acetate. The organic extract was dried over Na_2SO_4 and evaporated to near dryness. The residual acetic acid was removed *in vacuo* over NaOH. The residue was recrystallized from ethyl acetate–benzene and sublimed at 240° and 0.01 mm. The product melted at 300–305° (evacuated capillary), $[\alpha]^{21}D$ +310° (*c* = 0.5, diox.), $\lambda_{max}^{CH_3OH}$ 295 mμ (3,600). Repeated recrystallization from methanol gave analytically pure material containing one mole of methanol of crystallization.

Anal. Calcd. for $C_{18}H_{22}O_3 \cdot CH_3OH$: C, 71.67; H, 8.23. Found: C, 71.55; H, 8.23.

1,4-Diacetoxy-1,3,5(10)-estratriene-17-one (XVI).—The crude hydroquinone XV from reduction of 60 mg. of quinone XIV was dissolved in 1.0 ml. of pyridine and treated with 0.20 ml. of acetic anhydride. After standing on the steam-bath 20 min. the solution was treated with 5 drops of water and allowed to stand at room temperature for 10 min. The reaction mixture was then worked up in the usual way. Sublimation and repeated crystallization from cyclohexane-benzene gave a product that melted at 163.0–163.6°, $[\alpha]^{22}D$ +273° (*c* 1.0, $CHCl_3$), $\lambda_{max}^{CH_3OH}$ 265 mμ (340).

Anal. Calcd. for $C_{22}H_{26}O_5$: C, 71.33; H, 7.07. Found: C, 71.36; H, 7.06.

6-Dehydroestrone (XXI).—A solution of quinol II (50 mg.) in 10 ml. of methylene chloride was treated with 0.20 ml. of PBr_3 and allowed to stand at room temperature for 16 hours. The solution was shaken with 10 ml. of water for 10 minutes and the organic phase was extracted with 10 ml. of 10% $KHCO_3$ solution. The combined aqueous extracts were acidified with concd. HCl and heated on the steam-bath for one hour. Extraction of the cooled solution with chloroform produced a quantity of crystalline solid contaminated with a purple pigment. Recrystallization followed by sublimation in vacuum and recrystallization from methanol gave 5 mg. of colorless crystals, m.p. 259–261.5° (evacuated capillary). The identity of the product was verified by comparison of infrared and ultraviolet spectra with those of authentic 6-dehydroestrone.[16] A trace of impurity having $\lambda_{max}^{CH_3OH}$ 291 mμ appeared to be present. The melting point was not depressed by admixture of 6-dehydroestrone.

SHREWSBURY, MASS.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, GEORGETOWN UNIVERSITY, THE NATIONAL INSTITUTE FOR ARTHRITIS AND METABOLIC DISEASES, AND THE NAVAL MEDICAL RESEARCH INSTITUTE]

The Acetylcholinesterase Surface. IX. Dependence of Competitive Inhibition by Diaminocyclohexane Derivatives on Substrate Level[1a]

BY D. S. MASTERSON,[1b] S. L. FRIESS AND B. WITKOP

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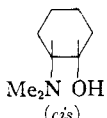
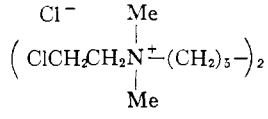
Certain features of the inhibition of the system acetylcholinesterase–acetylcholine by the *cis* and *trans* isomers of 2-dimethylaminocyclohexyltrimethylammonium iodide (III and IV) have been shown to depend markedly on the relative substrate levels employed. At *p*H 7.4 and 25° with a protein concentration of the order of 2×10^{-4} mg. per ml., inhibition by each of these isomers is apparently competitive at substrate levels of 1.5×10^{-3} *M* and lower, but is found to deviate from the competitive relation at higher levels. A possible model to account for this behavior as well as the feature of inhibition of the system by excess substrate has been discussed. The amide V in the *trans* series of diamine derivatives has been found to be inert to the catalytic action of the enzyme in hydrolysis reaction.

Previous kinetic studies of the system acetylcholinesterase–acetylcholine (AChE–AC) have pointed to the competitive nature of the inhibition process characteristic of such reversible inhibitors as eserine[2] and certain substituted ethylenediamines.[3] However, for the tertiary and quaternary compounds I and II reversible inhibition in the

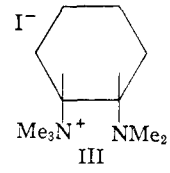


I

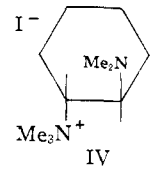


II

substrate concentration range up to 3×10^{-3} *M* was found to be clearly non-competitive,[4] with the intrinsic inhibitory power of either compound at *p*H 7.4 independent of the initial acetylcholine concentration employed. These observations and their implications with respect to surface equilibria, coupled with the marked ability of the surface to distinguish between stereochemical configurations of diamines[5] and aminoalcohols and acetates,[6] made it a matter of considerable interest to investigate the inhibitory properties of the *cis*- and *trans*-diamine derivatives III and IV, particularly



III



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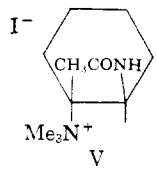


V

in regard to the competitive or non-competitive character of their inhibition. These compounds possess both the quaternary ammonium function and the center of high electron density, with appropriate separation distances between the two, that might be expected[3a] to lead to significant inhibitory activity in the AChE–AC system. This study has been carried out at several substrate concentrations on the low branch of the $[substrate]_0$ *vs.* activity profile, and has been supplemented briefly by enzymatic hydrolysis experiments with the closely related amide V.

Results

Inhibition data from kinetic experiments involving III and IV were fitted to linear v/v_I *vs.* [I] plots

(1) (a) The opinions in this paper are those of the authors and do not necessarily reflect the views of the Navy Department. (b) Taken in part from the M.S. thesis of D. S. Masterson, Georgetown University, 1957.

(2) K. B. Augustinsson and D. Nachmansohn, *J. Biol. Chem.*, **179**, 543 (1949).

(3) (a) S. L. Friess and W. J. McCarville, THIS JOURNAL, **76**, 1363 (1954); (b) S. L. Friess and H. D. Baldridge, *ibid.*, **78**, 199, 966 (1956).

(4) S. L. Friess, *ibid.*, **79**, 3269 (1957).

(5) S. L. Friess, E. R. Whitcomb, Bart T. Hogan and P. A. French, *Arch. Biochem. and Biophys.*, **74**, 451 (1958).

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

by the method of least squares, for the calculation *via* observed slopes of K_I values for dissociation of the enzyme–inhibitor complexes. Equations 1 and 2 for competitive and non-competitive inhibition,[7] respectively, are linear in v/v_I *vs.* [I], but (1) furnishes a slope dependent on $[S]_0$ whereas the slope in (2) does not contain substrate dependence.

$$v/v_I = 1 + \frac{K_m[I]}{K_I(K_m + [S]_0)} \quad (1)$$

$$v/v_I = 1 + ([I]/K_I) \quad (2)$$

where v = uninhibited velocity and v_I = inhibited velocity. Each observed slope was used to calculate the corresponding K_I values at the given substrate level, according to 1 and 2. The value of K_m required in (1) was experimentally determined to be $(2.23 \pm 0.02) \times 10^{-4}$ under the present conditions, from four series of kinetic runs over the AC range $2 - 10 \times 10^{-4}$ M and the application of the familiar Lineweaver–Burk equation[8] for a system obeying Michaelis–Menten kinetics.

The values of K_I calculated for competitive and non-competitive inhibition by the diamine derivatives III and IV are summarized in Table I.

TABLE I

INHIBITION OF AChE-AC BY COMPOUNDS III AND IV AT 25.00 ± 0.05° AND *p*H 7.40

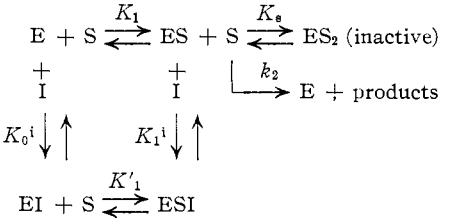
Series	Compound	[Substrate]$_0$ $M \times 10^3$	Competitive $K_I \times 10^5$	Non-competitive $K_I \times 10^4$
1	III	1.00	3.42 ± 0.02	1.88 ± 0.02
2	III	1.50	3.60 ± .07	2.79 ± .05
3	III	1.50	3.60 ± .05	2.79 ± .04
4	III	3.00	2.44 ± .03	3.53 ± .04
5	III	3.00	2.35 ± .03	3.40 ± .05
6[a]	III	3.36	2.06 ± .08	
7	IV	1.00	3.69 ± .05	2.03 ± .03
8	IV	1.50	3.73 ± .04	2.89 ± .03
9	IV	1.50	3.90 ± .02	3.02 ± .02
10	IV	3.00	2.80 ± .02	4.06 ± .03
11	IV	3.00	3.09 ± .02	4.48 ± .03

[a] This series was carried out at 25.14 ± 0.03° in a reaction volume of 6.40 ml. at *p*H 7.4.

Several interesting points emerge from the data of Table I. First, on the basis of either the competitive or non-competitive strength index at each substrate level, the *cis* compound III is a slightly more potent inhibitor (with smaller K_I value) than the *trans* derivative IV. This is in accord with previous observation[6,9] on the cyclic 1,2-aminoalcohols and acetates, in which the small separation distance between the two polar functions of *cis* derivatives appears to offer a better fit to the bifunctional[10] catalytic unit on the enzymatic surface than that in the corresponding *trans* compounds with their larger inter-group spacings, and is also in line with the generally accepted picture of binding of inhibitors or substrates by two-pointed interaction with the AChE surface.

More striking than this, however, is the observation that in the substrate concentration range up to 1.5×10^{-3} M the inhibition by each diamine derivative appears to be competitive in nature, since the operational K_I (comp.) values calculated by equation 1 for each compound are sensibly constant with changing [substrate] levels, while the K_I (non-comp.) values derived from (2) steadily rise with varying substrate. However, when $[substrate]_0$ is raised to the 3×10^{-3} M level this apparent competitive behavior breaks down, as indicated by a dropping of K_I (comp.) values from their previously constant levels and the continued rise of K_I (non-comp.) values. However, although neither law appears to be obeyed uniquely near this substrate level, the v/v_I *vs.* [I] plots are still quite linear with intercepts of unity, as required by both equations 1 and 2.

This behavior pattern covering the low concentration branch of the bell-shaped activity *vs.* [S] profile[11] for the AChE–AC system can be rationalized in terms of at least one general scheme (all K's are association constants).



This model employs the current assumption[10] that inhibition by excess substrate is mediated by the formation of the inactive supercomplex ES_2.

For this scheme at quasi-equilibrium it can readily be shown that

$$\frac{1}{v_I} = \left(\frac{\phi_0}{K_1[S]} + \phi_1 + K_s[S]\right)\frac{1}{k_2[E_0]} \quad (3)$$

where

$$\phi_0 = K_0^i[I] + 1, \text{ and} \quad (4)$$

$$\phi_1 = K_1^i[I] + 1 \quad (5)$$

and where the uninhibited rate v is given by (3) with $\phi_0 = \phi_1 = 1$. Under these conditions the operational ratio $v/v_I = r$ is given by

$$r = 1 + \frac{K_0^i(1 + pK_1[S])}{1 + K_1[S] + \bar{K}[S]^2} \times [I] \quad (6)$$

where $p = K_1^i/K_0^i$, and $\bar{K} = K_1K_s$.

Therefore in a linear plot of r *vs.* [I] at fixed initial [S] the slope m is given by

$$\text{slope} = m = \frac{K_0^i(1 + pK_1[S])}{1 + K_1[S] + \bar{K}[S]^2} \quad (7)$$

and for the operational equation 1 in classical competitive inhibition with $K_m = (1/K_1)$ for quasi-equilibrium

$$K_I(\text{comp.}) = \frac{K_m}{m(K_m + [S])} = \frac{1}{m} \times \frac{1}{1 + K_1[S]} \quad (8)$$

while for the non-competitive situation 2

$$K_I(\text{non-comp.}) = (1/m) \quad (9)$$

Two interesting, limiting kinetic cases can be drawn from equations 7, 8 and 9.

Case (i), $K_1[S] >> \bar{K}[S]^2$ (substrate inhibition negligible): (a) If inhibitor is strictly competitive,

(7) See P. W. Wilson in "Respiratory Enzymes," H. A. Lardy, ed., Burgess, Minneapolis, Minn., 1949, p. 24.

(8) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

(9) S. L. Friess and H. D. Baldridge, *ibid.*, **78**, 2482 (1956).

(10) D. Nachmansohn and I. B. Wilson, *Adv. in Enzymol.*, **12**, 259 (1951).

(11) K. B. Augustinsson, *Arch. Biochem. and Biophys.*, **23**, 111 (1949).

$K_1' = 0 = p$. Then K_I(non-comp.) from (9) would increase almost linearly with [S] at low [S]; K_I(comp.) would be constant with changing [S].

(b) If inhibitor is strictly non-competitive, $p = 1$. Then K_I(non-comp.) would be constant with changing [S] and K_I(comp.) would decrease as [S] increases. This situation is contrary to the observed data on both counts for both inhibitors, and can be discarded.

(c) If inhibitor is neither strictly competitive nor strictly non-competitive in its action, $p \neq 0, \neq 1$. Then K_I(non-comp.) increases with [S] if $p < 1$, and decreases with [S] if $p > 1$, and K_I(comp.) always decreases with increasing [S].

It would appear therefore that in case i of the general model, situation (a) could well describe the observed kinetic behavior at low [S], while the situation (c) could adequately account for the departure from competitive behavior at high [S] levels. To avoid an abrupt change in p required by a transition from (a) to (c) as [S] varies, however, the situation (c) could hold throughout the range if the parameters were such as to lead to relatively small decreases in K_I(comp.) with changing [S] at the low [S] levels, and larger decreases at the higher levels.

Case (ii), $\bar{K}[S]^2$ appreciable, so that inhibition by substrate cannot be neglected: This would apply to the present experimental conditions where [S] $\rightarrow 3 \times 10^{-3}$ M and higher. (a) When $p = 0$, in summary: as [S] increases, both K_I(comp.) and K_I(non-comp.) increase. This situation is ruled out for III and IV by the K_I(comp.) data.

(b) When $p = 1$: as [S] increases, K_I(non-comp.) increases, and, K_I(comp.) drops with increasing [S] over the 1–3 $\times 10^{-3}$ M range for $K_1 \simeq 10^4$ and $K_1 \simeq K_s$ or $K_1 >> K_s$; for $K_s >> K_1$, K_I(comp.) increases with increasing [S].

(c) When $p \neq 0, \neq 1$: as [S] increases, K_I(non-comp.) decreases with small [S] for $p > 1$ (and $K_1 \simeq K_s$) and then increases; it increases with all [S] for $p < 1$. K_I(comp.) decreases with small [S] for $p < 1$ (and $K_1 \simeq K_s$) and then increases well before the 3×10^{-3} M substrate level is reached.

It is accordingly possible that case ii, situation (b), of the model could also describe adequately the observed inhibitory behavior of III and IV, with suitable values of the parameters K_1, K_s and p.

This general model is formulated with the assumption that ES_2 is an important species in solution, particularly at high [S] levels where inhibition by substrate is significant. It is of some interest to note that this proposition is in agreement with the proposal of Hardegg, *et al.*,[12] that multiple adsorption of the substrate AC best accounts for the kinetic behavior of this enzyme system.

As an additional point to be made from the data of Table I, it is of interest to note that both the *cis* and *trans* derivatives III and IV are of the order of 3×10^2 weaker than the corresponding open-chain analog[13] in AChE inhibition, whereas the 1,2-cyclic analogs of choline[6,9] are all slightly better as inhibitors than the open-chain cation. To a considerable extent, this may be a reflection of steric difficulty in accommodating two polymethylated nitrogen functions in the 1,2-relation in the cyclohexane ring, with a sufficient degree of flexibility for optimal interaction with the bifunctional site as previously mapped by the group spacings in the choline analogs. Examination of models indicates that the crowding in the cyclic aminoalcohols is by no means as serious as that evidenced in the cyclic derivatives III and IV.

In view of the effectiveness of the *trans* compound IV as an AChE inhibitor, several hydrolysis experiments with the stereochemically similar amide V were next attempted in a further probing of the catalytic power of the AChE surface. With a 2×10^{-4} mg. per ml. enzyme concentration at pH 7.40 and concentrations of V ranging from 0.8 to 3.0×10^{-3} M, no detectable hydrolysis of amide was observed. The same lack of catalytic activity was found at fivefold higher enzyme concentration and compound V levels up to 5×10^{-3} M. It would appear therefore that, although the steric disposition of groups in V may be suitable for binding at the surface catalytic unit (as inferred from the activity of IV), AChE is relatively incapable of catalyzing the amide hydrolysis step. It remains to be seen whether or not the amide might function as an inhibitor of AC hydrolysis, taking advantage of its potentialities in the binding process alone.

Experimental

Kinetic experiments were conducted with an enzyme preparation, derived from electric eel tissue essentially according to the procedures of Rothenberg and Nachmansohn,[14] possessing a specific activity of 3.96×10^3 μmoles AC hydrolyzed/hr./mg. dry weight protein at a substrate level of 1.0×10^{-3} M and pH 7.4. Doubly recrystallized acetylcholine chloride and triply distilled water were used throughout. Enzymatic activities in inhibited and non-inhibited determinations were measured by the constant-pH titration technique,[3a] with a water-jacketed cell designed to use 60-ml. reaction volumes and fitted with gas inlets to permit expulsion of dissolved CO_2 by N_2 gas prior to a run, and stirring under a blanketing N_2 atmosphere in the course of a given determination. The reaction temperature was set at 25.00 ± 0.05°, with the pH fixed at 7.40 ± 0.02 in a buffer 1.0×10^{-3} M in phosphate, 0.01 M in magnesium chloride and 0.1 M in sodium chloride, and inhibitory rate series in duplicate were run at three initial substrate concentrations over the range 1.0–3.0 $\times 10^{-3}$ M. Protein levels were of the order of 2.1×10^{-4} mg. (dry weight) per ml. in final reaction mixtures.

Compounds III, IV and V were obtained from J. R. Geigy Co. as white, crystalline solids with the following m.p. values: III, 199–201°; IV, 229–230°; V, 212–214°. Water solutions of these materials were made freshly before use and stored at 4° in the intervals between removal of aliquots. Duplicate determinations of initial rates (up to 10–15% of total reaction) in each inhibition series were made at each substrate level employed, except for the 1.0×10^{-3} M AC runs, over a tenfold range of inhibitor concentration. A precision of the order of ±5% was observed in K_I values calculated from duplicate series.

Acknowledgment.—We are indebted to Dr. S. Häfliger and the J. R. Geigy Co., Basle, for their generous aid in furnishing the diamine derivatives used in this study, and to Dr. J. Z. Hearon for much helpful discussion.

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(12) See, for example, W. Hardegg, D. Bechinger and R. Dohrmann, *Pflügers Arch. ges. Physiol.*, **263**, 33 (1956).

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

(14) M. A. Rothenberg and D. Nachmansohn, *J. Biol. Chem.*, **168**, 223 (1947).