[CONTRIBUTION FROM THE CHEMICAL RESEARCH AND BIOLOGICAL ASSESSMENT DIVISIONS, U. S. ARMY CHEMICAL WARFARE LABORATORIES]

The Stereochemistry of Asymmetric Phosphorus Compounds. II. Stereospecificity in the Irreversible Inactivation of Cholinesterases by the Enantiomorphs of an Organophosphorus Inhibitor

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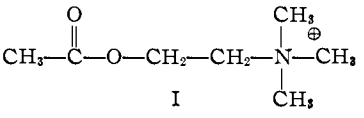
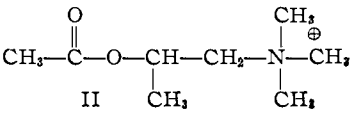
RECEIVED AUGUST 9, 1957

The *d*- and *l*-enantiomorphs of an asymmetric organophosphorus inhibitor of cholinesterases have been synthesized. The *levo* isomer reacted from ten to twenty times faster than the *dextro* in the irreversible inactivation of four cholinesterase preparations studied.

The stereospecificity exhibited by enzymes in their reactions with asymmetric carbon compounds is a well recognized phenomenon.[1] Such specificity is a consequence of the asymmetric nature of the enzyme surface. Therefore, although the natural substrate I of the acetylcholinesterases is optically inactive, it is not unreasonable to suspect that reactions of these enzymes with either asymmetric substrates or asymmetric inhibitors might be sensitive to the difference in the respective configurations of such substances.



I

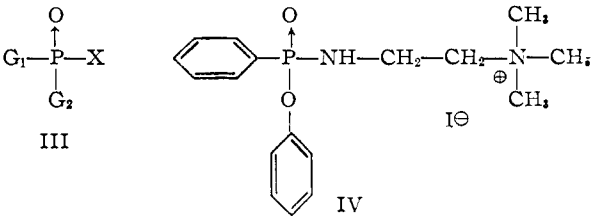


II

The use of optically active acetyl-β-methylcholine (II) as an asymmetric substrate to investigate the stereochemical specificity of serum cholinesterases has been reported.[2,3] Only the *d*-isomer was detectably hydrolyzed by these enzymes. Acetyl-β-methylcholine, however, is a very poor substrate for hydrolysis by serum cholinesterase. Indeed, the inability of cholinesterase enzymes of the non-specific or serum class to hydrolyze II has been shown[4,5] to be one of the distinguishing differences between the non-specific and the specific acetylcholinesterase types. The above results might have been more significant, therefore, if these stereoisomers had been tested with an enzyme of the true acetylcholinesterase class. An investigation along these lines but with *d,l*-acetyl-β-methylcholine was reported recently,[6] and a marked specificity of the enzyme toward one of the stereoisomers was indicated from the fact that only one-half of the substrate was hydrolyzed by the enzyme.

A stereospecificity has been reported for the inhibition of cholinesterases by the respective stereoisomers of asymmetric inhibitors. The inhibitors thus investigated have all been of the reversible type. For example, the *l*-isomers of amidone (2-dimethylamino-4,4-diphenyl-5-heptanone) and isoamidone (1-dimethylamino-2-methyl-3,3-diphenyl-4-hexanone) were reported[7] to be more effective inhibitors of cholinesterases than were the corresponding *d*-isomers. Specificity in the inhibition of an acetylcholinesterase by amino acids also has been reported.[8] Here, L-amino acids were shown to be weak reversible inhibitors of the enzyme; the D-forms, when tested, had a lower degree of activity or were completely inactive. These results may be contrasted, however, with those which report[9] that an enhancement in the activity of serum cholinesterase was obtained in the presence of certain of the same amino acids. The fact that the former[8] group of investigators used a specific and the latter group a non-specific cholinesterase, however, may account for the apparent discrepancy in these reports.

We have been investigating the stereochemistry of the inhibition of cholinesterase enzymes by irreversible inhibitors of the organophosphorus class (III). Compounds of this class are inhibitors by virtue of their ability to phosphorylate the enzyme.[10] Their action is termed irreversible, since an enzyme thus inactivated is able to regain its activity only slowly (or perhaps not at all), depending upon the structure of the groups attached to the phosphorus atom.[11] Since tetracovalent phosphorus atoms containing four dissimilar substituents are known to be optically active,[12] appropriately substituted organophosphorus inhibitors of the general class III can exist as optical stereoisomers. Evidence that potentially resolvable inhibitors of this class can show a stereo-



III IV

(1) See, for example, B. Helferich in "The Enzymes," edited by J. B. Sumner and K. Myrback, Academic Press, New York, N. Y., 1950, p. 79.

(2) D. Glick, *J. Biol. Chem.*, **125**, 729 (1938).

(3) A. Simonart, *Arch. intern. pharmacodynamie*, **60**, 209 (1938).

(4) G. A. Alles and R. C. Hawes, *J. Biol. Chem.*, **133**, 375 (1940).

(5) B. Mendel, D. B. Mundell and H. Rudney, *Biochem. J.*, **37**, 473 (1943).

(6) F. C. G. Hoskin and G. S. Trick, *Can. J. Biochem. Physiol.*, **33**, 963 (1955).

(7) M. E. Grieg and R. S. Howell, *Proc. Soc. Exp. Biol. Med.*, **68**, 352 (1948).

(8) F. Bergmann, I. B. Wilson and D. Nachmansohn, *J. Biol. Chem.*, **186**, 693 (1950).

(9) E. Aron, A. D. Herschberg and E. Frommel, *Helv. Physiol. Pharmacol. Acta*, **2**, 495 (1944).

(10) See, for example, B. J. Jandorf, H. O. Michel, N. K. Schaffer, R. Egan and W. H. Summerson, *Disc. Faraday Soc.*, **20**, 134 (1955).

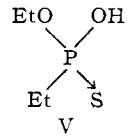
(11) I. B. Wilson, *ibid.*, 119 (1955).

(12) J. Meisenheimer and L. Lichtenstadt, *Ber.*, **44**, 356 (1911).

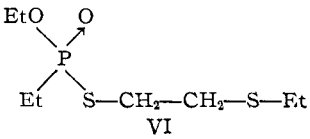
specificity in the inactivation of cholinesterases[13] and in hydrolytic cleavage by a phosphorylphosphatase[6] has been recently presented.

Resolved irreversible inhibitors of the organophosphorus class, however, have never been reported.[13a] The closest approach to the resolution of such an inhibitor may be found in the recent report[14] of the resolution of an organophosphonamidate[15] (IV). In this case, the cationic function present in the molecule was used as a handle to effect the resolution. At best, therefore, this method is limited to compounds which possess some functional group that can be used in a similar manner.

The recent resolution of an organophosphonothioic acid[16] (V), however, has served to provide us with a new approach to the resolution of an inhibitor of the desired type. In the method used, the optical isomers of O-ethyl S-(2-ethylthioethyl) ethylphosphonothiolate (VI) were synthesized di-



V



VI

rectly from the respective enantiomorphs of the parent acid V. The phosphonothiolate (VI) is an irreversible inhibitor of both specific and non-specific cholinesterases, and is very similar in structure to the O,O-diethyl S-(2-ethylthioethyl) phosphorothioate (VII) previously reported.[17] The method, therefore, has been used to synthesize the enantiomorphs of an asymmetric organophosphorus inhibitor which would be difficult (if not impossible) to resolve by classical methods.

The observed rotations (neat, 1 dcm.) of the optical isomers of VI were $-49.36°$ and $+47.15°$, respectively. This difference in the optical purity of the synthetic product is directly related to the difference in the optical purity of the parent acids (neat rotations, 1 dcm., of $-13.56°$ and $+13.21°$, respectively) used in the syntheses. The *d,l*-, *l*- and *d*-isomers of VI were shown to be free of any detectable traces of pyrophosphonate impurities by titrimetric methods. In addition, P^{31} nuclear spin resonance measurements (on the *d*-isomer) could detect no signal for a chemical shift[18] in the neighborhood of $\delta = -100$, a value which has been correlated[19] with the isomeric phosphonothionate (VIII) type structure. An expected[19] value of $\delta = -55$ was obtained for VI.

(13) H. O. Michel, *Federation Proc.*, **14**, 255 (1955).

(13a) NOTE ADDED IN PROOF.—After this article had been submitted for publication, a communication (G. Hilgetog and G. Lehmann, *Angew. Chem.*, **69**, 506 (1957)) was published on the resolution of O-methyl O-(*p*-nitrophenol) phosphorothioic acid anion, which describes several asymmetric ester derivatives prepared from the resolved anion for the purpose of investigating differences in physiological behavior of the stereoisomers. These esters were not too stable, however, and no mention was made that any physiological testing had been carried out using these compounds.

(14) K. L. Marsi, C. A. VanderWerf and W. E. McEwen, THIS JOURNAL, **78**, 3063 (1956).

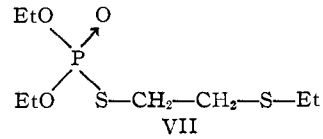
(15) The pharmacology of the stereoisomers of IV reportedly[14] has been determined.

(16) H. S. Aaron, T. M. Shryne and J. I. Miller, THIS JOURNAL, **80**, 107 (1958); see also H. S. Aaron and J. I. Miller, *ibid.*, **78**, 3538 (1956).

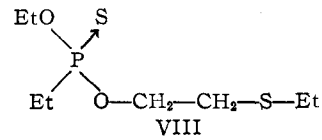
(17) T. R. Fukuto and R. L. Metcalf, *ibid.*, **76**, 5103 (1954).

(18) N. Muller and J. Goldenson, *ibid.*, **78**, 5182 (1956).

(19) H. Finegold, unpublished data.



VII



VIII

The kinetics were determined for the reactions of the *d,l*-, *l*- and *d*-isomers of VI with three specific and one non-specific acetylcholinesterase preparations. The rates of inactivation were all first order with respect to the enzyme concentration and proportional to the concentration of inhibitor. Since the ratios of the concentrations of inhibitor to enzymes were 10^4 or greater, second-order velocity constants (k) were computed from the equation

$$k = \frac{2.303}{Ct} \log A_0/A_t$$

where C = concentration of inhibitor, t = time of reaction, A_0 and A_t = cholinesterase activities at time zero and time t, respectively. The values of the velocity constants obtained in this study are given in Table I. Activation energies and $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values computed from the data for the inactivation of eel acetylcholinesterase at 25° are 12.8 kcal., 12.2 kcal. and −8.6 e.u. and 11.5 kcal., 10.9 kcal. and −7.2 e.u. for the *d*- and *l*-isomers, respectively.

TABLE I

VELOCITY CONSTANTS FOR INACTIVATION OF CHOLINESTERASES AT *p*H 7.4 BY *d,l*-, *d*- and *l*-O-ETHYL S-(2-ETHYLTHIOETHYL) ETHYLPHOSPHONOTHIOATE (VI)

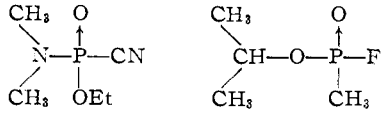
Enzyme type	Temp., °C.	$k \times 10^{-2}$ in l./mole·sec. *d,l*	*l*	*d*	*l*/*d*
Eel ChE	25	7.7	14.3	0.78	18
Eel ChE	15.1	..	7.3	0.37	20
Human red cell ChE	25	7.1	13.2	1.37	9.6
Bovine red cell ChE	25	4.9	9.0	0.94	9.6
Horse serum ChE	25	2.6	5.0	0.25	20

Discussion

The data of Table I show that the *levo*-isomer of VI reacts from ten to twenty times faster than the *dextro* with the enzyme preparations studied. Proof that this difference is not due to traces of a more active impurity present with the *levo*-isomer may be found in the fact that the velocity constants for the racemic mixture are, in each case, the mean of the values for the two resolved forms. The slight difference in the optical purity of the two enantiomorphs used was not enough to affect the kinetic results.

In earlier kinetic studies,[13] evidence for the presence of optical isomers in sarin having greatly different rates of reaction with eel cholinesterase was obtained. It was not possible to obtain the separated isomers of sarin, and their presence could only be inferred from the kinetics of the reactions. The results of the present work, therefore, strongly support the interpretation given to the previous findings.

The rate of phosphorylation of these enzymes by the stereoisomers of an organophosphorus inhibitor, therefore, possesses a specificity which is sensitive to the configuration at the phosphorus atom. The degree of specificity will vary with the structure of the inhibitor, on the one hand, and that of the enzyme, on the other. An example of the effect of a change in the structure of an organophosphorus substrate upon the stereospecificity of (in this case) enzymatic hydrolysis may be found in evidence presented to show that a phosphorylphosphatase selectively hydrolyzes one of the stereoisomers of tabun[6] (IX) but apparently shows little or no specificity for those of sarin[20] (X).



IX X

The structure of the enzyme will determine its inherent sensitivity to stereo differences between the two opposite configurations of an inhibitor. Based on the one compound VI examined, the ratios of the velocity constants of the two enantiomorphs summarized in Table I indicate that while differences may exist between the acetylcholinesterases, depending upon their source, there is apparently no clear-cut class distinction between the non-specific and the specific cholinesterases in the degree of their specificity for a particular configuration of an organophosphorus inhibitor. The structural differences that do exist between various cholinesterase enzymes, however, might well lend themselves to classification in terms of a stereochemical specificity when new asymmetric inhibitors are similarly examined.

Acknowledgment.—We wish to thank Mr. Harold Finegold for the determination and interpretation of the nuclear spin resonance measurements.

Experimental

***d,l*-O-Ethyl S-(2-Ethylthioethyl) Ethylphosphonothioate.**—*d,l*-O-Ethyl ethylphosphonothioic acid (0.69 g., 0.0045 mole), 20 ml. of 0.223 *N* sodium methoxide in methanol and 0.56 g. (0.0045 mole) of 2-(ethylthio)-ethyl chloride were combined and refluxed for two hours, then poured into water and extracted with ether. The ether layer was dried over Drierite, filtered and concentrated to a yellowish residue which was distilled to yield 0.59 g. of product, b.p. 102° (0.21 mm.), $n^{25}D$ 1.5093. An additional 0.17 g. was collected as a forerun to bring the total yield to 70%.

Anal. Calcd. for $C_8H_{19}O_2PS_2$: C, 39.7; H, 7.9; P, 12.8. Found: C, 39.9, 39.7; H, 8.1, 7.9; P, 12.5.

***l*-O-Ethyl S-(2-Ethylthioethyl) Ethylphosphonothioate.**—A mixture of 1.540 g. (0.0100 mole) of *l*-O-ethyl ethylphosphonothioic acid ($\alpha_{obs.}$ −13.56°, neat, 1 dcm.), 44.8 ml. of 0.223 *N* sodium methoxide in methanol and 1.25 g. (0.010 mole) of 2-(ethylthio)-ethyl chloride was refluxed for two hours. The solvent was then removed under reduced pressure, and the product was taken up in ether, washed with water, 2% bicarbonate solution, water and then dried over Drierite. After distilling off the solvent, the residue was vacuum distilled to yield 1.69 g. (0.0070 mole, 70% yield) of product, b.p. 104° (0.24 mm.), $n^{25.5}D$ 1.5095, $\alpha_{obs.}$ −49.36 ± 0.02° (neat, 1 dcm.), $[\alpha]D$ −35.6 ± 0.4° ($\alpha_{obs.}$ −0.626 ± 0.007°, methanol, 1 dcm., *c* 1.762), $[\alpha]D$ −43.1 ± 0.4° ($\alpha_{obs.}$ −0.718 ± 0.006°, acetone, 1 dcm., *c* 1.668).

Anal. Calcd. for $C_8H_{19}O_2PS_2$: C, 39.7; H, 7.9; P, 12.8. Found: C, 39.8, 39.8; H, 8.2, 8.1; P, 12.8.

***d*-O-Ethyl S-(2-Ethylthioethyl) Ethylphosphonothioate.**—A mixture of 1.541 g. (0.0100 mole) of *d*-O-ethyl ethylphosphonothioic acid ($\alpha_{obs.}$ +13.21°, neat, 1 dcm.), 44.8 ml. of 0.223 *N* sodium methoxide in methanol and 1.25 g. (0.010 mole) of 2-(ethylthio)-ethyl chloride was refluxed for two hours, then worked up as described for the levorotatory isomer, above. The product was distilled to yield 1.82 g. (0.0075 mole, 75%) b.p. 107–108° (0.28 mm.), $n^{25.6}D$ 1.5091, $\alpha_{obs.}$ +47.15 ± 0.01° (neat, 1 dcm.), $[\alpha]D$ +41.6 ± 0.4° ($\alpha_{obs.}$ +0.820 ± 0.008°, acetone, 1 dcm., *c* 1.974). Analysis of this product by a nuclear spin resonance measurement showed an absence of any impurity of the isomeric thionate structure VIII.

Anal. Calcd. for $C_8H_{19}O_2PS_2$: C, 39.7; H, 7.9; P, 12.8. Found: C, 39.7; H, 7.9; P, 12.6.

Method of Examination of the *d,l*-, *l*- and *d*-Products for Pyrophosphonate Impurity.—An accurately weighed sample (0.1 g.) of VI was dissolved in 5 ml. of acetone, 15 ml. of water was added, and the solution was stirred with a magnetic stirrer. After checking the apparent *p*H, the solution was titrated with 0.010 ml. increments of 0.022 *N* base; the apparent *p*H was recorded after each titration. A solvent blank was similarly examined. No acid was detectably formed in these solutions on stirring for up to 15 minutes at room temperature. Impure samples of product prepared by an alternate method and known to contain a few per cent. of pyrophosphonate impurities rapidly formed the theoretical amount of acid under these conditions. After standing overnight at room temperature, an amount of acid corresponding to less than 2 mole-parts per 1000 was formed in these solutions, most probably due to a solvolysis of the product.

Kinetic Measurements.—The kinetics were determined for the inactivation of four purified[21] cholinesterase preparations. The enzymes, with specific activities given in units of micromoles of acetylcholine hydrolyzed per second per mg. of protein, were as follows: electric eel, 13; human red blood cell, 0.030; bovine red blood cell, 0.065 and horse serum, 0.055. The concentrations of the cholinesterases, which were estimated by methods described previously,[23] ranged from 7×10^{-10} to 8×10^{-9} *M* in the kinetic determinations. The reactions of the enzymes with the phosphonothioates were run in solutions containing 0.357 *M* potassium chloride, 0.1% gelatin, 0.0018 *M* tris-(hydroxymethyl)-aminomethane buffer at *p*H 7.40 and 25°, except that in the case of horse serum cholinesterase, 0.107 *M* sodium chloride was used in place of 0.357 *M* potassium chloride. After allowing the reactions to proceed from 0 to 3 minutes, 0.11 *M* acetylcholine chloride was added to give a final concentration of 0.0073 *M*. Residual cholinesterase activity was then determined by titration with 0.03 *N* sodium hydroxide at a constant *p*H of 7.40 and 25°. Stirring of the solution was accomplished by means of a small magnetic stirrer. The addition of acetylcholine almost completely stops the reaction between the enzyme and anticholinesterase. However, the effect on the computed velocity constants of the slow reaction occurring between the enzymes and inactivators after the addition of acetylcholine was eliminated by titrating for a constant time interval. The rate of hydrolysis of acetylcholine catalyzed by the cholinesterases was directly proportional (with a standard deviation of ±2%) to the enzyme concentration down to over a ten-fold decrease in the initial enzyme concentration used. The reactions were followed over the range of 0.5 to 5% of the total substrate hydrolyzed.

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(20) P. A. Adie, F. C. G. Hoskin and G. S. Trick, *Can. J. Biochem. Physiol.*, **34**, 80 (1956).

(21) The electric eel acetylcholinesterase was obtained from Dr. D. Nachmansohn; the human red cell acetylcholinesterase was prepared by Dr. C. A. Zittle; the horse serum cholinesterase was prepared by the method of Strelitz[22]; the bovine red cell acetylcholinesterase was obtained commercially.

(22) F. Strelitz, *Biochem. J.*, **38**, 86 (1944).

(23) (a) H. O. Michel and S. Krop, *J. Biol. Chem.*, **190**, 119 (1951); (b) H. O. Michel, *Federation Proc.*, **11**, 259 (1952).