[CONTRIBUTION FROM THE U. S. NAVAL MEDICAL RESEARCH INSTITUTE]

The Acetylcholinesterase Surface. V. Some New Competitive Inhibitors of Moderate Strength[1,2]

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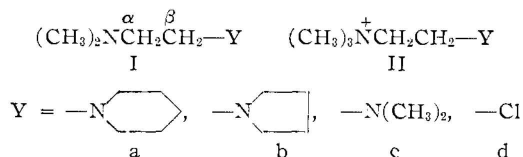
Diamines of the type (Ia) and its monomethiodide previously have been shown to be strong *in vitro* inhibitors of the enzyme acetylcholinesterase, when the α- and β-carbons are unsubstituted. It has now been observed that substitution of a single methyl group at either of these two carbon atoms lowers the strength of inhibition sharply, with enzyme–inhibitor dissociation constants rising by a factor of more than 10^2, and that substitution at the β-carbon is slightly more potent than at the α-position in this respect. These facts are in accord with the results of others on the effectiveness of certain acetylcholine analogs, investigated as a function of chain branching at the two methylene groups. As a further finding on the requirement of a polymethylated nitrogen atom for effective binding at the catalytic surface, the two methyl groups of Ia were replaced by *ortho* ring carbon atoms in the second ring of ethylenedipiperidine (VIII), resulting in a large drop in enzymatic inhibitory power. The addition of a single quaternizing methyl group as in IX again raises the inhibitory strength by a considerable factor. These results are discussed briefly in terms of the complementary structure of the catalytic surface.

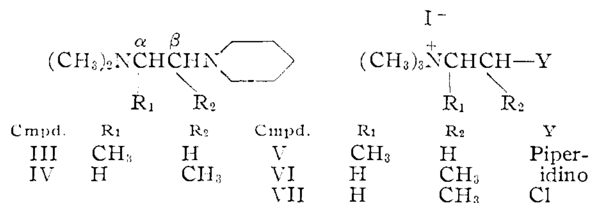
Introduction

Previous work[3] on inhibition of acetylcholinesterase (AChE) derived from tissue of the electric eel has shown that compounds with the structures I and II function as strong competitive inhibitors of



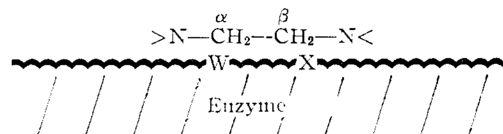
the enzyme. The enzyme–inhibitor dissociation constants (K_I) for these compounds deduced from the Wilson equation[4] were found to be of the order of 10^{-7} to 10^{-8} at *p*H 7.4 and 25°, and this relatively high potency was attributed in part to strong binding between a locus of high electron density in the inhibitor molecule (*e.g.* $-\ddot{N}<$) and the esteratic site of a catalytic unit on the enzyme's surface.

Now, in a preliminary attempt to probe some of the steric features of that portion of the enzymatic surface underlying the two methylene units when inhibitors like I or II are bound to sites sought competitively by substrate molecules, substitution of single methyl groups on the α- and β-carbon atoms of these inhibitors has been effected and the resulting compounds III–VI tested for this inhibitory power at *p*H 7.4 and 25°.



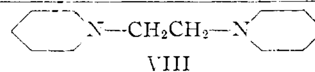
Cmpd.	R_1	R_2	Cmpd.	R_1	R_2	Y
III	CH_3	H	V	CH_3	H	Piper-
IV	H	CH_3	VI	H	CH_3	idino
			VII	H	CH_3	Cl

This procedure stems from a number of literature observations on the permissive structural variations in the α- and β-carbon atoms of acetylcholine-like substrates, including the representative study by Glick[5] on serum esterase showing that a methyl group in the β-position produces a greater drop in enzymatic hydrolysis rate than does substitution into the α-position. Using inhibitors as the effective surface mapping agents in the present work, it was therefore of some special interest to note any regular difference in degree of response of the enzyme to α- and β-substitution in the adsorbed molecules, to be interpreted as reflecting a difference in steric requirements of the corresponding areas W and X on the surface. Further, the effect of β-sub-



stitution as a phenomenon independent of the diamine structure also has been checked by comparing the inhibitory properties of the chloro compounds IId and VII.

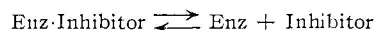
As an additional insight into the requirement of a polymethylated nitrogen function for strong binding (involving dispersion forces that act between methyl groups and the anionic site[6]), variation in the basic structures Ia and IIa to include the closure of two of the N-substituted methyl groups into the second ring of compound VIII and its monomethio-



dide IX was carried out, and the inhibitory activities of these species noted for correlation with this structural change.

Results

Inhibition studies with the series of compounds III–IX were conducted at *p*H 7.4 and 25.14° in the presence of 0.01 *M* Mg(II) ion, essentially as previously described.[3] With freshly prepared inhibitor solutions, each member of the series conformed to linearity of the standard v/v_I *vs.* [Inhibitor] plot[4] over a ten to twenty-fold variation in concentration, and the least-squares slopes of these plots were used to obtain the K_I values for the dissociation process.



$$\text{Enz·Inhibitor} \rightleftarrows \text{Enz} + \text{Inhibitor}$$

(1) The opinions in this paper are those of the authors and do not necessarily reflect the views of the Navy Department.

(2) Presented in part at the Third International Congress of Biochemistry, Brussels, Belgium, Aug. 1–6, 1955.

(3) S. L. Friess and W. J. McCarville, THIS JOURNAL, **76**, 1363, 2260 (1954).

(4) P. W. Wilson, "Respiratory Enzymes," H. A. Lardy, ed., Burgess Publishing Co., Minneapolis, Minn., 1949, p. 24.

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

(6) S. A. Bernhard, THIS JOURNAL, **77**, 1966 (1955); I. B. Wilson, *J. Biol. Chem.*, **197**, 215 (1952).

In the case of compound VII, the effects of small amounts of solvolysis of the secondary chloride function (leading to the weakly-inhibitory choline derivative) which might occur during the course of kinetic runs employing this inhibitor were minimized by the use of initial (5% of reaction) rates. The uncertainties noted in the K_I values summarized in Table I are the standard errors estimated from the least-squares fit of the Wilson plots.

TABLE I

DISSOCIATION CONSTANTS FOR AChE–INHIBITOR COMPLEXES

25.14 ± 0.03°, *p*H 7.4, ionic strength = 0.125

Inhibitor	$K_I \times 10^5$	Inhibitor	$K_I \times 10^5$
III	1.00 ± 0.07	VIII	1.47 ± 0.03
IV	1.04 ± .06	IX	0.11 ± .004
V	0.91 ± .03	Ia	.0064[a]
VI	1.45 ± .07	IIa	.0016[a]
VII	1.68 ± .07	IId	.012[a]

[a] See reference 3.

Using the K_I values of Table I as indices of inhibitory power, with increasing K_I denoting a decrease in inhibitory strength, several interesting comparisons can be made. First, the introduction of either an α- or a β-methyl substituent into the parent Ia or IIa structures raises the K_I value of the resulting inhibitors by a factor of roughly 2–10 $\times 10^2$, corresponding to a very considerable decrease in effectiveness. A similar rise of about 10^2 in K_I is also seen (in VII) on introduction of a β-methyl substituent into the chloro-derivative IId. These compounds (III–VII) as a group fall into an intermediate range of effectiveness below that of the potent inhibitors (eserine, Ia, IIa; $K_I \simeq 10^{-8}$) but still a power of ten better than weak difunctional inhibitors of the choline[7] type ($K_I \simeq 10^{-4}$). Further comparison of K_I values in the series III–VI referred to the unsubstituted structures Ia and IIa reveals the interesting point that introduction of an α- or β-methyl group (V or VI) into the monoquaternary system IIa results in a K_I change about 3–5 times greater than the corresponding substitutions produce in the ditertiary structure Ia (*cf.* compounds III and IV). It would reasonably appear from these evoked responses that the steric requirements of the enzymatic regions W and X are expressed more vigorously when a trimethylated nitrogen function adjacent to the α-methylene group is tightly bound[6] to the anionic site, than when the less tightly bound but possibly charged[8] dimethylamino group of Ia and its derivatives figures in the interaction between inhibitor and enzyme surface.

The picture of the relative effects of α- *vs.* β-methyl substitution in these inhibitors is not a striking one, as shown by comparisons of K_I within the sequences III–IV–Ia and V–VI–IIa. Little or no difference is observed between the two types of substitution in the ditertiary structure Ia, but for the monoquaternary base IIa the α-methyl derivative V is a slightly better inhibitor than the β-methyl compound VI, implying that a β-methyl substituent interferes somewhat more seriously with the enzymatic terrain X below it than an α-methyl group interferes with region W. This latter behavior is consistent with the observations of Alles and Hawes[9] on hydrolyses catalyzed by human red-blood cell esterase in which it was found that at equivalent substrate concentrations over the *p*H range 7–9 the compound acetyl-α-methylcholine displays higher hydrolysis rates than those for acetyl-β-methylcholine, and also with the work of Adams and Whittaker[10] in which branching studies on choline esters and certain aliphatic esters using blood cell esterase point to a greater hindering effect of a β-substituent. Apparently the true acetylcholinesterase and the serum esterases are alike in this steric response to α- and β-branching, in view of the previously cited work of Glick[5] on the serum enzyme.

In the final structure variation of the present study, compound VIII and its methiodide IX were prepared and tested for their inhibitory activities, with acetylcholine as substrate. The effective change here was the closure of two of the N-substituted methyl groups into the ring structure of piperidine, resulting in a symmetrical diamine. In VIII, either piperidine moiety can serve for binding at the esteratic site (as in I, II, etc.) but the other (in monoprotonated form[11] at *p*H 7.4) is constrained to function at the anionic site. The net result is an inhibitor of quite moderate strength ($K_I \simeq 10^{-5}$), falling in with the category of inhibitors III–VII. Replacement of the protonated form of VIII by the monomethylated quaternary derivative IX however results in a large increase in inhibitory power, with K_I decreasing by a factor of 13. This change may well reflect the need[6] for one or more methyl groups (in addition to a charged nitrogen atom) to give fairly strong binding at the anionic site. It is worth noting in this regard though that compounds III–VI, which possess all the attributes required for binding at this site in addition to the piperidine function for binding at the esteratic locus, still cannot match the inhibitory power of IX because of the blocking α- and β-substituents that prevent close conformation to the surface between these sites.

Experimental[12]

Enzymatic Rates and Inhibition.—Rates of enzymatic hydrolyses in the presence and absence of inhibitors were determined by the constant-*p*H titration method, as previously described.[3] Triply recrystallized acetylcholine chloride was employed, and freshly prepared solutions of this substrate and of the inhibitors described below were used throughout for the rate runs. These determinations were made at constant initial ionic strength of 0.125, achieved by regulation of the amount of sodium chloride added, and in the presence of dilutions of a single enzyme preparation[13]

(7) H. D. Baldridge, W. J. McCarville and S. L. Friess, THIS JOURNAL, **77**, 739 (1955).

(8) For the possible state of charge of Ia at *p*H 7.4 see: S. L. Friess and H. D. Baldridge, *ibid.*, **78**, 199 (1956).

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

(10) D. H. Adams and V. P. Whittaker, *Biochim. Biophys. Acta*, **3**, 358 (1949).

(11) For pK_a' values of 6.25 and 9.47 indicative of an essentially monoprotonated species at *p*H 7.4 see: A. Gero, THIS JOURNAL, **76**, 5158 (1954).

(12) Melting points and boiling points are uncorrected. Analyses by courtesy of Dr. W. C. Alford, Microanalytical Laboratory, National Institutes of Health, Bethesda, Md.

(13) Prepared essentially according to the method of M. A. Rothenberg and D. Nachmansohn, *J. Biol. Chem.*, **168**, 223 (1947).

from *Electrophorus electricus* that assayed at about 2.8 × 10^6 μmoles acetylcholine hydrolyzed/hr./mg. protein per ml. Initial substrate concentrations were confined to a range near the optimum for the enzyme dilution employed, and the kinetics of substrate hydrolysis were observed at 25.14 ± 0.03°. Initial rates corresponding to no more than 10% completion of reaction were used as velocity values for inhibited and uninhibited hydrolyses, so that no appreciable effect from accumulation of the weakly-inhibitory product choline could occur in this interval.

Preparation of Inhibitors. β-Piperidinoisopropyl Alcohol.—Over a period of an hour, 47 g. of piperidine was added to a mechanically-stirred, refluxing mixture of 49 g. of propylene oxide in 45 ml. of absolute methanol. Stirring was continued for a total of three hours, and the resulting mixture was allowed to stand at room temperature overnight. Fractionation of the reaction mixture gave 69 g. (86%) of β-piperidinoisopropyl alcohol, b.p. 107–109° (50 mm.), $n^{25}D$ 1.4579.

β-Piperidinoisopropyl Chloride Hydrochloride.—To a cold (ice-bath) stirred mixture of 68 g. of thionyl chloride and 300 ml. of chloroform there was added over a period of one hour 68 g. of β-piperidinoisopropyl alcohol. The resulting mixture was heated under reflux with continued stirring for about 30 minutes, cooled, and filtered. The product was recrystallized twice from absolute ethanol to give 68 g. (72%) of β-piperidinoisopropyl chloride hydrochloride, m.p. 202–204° (lit.[14] value 203–204°).

Dimethyl-β-piperidinoisopropylamine (III).—A mixture of 37.4 g. of β-piperidinoisopropyl chloride hydrochloride, 150 g. of 25% aqueous dimethylamine and 150 ml. of absolute ethanol was heated in a sealed bottle at 100° overnight. The cooled reaction mixture was then made strongly basic with potassium hydroxide, saturated with potassium chloride, and extracted with ether. The ether extract was dried over anhydrous magnesium sulfate and fractionated to yield 21 g. (63%) of dimethyl-β-piperidinoisopropylamine, b.p. 108–110° (34 mm.), $n^{25}D$ 1.4583; picrate, m.p. 194–196°.

Trimethyl-β-piperidinoisopropylammonium Iodide (V).—A mixture of 5 g. (0.029 mole) of dimethyl-β-piperidinoisopropylamine and 5 g. (0.035 mole) of methyl iodide in 200 ml. of anhydrous ether was mechanically shaken at room temperature for 25 hours. The product was collected by suction filtration and twice recrystallized from methanol-ether to give 4.5 g. of trimethyl-β-piperidinoisopropylammonium iodide, m.p. 182–184°; picrate, m.p. 187–188°. Assignment of this structure to the monoquaternary derivative of the diamine III is based on the analogous monoquaternization of dimethyl-β-piperidinoethylamine (Ia) under these conditions, yielding only trimethyl-β-piperidinoethylammonium iodide (IIa) identical with that prepared previously[3] in another way, and remains to be confirmed by Hofmann degradation.

Anal. Calcd. for $C_{11}H_{25}N_2I$: C, 42.31; H, 8.07; N, 8.97; I, 40.65. Found: C, 42.82; H, 8.20; N, 8.82; I, 40.41.

Dimethyl-β-piperidinopropylamine (IV).—A mixture of 51 g. of piperidine and 15.8 g. of dimethyl-β-chloropropylamine hydrochloride was heated under reflux at 120–130° (oil-bath) for 45 hours. The excess piperidine was removed *in vacuo* on the steam-bath. The residue was dissolved in water and the resulting solution made basic with potassium hydroxide, saturated with salt, and extracted with ether. The ether extract was dried over anhydrous potassium carbonate, filtered, and fractionated to give 9.1 g. (54%) of dimethyl-β-piperidinopropylamine, b.p. 109–110° (35 mm.) and 105–106° (30 mm.), $n^{25}D$ 1.4579; picrate, m.p. 86–88°.

Trimethyl-β-piperidinopropylammonium Iodide (VI).—A mixture of 4.6 g. (0.027 mole) of dimethyl-β-piperidinopropylamine and 4.6 g. (0.032 mole) of methyl iodide in 100 ml. of absolute ether was mechanically shaken overnight at room temperature. The product was filtered and twice recrystallized from isopropyl alcohol, then dried at 100° *in vacuo* overnight to give 2.6 g. of trimethyl-β-piperidinopropylammonium iodide, m.p. 168–169° dec.; picrate, m.p. 150–151°. This structural assignment is again in conformity with the exclusive monomethylation found at the dimethylamino group of compound Ia.

Anal. Calcd. for $C_{11}H_{25}N_2I$: C, 42.31; H, 8.07; N, 8.97; I, 40.65. Found: C, 42.61; H, 7.95; N, 8.99; I, 40.58.

Trimethyl-β-chloropropylammonium Iodide (VII).—An aqueous solution of 20 g. of recrystallized Eastman Kodak Co. dimethyl-β-chloropropylamine hydrochloride was made basic with potassium hydroxide, saturated with salt, and extracted with ether. The ether extract was dried over anhydrous magnesium sulfate and filtered. To the ether solution was then added 36 g. of methyl iodide, and the mixture allowed to stand at room temperature for three days. The crude product was filtered, twice recrystallized from acetone, and dried at 100° *in vacuo* overnight to give 26.4 g. of trimethyl-β-chloropropylammonium iodide, m.p. 186–187° dec. (lit.[15] value, 185–186°); picrate, m.p. 156–157°.

N,N'-Ethylenedipiperidine (VIII).—This material was prepared from ethylene dibromide and an excess of piperidine, essentially according to the method of Brühl[16]; b.p. 132–134° (15 mm.), lit. value[16] 132.5° (14 mm.).

N,N'-Ethylenedipiperidine Dihydrochloride.—Anhydrous hydrogen chloride was passed through a solution of 3.64 g. of ethylenedipiperidine in dry ether. The product was filtered, recrystallized once from methanol–ether mixture, and again from 95% ethanol. It was then dried *in vacuo* at 100° to give 3.88 g. (78%) of the dihydrochloride; sublimes without melting on heating above 250°.

Anal. Calcd. for $C_{12}H_{26}N_2Cl_2$: C, 53.52; H, 9.73; N, 10.40. Found: C, 53.45; H, 9.76; N, 10.30.

N,N'-Ethylenedipiperidine Monomethiodide (IX).—A solution of 4.91 g. (0.025 mole) of ethylenedipiperidine and 3.54 g. (0.025 mole) of methyl iodide in 100 ml. of anhydrous ether was mechanically shaken overnight to give 6.09 g. (72%) of the crude monomethiodide. Two recrystallizations from ether–methanol gave the pure monomethiodide, m.p. 156° (lit. value[17] 155.5°).

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(14) F. F. Blicke and C. E. Maxwell, This Journal, **64**, 428 (1942).

(15) B. M. Schultz and J. M. Sprague, *ibid.*, **70**, 50 (1948).

(16) W. Brühl, *Ber.*, **4**, 738 (1871).

(17) O. Aschan, *ibid.*, **32**, 988 (1899).