to *trans,trans* configurational change takes place in a reaction which obviously involves a multitude of prototropic shifts.

Acknowledgments.—The authors wish to express deep appreciation to Dr. Wilbur A. Lazier for his active interest in the investigation. We are grateful to Dr. R. C. Lord for helpful discussion. We are indebted to Dr. John Means and Mr. Glenn B. Hess for the spectral data.

BROOKLYN 6, N. Y.

[CONTRIBUTION FROM THE SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH]

The Steric Configuration of β-Benzoylpropionate Ion in Aqueous Solution as Determined by Immunochemical Means[1,2]

BY DAVID PRESSMAN AND MALCOLM SIEGEL

RECEIVED NOVEMBER 1, 1952

Immunochemical evidence is presented for the existence of a coiled or *cis* configuration of the β-benzoylpropionate ion in aqueous solution. A study of other structural features important in the combination of haptens with antibodies prepared against the β-benzoylpropionate ion shows that the antibody fits closely around the homologous ion.

It has been postulated that β-benzoylpropionate ion either exists in or readily assumes a coiled (*cis*) configuration in aqueous solution.[3] This postulate was based on the observation that β-benzoylpropionate ion combines strongly with antibodies formed against the *p*-azosuccinanilate ion grouping. These antibodies are known to be complementary to the *cis* configuration of the succinanilate ion since they combine strongly with the maleanilate ion (which must exist in the *cis* configuration), and do not combine with the fumaranilate ion (which must exist in the *trans* configuration). In order to determine whether antibodies prepared against the β-benzoylpropionate ion grouping also reflect this *cis* configuration a study was made of antisera prepared against the β-(*p*-azobenzoyl)-propionate ion grouping. The results are reported here. The study also included the determination of other structural features which are important in the combination of hapten with these antibodies.

Experimental Methods

Materials.—With two exceptions the simple substances used have been described previously,[3,4] or were commercial preparations crystallized to the correct melting point and neutral equivalent. β-Benzoylacrylic acid was prepared according to the method of Papa, Schwenk, Villani and Klingsberg.[5] On recrystallization from water it melted at 63–65°.

β-(*p*-Aminobenzoyl)-propionic acid was prepared as follows: **β-(*p*-acetaminobenzoyl)-acrylic acid** was prepared according to the method of Papa, Schwenk, *et al.* 6.2 g. of β-(*p*-acetaminobenzoyl)-acrylic acid was dissolved in 95% ethanol by the addition of 4 *M* sodium hydroxide. The mixture was catalytically reduced (1 g. of 5% Pd on $BaSO_4$) for 1.75 hours at an initial hydrogen pressure of 42.3 lb./in.^{2} On acidification with HCl 2.7 g. (43% yield) of **β-(*p*-acetaminobenzoyl)-propionic acid** was obtained, m.p. 197–198°. **β-(*p*-Aminobenzoyl)-propionic acid** was prepared by refluxing the acetyl derivative for 2 hours in 10% HCl. A 61% yield of crystals melting at 184–186° was obtained.

Whole beef serum was stored in the lyophilized form. Ovalbumin was the crystallized Armour preparation.

Protein Antigens.—The antigen used for injection was prepared by coupling the diazotization product from 0.54 g. (0.0028 mole) of β-(*p*-aminobenzoyl)-propionic acid with 4 g. of regenerated lyophilized beef serum at *p*H 10. After standing overnight at 3–5° the azoprotein was dialyzed *vs.* cold saline until a colorless dialysate was obtained.

The test antigen was prepared by similarly coupling 350 mg. of ovalbumin at *p*H 10 with the diazonium salt from 54 mg. of β-(*p*-aminobenzoyl)-propionic acid. The azoprotein was purified by dialyzing against cold saline, precipitating three times at *p*H 3.85, washing with 4 portions of cold 60% acetone, and redissolving in 50 ml. of saline at *p*H 7.4. The last traces of acetone were removed by dialysis against saline.

Preparation of Antisers.—Antisera were prepared by methods described previously.[4] High titer serum from rabbits injected with each antigen were pooled.

Reaction of Antiserum with Antigen and Hapten.—Equal volumes of antigen, antisera and hapten were mixed and incubated for about 1 hour at 37° (or room temperature) then allowed to stand for 2–4 days at 3–5°. A concentration of antigen diluted with borate buffer[6] was used which yielded a maximum amount of precipitate. The precipitates were washed 3 times with 8 ml. of saline, dissolved in 1 *M* NaOH, and analyzed by a modified Folin procedure.[7]

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

Binding Measurement.—10.0-ml. portions of a threefold borate buffer dilution of normal rabbit serum (19.2 mg. protein/ml. by Nessler analysis) were placed in dialysis bags and immersed in vials containing 10.0-ml. portions of a borate solution of hapten. The vials were rocked for 4 days to reach equilibrium. The concentration of the hapten both inside and outside the dialysis bag was determined by measurements of optical density. The per cent. of the hapten inside the dialysis bag bound to the protein was calculated either from the difference in optical density of the hapten inside and outside the bag or from the change in optical density of the outer phase.

Results

The Extent of Combination of Haptens with Antibody.—The extent of combination of hapten with antibody was determined by measuring the ability of hapten to inhibit the precipitation of anti-BzP antibody (antibody against the β-benzoylpropionate ion grouping) with BzP–oval (β-benzoylpropionate ion coupled to ovalbumin).

(1) Presented at the 122nd Meeting of the American Chemical Society, Atlantic City, N. J., September, 1952.

(2) This research was jointly supported by the Office of Naval Research and the U. S. Atomic Energy Commission.

(3) D. Pressman, J. H. Bryden and L. Pauling, THIS JOURNAL, **70**, 1352 (1948).

(4) D. Pressman, J. H. Bryden and L. Pauling, *ibid.*, **67**, 1219 (1945).

(5) D. Papa, E. Schwenk, F. Villani and E. Klingsberg, *ibid.*, **70**, 3356 (1948).

(6) D. Pressman, D. H. Brown and L. Pauling, *ibid.*, **64**, 3015 (1942).

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

TABLE I

THE EFFECT OF HAPTENS ON THE PRECIPITATION OF ANTI-BENZOYLPROPIONATE SERUM WITH BzP–OVALBUMIN

Anti-BzP serum, 1.00 ml.; BzP–Ovalbumin in borate buffer, 1.00 ml. (1.6 mg. protein); hapten in saline, 1.00 ml. 75 minutes at 37° and 2 days at 5°

Hapten		Hapten concn., molar × 10^5 10.3	41.7	83.3	167	333	667	1330	2670	σ	K_0'[b]	ΔF_{rel}, cal.
		Amount of precipitate[a]										
β-Benzoylpropionate	$C_6H_5COCH_2CH_2CO_2^-$	850	600		30					1.5	1.00	0
Levulinate	$CH_3COCH_2CH_2CO_2^-$			620		470		80		2	0.12	1200
Succinanilate	$C_6H_5NHCOCH_2CH_2CO_2^-$			940		740		340		1	.04	1800
N-Methylsuccinanilate	$C_6H_5NCH_3COCH_2CH_2CO_2^-$			580		470		60		2	.15	1000
γ-Phenylbutyrate	$C_6H_5CH_2CH_2CH_2CO_2^-$								450		.02	2200
Hydrocinnamate	$C_6H_5CH_2CH_2CO_2^-$						700		560		.01	2500
δ-Phenylvalerate	$C_6H_5CH_2CH_2CH_2CH_2CO_2^-$				1020		850		490	1	.01	2500
γ-Benzoylbutyrate	$C_6H_5COCH_2CH_2CO_2^-$				720		550		70	1	.04	1800
Valerate	$CH_3CH_2CH_2CH_2CO_2^-$								840		≪.005	≫2900
Hippurate	$C_6H_5CONHCH_2CO_2^-$		920		750		390			1.5	.08	1400
Phenylhydantoate	$C_6H_5NHCONHCH_2CO_2^-$			850		800		570		2.5	.01	2500
Benzenesulfonylglycinate	$C_6H_5SO_2NHCH_2CO_2^-$								750		<.005	>2900
β-Benzoylacrylate	$C_6H_5COCH{=}CHCO_2^-$			970		770		320		1	.04	1800
Maleanilate	*cis*-$C_6H_5NHCOCH{=}CHCO_2^-$			790		620		360		2.5	.04	1800
Fumaranilate	*trans*-$C_6H_5NHCOCH{=}CHCO_2^-$								640		.005	2900

[a] The amount of precipitate is reported in parts per mille of the amount present in the absence of hapten; 108 μg. These are averages of triplicate analyses with mean deviation of 3%. [b] The value of K_0' is corrected for albumin binding. In the case of levulinate ion correction is made only for the binding of the reference benzoylpropionate ion.

The amount of antigen used was that which gave the optimum amount of precipitate at *p*H 8. As in other antigen–antibody systems,[3,4,6] the antiserum gave the largest amount of precipitate with an optimum amount of antigen and less with either more or less of the antigen. The experiments were run at *p*H 8 since the carboxylic acids are nearly completely dissociated at this *p*H, and several other systems have been investigated under the same conditions. The results of the hapten inhibition and precipitation are given in Table I. Values of the relative hapten–antibody combination constants, K_0' (relative to the value for unsubstituted benzoylpropionate ion) and the heterogeneity index, σ, obtained on application of the theory of heterogeneous antisera[3] are given. There is also listed a value for the relative free energy change ΔF_{rel}, accompanying the combination of hapten with antibody. ΔF_{rel} is the difference in free energies of combination of antibody with any hapten and of antibody with the unsubstituted homologous hapten. It is defined by the equation

$$-\Delta F_{rel} = RT \ln K_0'$$

Since these experiments were carried out with whole serum, corrections were made as described previously[9] for the extent of binding of hapten to the serum albumin present. Nearly all of the haptens used were at concentrations so high as to make any binding to serum albumin a minor factor.[10] In the case of those few haptens for which albumin binding might be appreciable, the binding was measured at two concentrations, one above and one below the fiducial concentration[8] of that hapten in inhibiting the precipitation of anti-BzP antibody. The binding at the fiducial concentration was calculated by linear interpolation between the above two points. The binding values for β-benzoylpropionate, N-methylsuccinanilate and hippurate ion appear in Table II.

TABLE II

BINDING OF HAPTENS AT FIDUCIAL CONCENTRATION TO RABBIT SERUM

Hapten	Equil. concn. in protein phase, molar × 10^4 (Threefold borate dilution of normal serum)	Bound, %	Fiducial concn., molar × 10^4	Calcd. % bound at fiducial concn.
β-Benzoylpropionate	3.7	65	5.5	51
	7.4	37		
N-Methylsuccinanilate	11	20	20	14
	30	8		
Hippurate	19	16	43	9
	52	6		

TABLE III

THE NON-SPECIFIC EFFECT OF HAPTENS ON THE PRECIPITATION OF ANTI-OVALBUMIN SERUM WITH OVALBUMIN

Anti-ovalbumin serum (threefold dilution with normal serum), 0.50 ml.; ovalbumin in borate buffer, 0.50 ml. (62.5 μg.); 0.08 *M* hapten in saline, 0.50 ml. One hour at room temperature and 4 days at 5°

Hapten	Hapten concn., 2670 × 10^{-5} *M* Amt. of ppt.[a]
Levulinate	920
Succinanilate	980
N-Methylsuccinanilate	1000
γ-Phenylbutyrate	910
Hydrocinnamate	890
δ-Phenylvalerate	980
γ-Benzoylbuturate	950
Hippurate	950
Maleanilate	990
Fumaranilate	1000
Phenylhydantoate	1010

[a] The amount of precipitate is reported in parts per mille of the amount present in the absence of hapten; 463 μg. These are averages of triplicate analyses with mean deviation of 6%.

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

(9) D. Pressman and M. Siegel, *ibid.*, **74**, 686 (1952).

(10) See discussion of reference 9.

The haptens were found to have little or no effect at the highest concentrations used on the amount of precipitate formed when anti-ovalbumin serum was precipitated with the optimum concentration of ovalbumin, showing that they had no non-specific effect. The results are given in Table III.

Discussion

The *cis* Configuration of β-Benzoylpropionate Ion.—Maleanilate ion combines much more strongly with antiserum against the benzoylpropionate ion than does fumaranilate ion (ΔF_{rel} = 1800 and 2900 cal., respectively). Therefore, the antibody appears to be more nearly complementary to a *cis* or coiled configuration of the $\overset{O}{\overset{\|}{C}}\text{--}CH_2\text{—}CH_2C\overset{O}{\underset{O}{}}$ grouping than to the *trans*. On the basis of this argument, part of the β-benzoylpropionate ion must have been in a *cis* configuration during the period of antibody formation. Such a *cis* configuration would be stabilized by the resonance structures shown in Fig. 1.

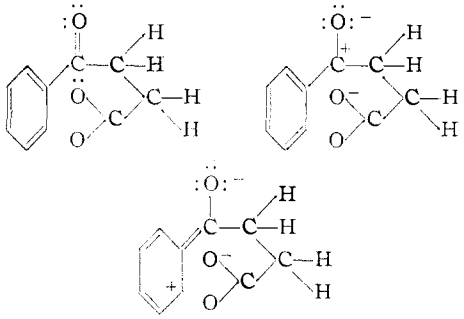
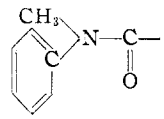


Fig. 1.—Resonance forms of β-benzoylpropionate ion which stabilize *cis* configuration.

Effect of Structural Changes in the Hapten.—The fit of the antibody about the β-benzoylpropionate ion must be very close since all the changes made in structure of that ion (*i.e.*, replacement of the phenyl, carbonyl or methylene groups by other groups) decreased the extent of combination with the antibody appreciably, thereby increasing the free energy of combination with antibody by over 1000 cal.

When the phenyl group of benzoylpropionate ion is replaced by the methyl, anilino or methylanilino groups (leaving the $-\overset{O}{\overset{\|}{C}}\text{—}CH_2CH_2C\overset{O}{\underset{O}{}}$ residue intact as in levulinate, succinanilate and N-methylsuccinanilate ion), the relative ΔF's of combination are increased. The increase of 1200 cal. which results from the replacement of the phenyl by methyl to give levulinate ion is probably due largely to the difference in the van der Waals attraction of the phenyl and methyl groups for the antibody. Replacement of the phenyl by an anilino group has a greater effect, increasing ΔF_{rel} by 1800 cal. This may be a steric effect, due to displacement of the phenyl group from the position next to the carbonyl group. Replacing the NH of succinanilate by a N-CH_3 group decreases ΔF_{rel}. This may be due to its branched structure



being more equivalent to the structure of the original benzene group which is attached to the carbonyl carbon in the homologous hapten. Another possibility is that the coiled configurations for the NH and NCH_3 compounds are different, and the configuration of the M-methyl compound is closer to that of the benzoylpropionate.

The replacement of a methylene group of the $COCH_2CH_2COO^-$ residue by the NH group to give hippurate increases ΔF_{rel} by 1400 cal. The effect is somewhat smaller, 700 cal., when the change is made from a heterologous ion, succinanilate ion, to phenylhydantoate ion. This is what would be expected since the change is made in an ion which does not fit as well as the homologous ion β-benzoylpropionate. The decrease of combining power when NH replaces the isosteric CH_2 is very large and indicates that the NH group is hydrated in aqueous solution. The increase in the size of the group due to attached water would cause a steric interference in the combination of the hapten with antibody larger than that which can be explained by van der Waals interaction or by bond angle. Evidence for the hydration of the NH group has already been mentioned in the case of similar compounds interacting with antibodies to the succinanilate ion group,[3] and also in connection with the interaction of annular nitrogen compounds with anti-benzoate antibodies.[11]

The replacement of the CH_2—CH_2 group by CH=CH to give β-benzoylacrylate ion increases ΔF_{rel} by 1800 cal. This large effect shows that benzoylacrylate does not fit the antibody well. The lack of fit is in agreement with the fact that β-benzoylacrylate probably exists in the fixed *trans* configuration while the antibody region appears to be complementary to a *cis* configuration. Unfortunately a *cis* benzoylacrylate ion was not available for comparison.

The similar change, saturation to unsaturation, as in going from succinanilate to maleanilate or to fumaranilate, involves no change in ΔF_{rel} to the *cis* and 1100 cal. to the *trans* isomer. These data confirm the *cis* configuration of succinanilate in aqueous solution.

The replacement of the carbonyl group by a methylene group increases ΔF by 2200 cal. indicating that the carbonyl group is very important for the combination of hapten with this antibody. It is probably important either as a proton acceptor in forming a hydrogen bond or as a factor in stabiliz-cyclization. The relative position of the carbonyl group and the carboxylate group is critical, since moving the benzoyl group one methylene group further from the carboxylate group (as in benzoylbutyrate) decreases the energy of combination by 1800 cals. Thus, benzoylbutyrate ion has difficulty assuming a coiled configuration similar to that of the benzoylpropionate ion.

Replacing the carbonyl by another oxygen con-

(11) D. Pressman and M. Siegel, Abstracts 116th Meeting, Amer. Chem. Soc., 1949.

taining group (*i.e.*, the sulfonyl group), as in the change from hippurate ion to benzenesulfonylglycinate, causes a very large decrease in the strength of combination. This large effect may be entirely steric and due to the larger space requirement of two oxygens.

In the absence of the carbonyl group, there seems to be some correlation between the combining power of the hapten and the distance between the benzene group and the carboxylate ion. Phenylbutyrate ion, in which the benzene and carboxylate have the same distance between them as in the homologous benzoylpropionate ion, is somewhat more effective in combining with anti-BzP antibodies than are the phenylvalerate and the phenylpropionate (hydrocinnamate) ions which contain one methylene group more or less than the butyrate.

The importance of the van der Waals interaction of the benzene ring is emphasized by a comparison of the combination of anti-BzP antibody with valerate and with phenylbutyrate, ΔF_{rel} for the combination with the aliphatic compound is at least 700 cal. greater.

NEW YORK, N. Y.

[CONTRIBUTION FROM VENEREAL DISEASE EXPERIMENTAL LABORATORY, U. S. PUBLIC HEALTH SERVICE, SCHOOL OF PUBLIC HEALTH, UNIVERSITY OF NORTH CAROLINA]

The Preparation of Some Organophosphorus Compounds Possessing Anticholinesterase Activity[1]

BY LEON D. FREEDMAN, HENRY TAUBER, G. O. DOAK AND HAROLD J. MAGNUSON

RECEIVED OCTOBER 20, 1952

Fifty phosphonic and phosphinic acids previously prepared in this Laboratory have been tested for anticholinesterase activity. The enzyme used in the present study was human plasma cholinesterase. Fourteen of the compounds were found to be active at a concentration of 0.003 *M*. The results obtained suggested the synthesis of other organophosphorus compounds. Since several disubstituted phosphonic and phosphinic acids were required, the diazo synthesis was extended to the preparation of such compounds. The reaction conditions were similar to those described previously. Reasonable yields were obtained in all cases. Several esters of phosphonic and phosphinic acids were also prepared. Most of the esters were more active than the free acids.

Recent communications[2] from this Laboratory have described the preparation of 50 phosphonic and phosphinic acids. Although these compounds were prepared primarily for testing against a variety of microörganisms,[3] the usefulness of certain organic phosphorus compounds as anticholinesterase agents[4] prompted us to investigate the value of aromatic phosphonic and phosphinic acids as enzyme inhibitors.

The enzyme used in the present study was human plasma cholinesterase prepared from Cohn Fraction IV-6. It possessed an activity 80 times that of whole plasma protein. All the phosphorus compounds were tested first at a concentration of 0.003 *M*; the majority caused little or no inhibition and were not further studied. A few, however, possessed significant anticholinesterase activity at this level and were further tested to determine the concentration required for 50% inhibition (I_{50}). The results obtained are shown in Table I. It is seen that 11 of the 14 active compounds were halogen derivatives. The two most active phosphonic acids and the three most active phosphinic acids contained a halogen atom in ortho position. The *m*-halo derivatives were less active; and all the *p*-substituted compounds were inactive.

These results suggested the synthesis of other organophosphorus compounds. It was of particular interest to prepare *o*-fluoro and *o*-iodo substituted phosphonic and phosphinic acids for comparison with the corresponding *o*-bromo and *o*-chloro derivatives. It seemed advisable also to prepare other compounds with ortho substituents since every *o*-substituted compound we had tested possessed significant anticholinesterase activity. Several disubstituted phosphonic and phosphinic acids were synthesized to determine the effect of a second ring substituent on the activity of the halogen-substituted acids.

Because the inhibition studies were made at *p*H 7, the phosphinic acids were present entirely as

TABLE I

ANTICHOLINESTERASE ACTIVITY OF PREVIOUSLY DESCRIBED COMPOUNDS

Compound	I_{50}[a] mole/l.
(*o*-BrC_6H_4)$C_6H_5PO_2H$	6×10^{-5}
(*o*-BrC_6H_4)$_2PO_2H$	1×10^{-4}
(*o*-ClC_6H_4)$_2PO_2H$	5×10^{-4}
(*m*-BrC_6H_4)$_2PO_2H$	2×10^{-3}
(*m*-ClC_6H_4)$_2PO_2H$	2×10^{-3}
(*m*-ClC_6H_4)$C_6H_5PO_2H$	3×10^{-3}
(*m*-BrC_6H_4)(*m*-$CH_3NHC_6H_4$)PO_2H	4×10^{-3}
(*m*-$CH_3NHC_6H_4$)$_2PO_2H$	4×10^{-3}
(*o*-BrC_6H_4)$C_2H_5PO_2H$	4×10^{-3}
o-$BrC_6H_4PO_3H_2$	4×10^{-3}
o-$ClC_6H_4PO_3H_2$	4×10^{-3}
m-$BrC_6H_4PO_3H_2$	5×10^{-3}
o-$NH_2C_6H_4PO_3H_2$	8×10^{-3}
m-$CH_3(CH_2)_3NHC_6H_4PO_3H_2$	9×10^{-3}

[a] The I_{50} values in this and succeeding tables were obtained from graphs in which % inhibition was plotted against the logarithm of the molar concentration of the compound.

(1) Presented before the Organic Division of the American Chemical Society in Atlantic City, N. J., September, 1952.

(2) (a) G. O. Doak and L. D. Freedman, THIS JOURNAL, **73**, 5658 (1951); (b) G. O. Doak and L. D. Freedman, *ibid.*, **74**, 753 (1952); (c) L. D. Freedman and G. O. Doak, *ibid.*, **74**, 2884 (1952); (d) G. O. Doak and L. D. Freedman, *ibid.*, **75**, 683 (1953).

(3) Some of these results have been reported, J. D. Thayer, H. J. Magnuson and M. S. Gravatt, *Antibiotics and Chemotherapy*, in press.

(4) *Cf.* G. B. Koelle and A. Gilman, *Pharmacol. Rev.*, **1**, 166 (1949).