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]

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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).

TABLE II

ARYLPHOSPHONIC AND DIARYLPHOSPHINIC ACIDS

Compound	Yield,[a] %	M.p.,[b] °C.	Phosphorus, % Calcd.	Phosphorus, % Found	Neutral equivalent[c] Calcd.	Neutral equivalent[c] Found	I_{50}, mole/l.
o-$IC_6H_4PO_3H_2$	22[d]	219–222	10.91	10.71	142.0	143.0	4×10^{-4}
o-$HOOCC_6H_4PO_3H_2$[e,f]	37[d]	175.5–179	15.33	14.94	67.4	[g]	4×10^{-3}
o-$CH_3OC_6H_4PO_3H_2$[h,i]	20[d]	202–203[j]	16.47	16.02	94.1	95.3	4×10^{-3}
2,5-$Cl_2C_6H_3PO_3H_2 \cdot H_2O$[k]	32[d]	193–196	12.64	12.27	122.5	123.4	6×10^{-3}
2-Br-5-$O_2NC_6H_3PO_3H_2 \cdot H_2O$[i,l]	15[m]	Dec. >230	10.33	10.12	150.0	149.0	7×10^{-2}
2-Cl-4-$NH_2C_6H_3PO_3H_2$[n,o]	86	>200[p]	14.93	14.66	103.8	104.0	1×10^{-2}
o-$FC_6H_4PO_3H_2$[q]	41[r]	146–149	17.59	16.99	88.0	87.7	$>1 \times 10^{-2}$
2-Br-5-$CH_3C_6H_3PO_3H_2$[i,s]	27[d]	199–203	12.34	12.00	125.5	124.3	$>1 \times 10^{-2}$
2-Cl-4-$O_2NC_6H_3PO_3H_2$[i,t]	20[d], 32[r]	221.5–224	13.04	12.91	118.8	118.8	$>1 \times 10^{-2}$
2-CH_3O-4-$O_2NC_6H_3PO_3H_2 \cdot H_2O$[i,u]	31[r,v]	226.5–227.5	12.34	12.03	125.6	125.0	$>1 \times 10^{-2}$
(*o*-IC_6H_4)$C_6H_5PO_2H$	28	206–207.5	9.00	9.01	344.1	339.0	1×10^{-3}
(2,5-$Cl_2C_6H_3$)$_2PO_2H$[w]	13[d]	232–233	8.70	8.54	356.0	359.5	1×10^{-3}
(*o*-$CH_3OC_6H_5$)$_2PO_2H$[x,y]	10[d]	234–238	11.13	10.87	278.2	276.5	4×10^{-3}
(*o*-FC_6H_4)$_2PO_2H$[w]	11[s]	220–222	12.19	11.90	254.2	254.2	1×10^{-3}
(2-Br-5-$CH_3C_6H_3$)$_2PO_2H$[w]	12[d]	265–271	7.67	7.53	404.1	390.5	7×10^{-3}
(2-Br-5-$O_2NC_6H_3$)$_2PO_2H$[w,z]	12[m]	>300	6.65	6.48	466.0	463.0	$>1 \times 10^{-2}$
(2-Cl-4-$O_2NC_6H_3$)$_2PO_2H$[w,aa]	2[d], 0[r]	Dec. 270–275	8.22	7.99	377.1	372.7	$>1 \times 10^{-2}$

[a] The yield data, in most cases, are the results obtained in a single experiment. Reaction conditions were chosen which were expected to give a reasonable yield of both phosphonic and phosphinic acids; *cf.* ref. 2d. Larger yields of either acid could probably be obtained under other conditions. [b] Melting points were taken as previously described; *cf.* ref. 2a. [c] The indicator used for the phosphonic acids was thymolphthalein; the indicator used for the phosphinic acids was phenolphthalein. [d] This yield was obtained by the use of ethyl acetate as the solvent, cuprous bromide as the catalyst, and phosphorus tribromide as the source of phosphorus. [e] Previously prepared by A. Michaelis, *Ann.*, **293**, 193 (1896); m.p. 172°. [f] Calcd.: C, 41.60; H, 3.49. Found: C, 41.90; H, 3.39. [g] Potentiometric titration (*cf.* ref. 6) of this compound showed the second dissociation constant of the phosphono group was too small (pK_a 9.17) to permit the use of thymolphthalein as the indicator. [h] Previously prepared by A. Burger, personal communication. [i] This compound was isolated and purified by procedure A as previously described; *cf.* ref. 2a. [j] M.p. varied slightly with the rate of heating. [k] Previously prepared by G. M. Kosolapoff, THIS JOURNAL, 69, 2020 (1947); m.p. 194–197°. [l] Calcd.: N, 4.67; H_2O, 6.00. Found: N, 4.69; loss at 100°, 6.20. [m] This yield was obtained by the use of isopropyl acetate as the solvent, cuprous bromide as the catalyst, and phosphorus tribromide as the source of phosphorus. [n] Recrystallized from 3 *N* hydrochloric acid. [o] Calcd.: N, 6.75. Found: N, 6.80. [p] M.p. varied greatly with the rate of heating. [q] This compound was isolated and purified by procedure B as previously described; *cf.* ref. 2a. [r] This yield was obtained by the use of ethyl acetate as the solvent, cuprous bromide as the catalyst, and phosphorus trichloride as the source of phosphorus. [s] This compound was dried *in vacuo* at 100° to remove solvent of crystallization. [t] Calcd.: N, 5.90. Found: N, 5.77. [u] Calcd.: N, 5.58; H_2O, 7.17. Found: N, 5.43; loss at 100°, 7.23. [v] No phosphinic acid was obtained from this reaction. [w] Recrystallized from aqueous alcohol. [x] Previously reported by G. M. Kosolapoff, *ibid.*, 71, 369 (1949); m.p. 227–228°. [y] Recrystallized from 6 *N* hydrochloric acid. [z] Calcd.: N, 6.01. Found: N, 5.99. [aa] Calcd.: N, 7.43. Found: N, 7.30.

singly charged anions,[5] and the phosphonic acids as mixtures of the ions $ArPO_3H^-$ and $ArPO_3^=$.[6] Since the receptors of the enzyme might be more accessible to a neutral molecule than to a negatively charged one,[7] we prepared esters of several of our active compounds.

Table II lists the new phosphonic and phosphinic acids prepared. With the exception of 2-chloro-4-aminobenzenephosphonic acid, which was prepared by catalytic reduction of the corresponding nitro derivative,[2b] the compounds listed were synthesized from the appropriate diazonium fluoborates.[2a] In general, the disubstituted phosphonic and phosphinic acids were obtained by the same procedures used for the monosubstituted compounds.

In the presence of copper salts, both *o*-iodobenzenephosphonic acid and (*o*-iodophenyl)-phenylphosphinic acid were unstable at high *p*H's. The instability is due, presumably, to replacement of the iodine by a hydroxyl group. Thus, if the crude iodo compound is dissolved in 10% sodium hydroxide, iodide ion can be detected in the resulting solution and the presence of a phenol can be demonstrated by the ferric chloride test. For this reason the *o*-iodo derivatives could not be purified in the usual way, one step of which requires the precipitation of the acid from alkaline solution. The methods used for purifying the *o*-iodo acids are given in the Experimental section.

Also shown in Table II are the concentrations necessary for the 50% inhibition of the plasma enzyme employed. Although most of the compounds listed possessed some activity, the results with the disubstituted phosphonic acids were disappointing. In every compound tested a second substituent in addition to the ortho group markedly reduces the inhibitory effect. The considerable activity of the *o*-methoxy and *o*-carboxy derivatives demonstrates that a halogen is not essential for appreciable anticholinesterase activity.

Table III shows the esters prepared and tested. The activity of tetraethyl pyrophosphate is given for comparison. As we had hoped, most of the esters were more active than the free acids. The activity of *o*-bromobenzenephosphonic acid, in particular, was greatly enhanced by esterification.

Aldridge and Davison[8] have pointed out that there are enzymes in mammalian tissues which can

(5) H. H. Jaffé, unpublished results.

(6) H. H. Jaffé, L. D. Freedman and G. O. Doak, paper presented before the American Chemical Society, Atlantic City, N. J., September, 1952.

(7) A. Hood and W. Lange, THIS JOURNAL, **72**, 4956 (1950), for example, have reported that the acidic half-esters of fluorophosphoric acid are much less active than the corresponding neutral esters.

(8) W. N. Aldridge and A. N. Davison, *Biochem. J.*, **51**, 62 (1952).

TABLE III

ESTERS

Compound	Yield,[a] %	B.p., °C.	Mm.	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	I_{50}, mole/l.
TEPP[b]								5×10^{-9}
$(o\text{-}BrC_6H_4)C_6H_5PO_2CH(CH_3)_2$	50	183–191	2.0	53.11	53.22	4.76	5.03	1×10^{-6}
$(o\text{-}BrC_6H_4)_2PO_2C_2H_5$[c,d]	38	186–194	0.1	41.61	41.48	3.24	3.42	3×10^{-6}
$(o\text{-}BrC_6H_4)C_6H_5PO_2C_2H_5$	74	157–165	.1	51.71	51.44	4.34	4.54	1×10^{-5}
$o\text{-}BrC_6H_4PO(OC_2H_5)_2$	75	115–121	.1	40.97	40.91	4.81	4.96	1×10^{-5}
$(o\text{-}BrC_6H_4)C_6H_5PO_2CH_3$	55	160–168	.1	50.18	49.47	3.89	3.96	8×10^{-5}

[a] These figures must be considered only approximate, since they are the results of a single run. [b] Tetraethyl pyrophosphate, Monsanto, 40% pure. [c] Calcd.: P, 7.67. Found: P, 7.66. [d] M.p. 115–118°.

hydrolyze certain phosphate esters. Therefore, it was important to learn whether the enzyme used in our tests could split esters of phosphonic or phosphinic acids. The Warburg manometric technique was used to determine the rate of hydrolysis of 0.001 *M* ethyl bis-(*o*-bromophenyl)-phosphinate in sodium bicarbonate buffer at *p*H 7.0.[8] In one hour there was no evidence of hydrolysis; and even after 18 hours the amount of carbon dioxide evolved indicated that less than 4% of the ester had hydrolyzed. It seems safe to conclude, therefore, that this ester does not hydrolyze under the experimental conditions used for determining anticholinesterase activity.

Experimental

***o*-Iodobenzenephosphonic Acid.**—This compound was prepared from *o*-iodobenzenediazonium fluoborate by the general method described previously.[2a] After the reaction mixture was steam distilled, the residual liquid was filtered. A small amount of phosphinic acid remained on the filter but could not be readily purified. The filtrate was evaporated on the steam-bath to incipient crystallization, cooled and filtered. The resulting crude phosphonic acid was recrystallized from dilute hydrochloric acid.

***o*-Carboxybenzenephosphonic Acid.**—This compound was prepared from *o*-carbomethoxybenzenediazonium fluoborate. No phosphinic acid was obtained. The phosphonic acid was isolated and purified by procedure A as previously described.[2d]

2,5-Dichlorobenzenephosphonic Acid and Bis-(2,5-dichlorophenyl)-phosphinic Acid.—These compounds were separated and purified by the procedure used for the *p*-bromo derivatives.[1,2d]

A previous paper[2a] from this Laboratory described an unidentified phosphonic acid isolated from the reaction of *o*-nitrobenzenediazonium fluoborate and phosphorus trichloride. This phosphonic acid has now been shown to be 2,5-dichlorobenzenephosphonic acid by analysis and mixed m.p. with an authentic sample. The replacement of a nitro group in a diazonium salt by a chlorine atom has been previously observed[9]; the origin of the second chlorine atom is more difficult to understand.

(*o*-Iodophenyl)-phenylphosphinic Acid.—*o*-Iodobenzenediazonium fluoborate (31.8 g., 0.1 mole) was suspended in 125 ml. of ethyl acetate and 13.5 ml. of phenyldichlorophosphine[10] and 0.8 g. of cuprous bromide[11] were added. The apparatus used has been described.[2a] A short lag period (about 30 minutes) was followed by a vigorous reaction. When the evolution of gases was complete, 25 ml. of water was added and the mixture was steam distilled until approximately 500 ml. of distillate had been collected. The residual liquid in the flask was transferred to a beaker and cooled. The precipitated phosphinic acid was removed by filtration and recrystallized several times from 95% alcohol.

The Esters.—The conditions used for the preparation of the esters were similar to those described below for diethyl *o*-bromobenzenephosphate. All the esters in Table III are liquids at room temperature except for ethyl bis-(*o*-bromophenyl)-phosphinate.

Diethyl *o*-Bromobenzenephosphonate.—*o*-Bromobenzenephosphonic acid (22.8 g.) and 44.0 g. (about a 10% molar excess) of phosphorus pentachloride were heated under reflux for 30 minutes. After the phosphorus oxychloride and excess phosphorus pentachloride had been removed *in vacuo* (water-pump), the phosphonyl chloride was dissolved in 50 ml. of dry carbon tetrachloride. To this solution was added 56 ml. of absolute ethanol. The mixture was refluxed for one hour, and the carbon tetrachloride and excess alcohol were stripped off at the water-pump. Distillation of the residue at 0.1 mm. gave the pure ester.

Inhibition Studies. Enzyme Solution.—0.9 mg. of the enzyme[12] was dissolved in 1.0 ml. of 0.6% sodium chloride solution. A trace of insoluble material was removed by filtration.

Substrate Solution.—0.016 *M* acetylcholine bromide in 0.1 *M* phosphate buffer of *p*H 7.0.

Inhibitor Solutions.—The free acids were dissolved in dilute sodium hydroxide solution and adjusted to *p*H 7.0 with dilute hydrochloric acid. The esters were dissolved in the corresponding alcohol and then diluted with water. The final concentration of alcohol was less than 4%. It was ascertained that this amount of alcohol had no effect on the activity of the enzyme.

Procedure.—1.0 ml. of 0.1 *M* phosphate buffer at *p*H 7.0 was mixed with 0.5 ml. of enzyme solution. The volume was made up to 3.0 ml. with inhibitor solution and distilled water. This mixture was kept at 23° for 20 minutes; then 1.0 ml. of the acetylcholine bromide solution was added. After an additional 30 minutes an aliquot of the mixture was analyzed for residual substrate by the method of Hestrin[13] employing the Klett-Summerson photoelectric colorimeter. The activity of the enzyme in the absence of the inhibitor was always determined simultaneously.

In the case of *o*-aminobenzenephosphonic acid, the colorimetric method failed because the compound reacted with the alkaline hydroxylamine reagent. Therefore, the Warburg manometric method was used for measuring the hydrolysis of acetylcholine by the enzyme. The inhibitor, dissolved in 3.6 ml. of 0.01 *M* sodium bicarbonate, was placed in the main compartment of the Warburg flask. After this solution was gassed with 95% nitrogen–5% carbon dioxide, 0.2 ml. of enzyme solution (which contained 0.28 mg. of enzyme in 0.01 *M* sodium bicarbonate) was tipped into the main compartment. The reaction was allowed to proceed for 20 minutes at 23° and then 0.2 ml. of 0.08 *M* acetylcholine bromide in 0.01 sodium bicarbonate was added. Thirty minutes later the carbon dioxide production was compared with that in the control vessel which contained no inhibitor.

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(9) *Cf.* J. F. Bunnett and R. E. Zahler, *Chem. Revs.*, **49**, 273 (1951).

(10) Kindly furnished by the Victor Chemical Works.

(11) The amount of catalyst used in this preparation was only 40% of the amount specified in previous papers.[2] We have found that such a decrease moderates the violence of the reaction without lowering the yield.

(12) Prepared from Cohn Fraction IV-6 by Cutter Laboratories.

(13) S. Hestrin, *J. Biol. Chem.*, **180**, 249 (1949).