believe that the normal configuration of the *p*-azosuccinanilate group is inferior to any other accessible configuration in acting as a template during antibody formation, a corresponding *cis* configuration, presumably B, is indicated for this group.





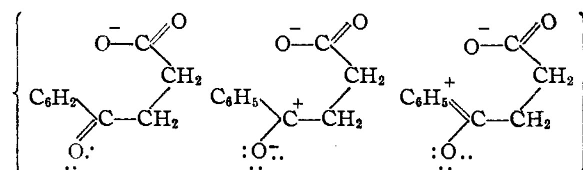
It seems to us likely that the *cis* configuration indicated for this haptenic group is that represented by B, with a hydrogen bond between the amide nitrogen atom and one of the oxygen atoms of the carboxyl group, and that it is largely the energy of this hydrogen bond which stabilizes the *cis* configuration. (The ring closed by the hydrogen bond is probably not coplanar; the two methylene groups may well have nearly the staggered rather than the eclipsed relative orientation.)

The Structure of the Succinanilate Ion, the Succinate Ion, and Related Ions.—The large value of K_0' for the succinanilate ion (1.00, four times the value for the maleanilate ion) indicates strongly that the *cis* configuration is for this ion, too, the normal configuration, and not just an easily accessible one. On the other hand, the value of K_0' for the succinate ion, 0.01, is considerably smaller than that for the maleate ion, 0.03, and it hence seems likely that the *cis* configuration with the two carboxylate groups nearly coplanar with the rest of the ring is not the normal or preferred one for the succinate ion in aqueous solution, but is instead only one of several readily accessible configurations, being itself represented by about 8% of the dissolved ions (the percentage being indicated by the product of ratios of the above K_0' values). The lack of preference for the *cis* configuration presumably is due in part to the inability of the ion to form a hydrogen bond and in part to the electrostatic repulsion of the two carboxylate groups.

The succinamate ion, however, can form a hydrogen bond stabilizing the *cis* configuration, and it contains only one charged group. It is accordingly not surprising that the value of K_0' for this ion (0.035) is considerably greater than that for the succinate ion, and this fact may be taken as verifying that the normal configuration of the succinamate ion, and also of its various monosubstituted derivatives, is the hydrogen-bonded *cis* configuration described above for the succinanilate ion.

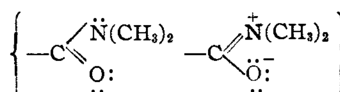
***cis* Configuration without Hydrogen-bond Stabilization.**—It is interesting that there is evidence for predominance of the *cis* configuration, also for some molecules in which this configuration is not stabilized by a hydrogen bond. Thus the large value, 0.59, of K_0' for the β-benzoylpropionate ion requires that the *cis* configuration predominate for this ion in solution, this being essentially the value that would be expected for the *cis* configuration. (The decrease of 41% from the succinanilate ion would be expected to result from the somewhat different orientation of the phenyl group than that for the immunizing haptenic group.)

A reasonable explanation of this observation is that the *cis* configuration for this molecule is stabilized by the electrostatic attraction of the negative charge of the carboxyl ion for a positive charge on the benzene ring and carbonyl carbon atom. The resonance structure places a significant



amount of positive charge in this region, the corresponding negative charge being on the carbonyl oxygen atom, and it is obvious that the electrostatic interactions would stabilize the *cis* configuration, less effectively, however, than would a hydrogen bond.

The sequence of values 0.035, 0.053, 0.134 for K_0' for succinamate ion, N-methylsuccinamate ion, and N,N-dimethylsuccinamate ion strongly indicates that the third of these substances has, like the other two, predominantly the *cis* configuration. For it the positive charge attracting the carboxyl ion is placed on the nitrogen atom by amide resonance



The *cis* configuration indicated for benzylsuccinate ion, $C_6H_5CH_2OCO(CH_2)_2COO^-$, by its rather large value of K_0' (0.134, as compared with 0.255 for N-benzylsuccinamate ion) has a similar explanation, the positive charge being on the oxygen atom to which the benzyl group is attached and on the adjacent carboxyl carbon atom.

The Structural Features Affecting Interaction with Antibody.—The electrically charged carboxyl group is without doubt the structural feature which is of greatest importance in the interaction of haptens and anti-S_p antibody; this feature was, however, not varied in the present investigation.[31] The other structural features which might be important are the imino group, the carbonyl group, the benzene ring, and the framework determining the relative positions of these groups and the carboxyl group.

The data given in Table II indicate that the imino group is not involved directly in attraction of the antibody (hydrogen-bond formation), but exerts only an indirect effect through stabilizing the *cis* configuration for some haptens.

The carbonyl group, on the other hand, makes an important contribution to the attractive forces between hapten and antibody, without doubt by serving as the proton receptor in a hydrogen bond with the antibody. This is strikingly shown by the relative values of K_0' for succinanilate ion (1.00) and γ-anilinobutyrate ion (0.01), and for β-benzoylpropionate ion (0.59) and γ-phenylbutyrate ion (0.01), and is indicated also by other comparisons among the data in Table II (levulinate ion, $CH_3COCH_2CH_2COO^-$ (0.066) and valerate ion (0.01)).

The data also show clearly that the antibody is not pliable, but is rigid: it cannot adjust itself to a change by as much as 1 Å. in the relative position of the carbonyl group and the carboxyl group, but requires for strong combination with a hapten that these groups be the same distance apart as in the haptenic group of the immunizing antigen. This is shown by the comparison of succinanilate ion ($K_0' = 1.00$) with malonanilate ion (0.03) and glutaranilate ion (0.03), and of β-benzoylpropionate ion (0.59) with γ-benzoylbutyrate ion (0.053).

The considerable effect of the van der Waals attraction of the antibody for the benzene ring of the haptenic group is indicated by the 30-fold increase in value of K_0' caused by introduction of a benzene ring in succinamate ion. The effect of the azo group and an additional benzene ring is, however, very small—*p*-(*p*-hydroxyphenylazo) succinanilate ion shows an increase in K_0' of only 38% over the succinanilate ion. We may accordingly conclude that the combining group of the antibody is complementary in structure to the succinamate group and also to the benzene ring, but that it does not extend much farther along the haptenic group.

The value of K_0' for N-cyclohexylsuccinamate ion (0.150), corresponding to decrease to one-seventh on replacing phenyl by cyclohexyl, is probably due to the smaller van der Waals attraction of cyclohexyl, resulting from its smaller polarizability and greater thickness[32] than for the phenyl group.

The value $K_0' = 0.00$ found for the tartranilate ion requires explanation. The great effect of the two hydroxyl groups can hardly be attributed to steric hindrance, since citraconate ion was found to be nearly as effective as maleate ion, and the methyl group is as large as the hydroxyl group. It seems probable that the small effectiveness of the tartranilate ion as an inhibiting hapten results from the fact that hydroxyl groups of the ion in solution are holding water molecules by hydrogen bonds, and that these molecules must be removed in order for the ion to fit into the antibody. This would reduce the free energy of combination with antibody by an amount equal to the free energy of hydration of the hydroxyl groups. This explanation is the same as that previously suggested[33] for the low values of K_0' for *p*-amino and *m*-amino substituted haptens. The same phenomenon explains the low value (0.102, only one-tenth that for the succinanilate ion) of K_0' for the phenylhydantoate ion, $C_6H_5NHCONHCH_2COO^-$. A steric explanation could hardly be invoked here, because the NH group is essentially equal in size to the methylene group which it replaces.

The Effect of Substituents in the Benzene Ring of the Succinanilate Ion.—The effect of various groups in the para position of the benzene ring of the succinanilate ion on the value of K_0' is in the order

$$NO_2 > HOC_6H_4NN > Br > NH_2 > H$$

The action of the nitro group to cause even greater combination than the homologous azo-group was observed previously with anti-R_p serum (antiserum specific for the *p*-azophenylarsonate ion group).[30]

The effect on K_0' of the position of the substituent is in the order $p > m > o$, in agreement with earlier observations,[5,28,30] on *p*-azohaptenic systems.

The magnitude of the effect of substituents in this system is less, however, than for other systems, the spread for the above groups in the para position being by a factor of less than 2 in K_0', as compared with 5 for these groups with anti-R_p serum, 15 with anti-R_p' serum (antiserum specific for the *p*-(*p*-azophenylazo)-phenylarsonate ion group), and 20 with anti-X_p serum (specific to the *p*-azobenzoate ion group). Also in changing a substituent from the *para* to the *ortho* position there is a factor of only 2.6 involved, which is a little larger than the value 2 for anti-R_p' serum but is much smaller than those for anti-R serum (5 to 90) and anti-X_p serum (20 to 1000). The small effect of the substituents in the present system

(31) See ref. 1 and earlier papers.

(32) The effect has been found also in the benzoic acid system, D. Pressman, S. M. Swingle, A. L. Grossberg, and L. Pauling, THIS JOURNAL, **66**, 1731 (1944).

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

must be due to a rather poor fit of the antibody to the benzene ring, which probably results from the greater distance from the part of the haptenic group which carries the electric charge.

The values of K_0' for N-α- and N-β-naphthylsuccinamate ions, 0.45 and 1.09, are reasonable when compared with the observed effects of substituents in the *o*-, *m*-, and *p*-positions.

Discussion of Other Haptens.—The order of effectiveness of various groups replacing one hydrogen atom on the nitrogen atom of the succinamate ion in increasing the value of K_0' is $C_6H_5 > C_6H_5CH_2 > C_6H_5(CH_3)CH >$ *cyc*-$C_6H_{11} > (CH_3)_2CH > CH_3 > H$. The range of values of K_0' from the benzyl group to hydrogen is through a factor of 8. Replacement of a hydrogen atom by a methyl group presumably increases the value of K_0' from 0.035 to 0.053 through the action of the increased van der Waals attraction, corresponding to the increase in polarizability of the group. Further increasing the size of the alkyl group causes additional increase in K_0'.

The larger value (0.165) of K_0' for N,N-pentamethylenesuccinamate ion than for N,N-diethylsuccinamate ion (0.122) is probably in the main due to the more compact structure of the pentamethylene group than of the two ethyl groups.

In the homologous series of ions of dibasic acids, succinic, glutaric, adipic, and sebacic, the last combines the most strongly with anti-S_p serum, as was reported also by Landsteiner and van der Scheer.[7] We checked this effect for larger amounts of haptens, up to 10^{-4} mole, in both the system S_p-ovalbumin:anti-S serum and the system ovalbumin:antiovalbumin, and found the effect to be specific to the anti-S_p serum. In general an increase in the hapten inhibiting effect would be expected for such a series with increase in the number of methylene groups, because of the increasing van der Waals attraction. In the S_p system, however, it might be expected that the succinate ion would have the maximum effect, because of its close relation to the immunizing para-azosuccinanilate ion haptenic group. It is very probable that the failure of the succinate ion to be active is, as discussed above, due to the predominance of the *trans* configuration for this ion, which does not bring an oxygen atom of the second carboxyl group into the position corresponding to the carbonyl group of the original immunizing antigen.

Asparagine, the aspartate ion, and the glutamate ion were all found to be ineffective as haptens. It is likely that this ineffectiveness is to be ascribed to the effect of the positively charged ammonium ion group in these haptens.

Conclusion.—In general, it has been found that the inhibiting power of haptens in the *para*-azosuccinanilate system depends upon the structural features found previously for other systems, principally the shape of the hapten, the polarizability of groups, and the distribution of charge. The previously recognized phenomenon of decrease in inhibiting power for haptens that are hydrated in solution and must have water removed for combination with antibody has been substantiated by several examples in this system. An interesting result of the studies has been the discovery that the *para*-azosuccinanilate group has the *cis* configuration in the azoprotein used as the immunizing antigen, and that a similar *cis* configuration is shown by the succinanilate ion, the succinamate ion, and many related substances in which this configuration can be stabilized by hydrogen bond formation. The *cis* configuration has also been found to predominate for some other substances in solution, the stabilizing influence presumably being the attraction of the negative charge of the carboxyl group for a positive charge produced elsewhere in the molecule by resonance.

It may be pointed out that the results presented in this paper, like those reported in the preceding papers of this series, strongly support the concept that the forces of attraction between antibody and antigen are interatomic forces operating through distances of a few ångströms, and that the specificity of the resultant integrated attraction depends upon a detailed complementariness in structure of antibody and antigen.

Acknowledgment.—This investigation was carried out with the aid of a grant from The Rockefeller Foundation. We wish to thank Mr. Dan Rice for assisting in the analytical work.

Summary

A quantitative study has been made of the precipitation reaction of S_p-ovalbumin and anti-S_p serum, prepared by injecting rabbits with an azoprotein made by coupling sheep serum with diazotized *p*-aminosuccinanilic acid, and of the inhibiting effect of fifty haptens on this precipitation. The data have been interpreted to show that the normal configuration of the *p*-azosuccinanilate ion haptenic group is a *cis* configuration, which is presumably stabilized by a hydrogen bond between the nitrogen atom of the amide group and an oxygen atom of the carboxyl group. They further indicate that a similar *cis* configuration is the predominant configuration for the succinamate ion and related ions, including some which are not stabilized by hydrogen-bond formation.

The results support the concept that complementariness in structure of antibody and antigen is responsible for their specific combination, and that the forces involved require approximation of the attracting molecules to within one or two ångströms. The values of the hapten inhibition constant show that the principal forces of attraction between the antibody and the hapten are the attraction for the negative charge of the carboxyl group, attraction for the carbonyl group (presumably by formation of a hydrogen bond), and van der Waals attraction for the benzene ring and other parts of the hapten.

PASADENA 4, CALIFORNIA RECEIVED OCTOBER 14, 1947