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

Nature of the Acetyl Cholinesterase Surface. III. Enzymatic Response to *cis–trans* Isomers in the Cyclohexane Series as Mapping Agents[1,2]

BY H. DAVID BALDRIDGE, JR., W. J. MCCARVILLE AND S. L. FRIESS

RECEIVED MAY 15, 1954

It has been found that the *cis* and *trans* isomers of the iodide salt of *d,l*-2-trimethylaminocyclohexyl acetate function as substrates of the enzyme acetyl cholinesterase, with the maxima in their activity *vs.* substrate concentration curves occurring at lower concentration values than that observed for the natural substrate acetyl choline. Similarly, the salts of the *cis–trans* isomers of *d,l*-2-trimethylaminocyclohexanol function as weak but reversible and competitive inhibitors of the enzymatic hydrolysis with an inhibitory strength somewhat greater than that of choline. In the inhibitors, the order of affinity of the enzyme for these molecules appears to be in the sequence: *cis* > *trans* > open chain choline derivative. The significance of these results with respect to the distance of separation between the two sites of a single catalytic unit on the enzyme's surface is discussed briefly.

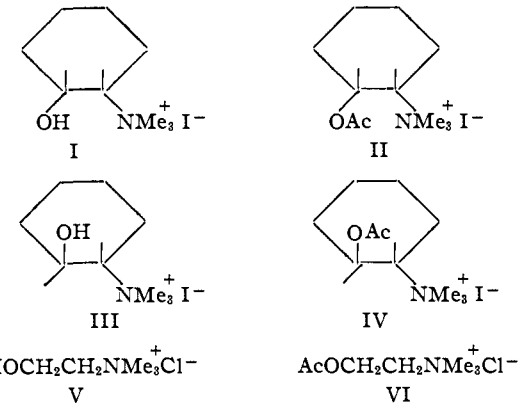
Introduction

Previous studies[3] on purified acetyl cholinesterase (AChE) derived from electric eel tissue have indicated that the structural features required for one class of potent competitive inhibitors of the enzyme are found in the unit

$$Me_3\overset{+}{N}—CH_2CH_2—\ddot{X}<$$

Here, X represents a locus of high electron density such as that found in a tertiary N function (preferably in a five- or six-membered heterocyclic ring for highest activity), or in a halogen atom such as chlorine. Presumably, these inhibitors function by competition with the natural substrate acetyl choline (AC) for adsorption on the two sites (esteratic and anionic) present[4] in each catalytic unit of the enzyme's surface. However, the general structure of this class of inhibitors does little to aid in defining the precise distance of separation between the sites adsorbing the quaternary and high-electron-density functions, respectively, because of the flexibility of the two-carbon chain and the resulting lack of knowledge of the particular molecular geometry assumed on its adsorption at the catalytic surface.

In a preliminary effort to remove this element of geometrical uncertainty and to obtain an estimate of the intersite separation distance, several pairs of isomeric compounds in the cyclohexane series, in which the separation between functional groups 1,2 to each other is comparatively well defined, have been tailored to serve as substrates and inhibitors. Thus, in the sequence of cyclic compounds I–IV which have been compared with the open-chain analogs V and VI, the separation distances between O and N atoms in the *cis–trans* pair I and III are known[5] within much more precise limits than those which can be estimated for choline (V), and similarly for the nitrogen–ether oxygen separation distance in compounds II and IV as compared with the acetylcholine ion (VI). In both sets of cyclic derivatives the ring carbons 1 and 2 function as the methylene groups of V or VI, but the ring itself serves to define the orientation of the substituents and limit their separation distance to a relatively small range.



Results

Inhibitors.—Compounds I and III were tested for their inhibitory strength on the enzymatically catalyzed hydrolysis of the natural substrate VI, and the resulting data compared with those determined for choline (V) by Wilson.[6] In each case the velocity of substrate hydrolysis at fixed enzyme and initial substrate concentrations was observed as a function of inhibitor concentration, and the data treated as previously[3] by the P. W. Wilson equation to yield the best least-squares value of the enzyme–inhibitor dissociation constant K_I. The results are summarized in Table I.

TABLE I

THE QUATERNARY AMINOALCOHOLS AS AChE INHIBITORS
$[AC]_0 = 3.34 \times 10^{-3} M$, temp. 25.12 ±0.02, *p*H 7.4

Compound	$K_I \times 10^4$	O → N Distance, Å.
I (*d,l,cis*)	1.1 ± 0.1	2.5–2.9[b]
III (*d,l-trans*)	2.1	2.9–3.7[b]
V	4.5[a]	2.3[c]

[a] See reference 6. [b] See reference 5. [c] Calculated using the Eyring formulation for a flexible chain.

(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 before the Division of Biological Chemistry, National Meeting of the American Chemical Society, Kansas City, Mo., March 26, 1954.

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

(4) For a discussion of their work leading to postulation of this duality of catalytic sites, see: D. Nachmansohn and I. B. Wilson, *Advances in Enzymol.*, **12**, 259 (1951).

(5) These separation distances in the *d,l*-1,2-aminocyclohexanols have been calculated by G. E. McCasland and D. A. Smith, THIS JOURNAL, **72**, 2190 (1950), from inspection of the molecular models and the known covalent bond radii. Limiting ranges for the distances in *cis* and *trans* isomers were set by consideration of both chair and boat forms of the ring, and equatorial and polar disposition of the substituents.

(6) I. B. Wilson, *J. Biol. Chem.*, **197**, 215 (1952).

It is seen from Table I that all of the quaternized aminoalcohols listed are relatively weak inhibitors, when compared with the monoquaternized diamines ($K_I \simeq 10^{-8}$) of the previous study.[3] However, if decreasing values of the dissociation constant K_I are taken as an index of increasing inhibitory strength, then in this series the *cis* derivative is a better inhibitor than the *trans*, which in turn is better than the open chain derivative V. In terms of conformation to the catalytic surface of the enzyme, this would imply that the O → N distance of the *cis* derivative (2.5–2.9 Å.) is the nearest approximation in the series to the distance between sites used competitively by these inhibitors and the substrate.

Substrates.—The hydrolysis of both of the acetates II and IV is catalyzed markedly by AChE, but the treatment of the data for these substrates is somewhat complicated by the nature of the enzyme. As first observed by Nachmansohn and Rothenberg,[7] the electric eel tissue enzyme is inhibited quite sharply by excess natural substrate VI, and the shape of the activity *vs.* substrate concentration curve is roughly bell-shaped with an activity maximum at about 3.3×10^{-3} *M* acetylcholine. This same general behavior is noted for the new substrates II and IV, namely, each of the hydrolyses is catalyzed by the enzyme, and there is inhibition by substrate added in excess of the respective optimum concentration values. The shapes of the activity *vs.* substrate concentration curves at *p*H 7.4, 25.12°, and constant enzyme concentration are shown in Fig. 1.

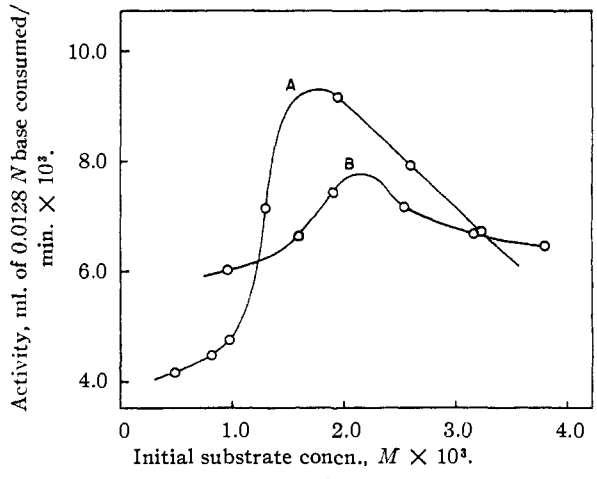


Fig. 1.—Activity *vs.* initial substrate concentration plots: A, compound II (*cis*-acetate); B, compound IV (*trans*-acetate).

It is seen from Fig. 1 that the position of the maximum on the concentration axis varies with the stereochemical nature of the substrate. Table II presents a summary of the data on these maxima, together with a tabulation of the relative activity of a fixed amount of the esterase in hydrolysis of the three substrates II, IV and VI. The last column gives this hydrolytic power, relative to 1.00 for acetylcholine, with each rate of hydrolysis having been measured at an initial substrate concentration which yields the optimum rate (V_{opt}, at the peak of the substrate concentration *vs.* activity curve) for that substrate.

(7) D. Nachmansohn and M. A. Rothenberg, *J. Biol. Chem.*, **158**, 653 (1945), *Science*, **100**, 454 (1944).

TABLE II

QUATERNARY ACETATES AS ACHE SUBSTRATES, *p*H 7.4, 25.12°

Compound	[Substrate] at optimum velocity, $M \times 10^3$	Relative V_{opt} value
II (*cis*)	1.8 ± 0.1	1.14 ± 0.04
IV (*trans*)	2.1	1.06
VI	3.3	1.00

Two curious points are to be noted from Table II. First, with respect to the concentrations of substrates required to attain their respective peak activity values, the compounds listed lie in the sequence *cis*-acetate II < *trans*-acetate IV < acetylcholine VI. Also, it is seen that in a comparison of V_{opt} values, the sequence is inverted and run II > IV > VI. If now the coupled phenomena of a low concentration of substrate required for peak activity together with a high value for that peak activity (V_{opt}) can be taken as criteria of close approach to conformity with the enzyme's surface, then the order of fitting deduced from Table II would be just that noted previously from the data of Table I, *i.e.*, *cis* derivative better than *trans*, which in turn is better than the open-chain compound. This agreement of the two correlations perhaps may be fortuitous in this group of compounds, however, and it will be interesting to test the generality of the procedure by observations on quaternary acetates and alcohols in the cyclopentane series.

Discussion

The data for the *cis–trans*-alcohols and perhaps the acetates in this cyclohexane series seem to point to an enzymatically preferred O → N separation distance close to or in the range of 2.5–2.9 Å. It will be most interesting to observe whether this range can be narrowed by study of the action of the *cis–trans* isomeric pairs of 1,2-acetates and alcohols in the cyclopentane series, where planarity of the ring would result in a more precise substituent orientation than that attainable with the cyclohexane derivatives.

One implicit feature in this process of fitting the enzyme by tailored substrates and inhibitors has been the assumption that major interaction occurs only between the polar substituents of the small molecules and the catalytic surface of the enzyme. To the extent that this procedure neglects a possible increment of interaction of the cyclohexane ring moiety with the protein, over that shown by the two-carbon chain in choline derivatives, it lacks quantitative significance.

A point of further interest lies in the attempt to match the most effective distance between functional groups in the substrate or inhibitor molecules with the distance between the actual regions in the two enzymatic sites responsible for adsorption of these functional groups. For the aminoalcohols I and III this separation distance on the enzyme

can be compared with definite atomic N → O distances in the inhibitors. However, with the substrates II and IV, the N → O separation distance may not effectively mirror the distance between sharply defined adsorption regions on the enzyme, since the possibility exists that the esteratic site[4] binds the –OCO– entity as a single locus of over-all high electron density,[3a] rather than concentrating its binding capacity on a single atom of the group. In the former event, the average distance between catalytic adsorption regions on the enzyme most probably would be reflected in the distance between the quaternary N and that geometrical portion of the –OCO– function characterized as the center of highest electron density.

Experimental[8]

Synthesis of *d,l-cis*-2-Dimethylaminocyclohexanol Methiodide (I) and its Acetate (II).—A supply of 2-chlorocyclohexanone was prepared essentially according to the procedure of Newman and co-workers[9]; m.p. 21–22° (lit. value[9] 23°). To a solution of 160 g. of 25% aqueous dimethylamine in a pressure bottle was then added 65 g. of the 2-chlorocyclohexanone, after which the sealed bottle was heated in an oil-bath at 130–140° for 16 hours.[10] The cooled mixture was then acidified and thoroughly extracted with ether to remove residual non-basic material, followed by the addition of sufficient sodium hydroxide to liberate the free bases present. After ether extraction of the bases, a 64% yield of 2-dimethylaminocyclohexanone was obtained; b.p. 54–56° (2 mm.), m.p. of picrate 116–118° (lit. value[10] for picrate 114–115°).

Because of its instability, a batch of freshly prepared aminoketone was taken without distillation and reduced directly to the *cis*-aminoalcohol, using the Pt catalyst in ethanol as recommended by Mousseron and Jullien.[10b]) The product obtained in 71% yield had the following constants: b.p. 84° (12 mm.), m.p. 35–37°, with a second modification melting 44–45°; lit. values[10b]; b.p. 98° (15 mm.), m.p. 43–44°.

A mixture of 2.2 g. of the *d,l-cis*-2-dimethylaminocyclohexanol above with 7.1 g. of methyl iodide and 100 ml. of anhydrous ether was shaken mechanically for 16 hours. The precipitated methiodide was recrystallized twice from ether–methanol to yield 1.8 g. of crystals of I, m.p. 244–245°.

Anal. Calcd. for $C_9H_{20}NOI$: C, 37.90; H, 7.07; N, 4.91; I, 44.50. Found: C, 37.77; H, 7.02; N, 4.97; I, 44.21.

The above sample of compound I was identical with that produced by the exhaustive methylation of an authentic sample of *d,l-cis*-2-aminocyclohexanol prepared by McCasland and co-workers[11] *via* the oxazoline route from the *trans*-aminoalcohol. A mixed m.p. of the two products was undepressed.

A solution of 5.3 g. of the *d,l-cis*-2-dimethylaminocyclohexanol in 100 ml. of anhydrous ether was treated with ketene for a period of three hours. The resulting cloudy mixture was filtered, concentrated, and the product distilled; yield of *d,l-cis*-2-dimethylaminocyclohexyl acetate, 4.4 g., b.p. 98–100° (13–15 mm.), $n^{25}D$ 1.4568; darkens on standing unless stored *in vacuo* in the cold. To a solution of 2 g. of this acetate in 100 ml. of anhydrous ether was then added 7 g. of methyl iodide, and the mixture shaken overnight. The precipitated product II was recrystallized repeatedly from ether–methanol as white crystals, m.p. 182–183°.

Anal. Calcd. for $C_{11}H_{22}O_2NI$: C, 40.37; H, 6.78; N, 4.28. Found: C, 40.29; H, 6.57; N, 4.46.

Synthesis of *d,l-trans*-2-Dimethylaminocyclohexanol Methiodide (III) and its Acetate IV.—A 5-g. sample of cyclohexene oxide prepared according to the procedure of Winstein[12] was sealed in a glass bomb with a fourfold excess of Eastman Kodak Co. anhydrous dimethylamine. The bomb was heated at 100° for four hours to effect *trans* ring opening, then chilled, opened, and the excess dimethylamine distilled off. The resulting *trans*-dimethylaminoalcohol was distilled ($n^{20}D$ 1.4703) and split into two parts for conversion to III and IV, respectively.

One portion of the *trans*-dimethylaminoalcohol was dissolved in 50 ml. of anhydrous ether and treated with ketene at room temperature, in stirred solution, for an hour. Approximately half the ether was then distilled to remove excess ketene, followed by the addition of 50 ml. of fresh ether and 20 g. of methyl iodide. The solution was shaken, and then allowed to stand for complete precipitation of the methiodide IV. The white, slightly hygroscopic solid was filtered and recrystallized repeatedly from methanol–ether mixture as shiny white crystals, m.p. 210–212°.

Anal. Calcd. for $C_{11}H_{22}O_2NI \cdot H_2O$: C, 38.27; H, 7.01. Found: C, 38.18; H, 7.01.

About 1 g. of the remaining dimethylaminoalcohol was dissolved in 10 ml. of anhydrous ether, a fivefold excess of methyl iodide added, and the mixture shaken. After standing overnight the methiodide III was filtered, and recrystallized four times from methanol–ether mixture; m.p. 212–213°; mixed m.p. with IV, 207–214°.

Anal. Calcd. for $C_9H_{20}NOI$: C, 37.90; H, 7.07; N, 4.91. Found: C, 37.84; H, 7.03; N, 4.97.

Enzymatic Rate Determinations.—The equipment and techniques employed in the enzymatic rate determinations were essentially those described in the previous study.[3] The standard phosphate buffer of *p*H 7.4 was used throughout in runs testing I and III as inhibitors as well as II and IV as substrates. Reaction temperatures were regulated at 25.12 ± 0.02°. Two stock enzyme solutions, from which high dilutions (1:12,500) were made for use in the actual rate runs, assayed at 32 and 11 g. of acetylcholine chloride hydrolyzed/hr./mg. of protein/ml., respectively.

Inhibitor and substrate solutions were freshly prepared before use. Recrystallized acetylcholine chloride was used as substrate at its optimum concentration (3.3×10^{-3} *M*) in testing compounds I and III as inhibitors. Both I and III functioned as weak but reversible and competitive inhibitors over the concentration range $1\text{–}10 \times 10^{-4}$ *M* as evidenced by the linear Wilson plot[13] of v/v_I *vs.* [I]. The slopes of these plots in conjunction with a K_m value[4] of 2.6×10^{-4} furnished the observed dissociation constant (K_I) values.

In runs with compounds II and IV as substrates, stock solutions of substrate were prepared just before use, and kept chilled during the brief storage periods between the removal of aliquots for rate runs. Rate plots were corrected for the small amounts of the uncatalyzed spontaneous substrate hydrolysis occurring. These plots were linear for at least the first 10% of reaction, before significant amounts of inhibitory alcohol product began to accumulate, and consequently this region was used to obtain velocity values. These values were in general reproducible to within ±5%.

Acknowledgment.—We are indebted to Dr. H. S. Polin for his aid in the procurement of electric eel tissue and its initial extracts.

BETHESDA, MARYLAND

(8) Melting points (Fisher–Johns apparatus) are uncorrected. Analyses by courtesy of: (a) Dr. W. C. Alford, Microanalytical Laboratory, National Institutes of Health, Bethesda, Md., and (b) Micro-Tech Laboratories.

(9) M. S. Newman, M. D. Farbman and H. Hipsher, *Org. Syntheses*, **25**, 22 (1945), John Wiley and Sons, New York, N. Y.

(10) Adapted from the procedures of: (a) M. Protiva and M. Borovicka, *Chem. Listy*, **44**, 91 (1950); *C. A.*, **45**, 7967 (1951); and (b) M. Mousseron and J. Jullien, *Compt. rend.*, **231**, 479 (1950).

(11) See ref. 5. We are indebted to Dr. McCasland for a generous sample of the hydrochloride of *d,l-cis*-2-aminocyclohexanol.

(12) S. Winstein, THIS JOURNAL, **64**, 2792 (1942).

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