all of the 17 disulfide bonds of HSA are reduced by 0.1 M β-mercaptoethylamine-HCl, whereas only 1 disulfide bond is split in the absence of the detergent.[8] Furthermore, the facilitation of the reduction of disulfide bonds in HSA in 0.1 M reducing agent increases as the maximum initial rotation of $-70°$ has been attained at 0.03M. The relative viscosity increases further and binding data indicate that the number of detergent molecules combined has not reached its maximum even at 0.1 M concentration.

Structural Considerations.—What structural change of the protein is reflected in the constant optical rotation of $-70°$? Since optical rotation in polypeptides is regarded as a function of the degree of secondary organization of the chain, it is reasonable to assume that this value expresses the maximal transformation of the secondary structure caused by the combination with decyl sulfate. The continuing increase in viscosity and in the availability of disulfide bonds for reduction reflects further loosening of the molecule on the level of its tertiary structure. Electrostatic repulsion between neighboring polypeptide segments would be expected to separate these segments and thus to contribute to both viscosity and accessibility of bonds situated in the interior of the molecule. The latter function is more indicative of such a process than is viscosity because of the difficulty of ascertaining how much of the viscosity rise is due just to the increase in molecular volume and asymmetry caused by the attachment of a large number of detergent molecules. Since detergents do not compete for the H-bonds of the secondary structure, the rotation of $-70°$ probably represents the extent to which the secondary structure of HSA can be distorted by charge effects. In this connection it is significant that in preliminary studies on the rotatory dispersion of HSA we found that λ_0, the extrapolated intercept of the curve with the wave length axis in linear Drude plots, was 2660 Å. for both native HSA and the same protein in 8% sodium "lorol" sulfate ($-[\alpha]_D$ 72.0), whereas 10 M urea shifted λ_0 to 2340 Å. If the value of λ_0 is indicative of the degree of secondary structure,[16] this finding would indicate that, unlike urea, the detergent did not interfere extensively with the helical organization.

In this connection it is significant that the optical rotation of HSA in 6 M urea and increasing concentrations of SDeS reaches the plateau of $-81°$ at the same SDeS concentration at which $-70°$ was reached in the absence of urea (Fig. 5). As mentioned before, the binding of detergent measured in the absence of urea continues beyond this concentration; the extent of disruption caused by 6M urea is probably limited therefore not by the number of detergent molecules bound, but by the structural state which is brought about by the amount of detergent bound at the molarity of 0.034.

(16) K. Linderstrøm-Lang and J. A. Schellman, *Biochim. Biophys. Acta*, **15**, 156 (1954).

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

The Acetylcholinesterase Surface. VIII. Further Observations on Bifunctional Inhibition of the Enzyme[1]

BY S. L. FRIESS

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It has been observed that the bifunctional aminoalcohol *cis*-2-dimethylaminocyclohexanol (I) at *p*H 7.4 acts as a mild inhibitor of the acetylcholinesterase system, with an unexpected strength about one order of magnitude greater than that of its methiodide derivative or the natural inhibitor choline. This inhibition appears to be of the reversible, non-competitive type at substrate concentration levels below 4×10^{-3} M, leading to the possibility that the Michaelis–Menten constant at these levels is a true equilibrium constant. This possibility is strengthened by the observation of Jenden, *et al.*, that a bis-quaternary salt III also displays non-competitive inhibition of AChE at the same substrate levels. The order of strength of I has been interpreted in terms of a zwitterion as the effective inhibitory species. Esterase inhibition by I is also found with the frog *rectus abdominis* preparation, with a discrepancy factor between *rectus* and *in vitro* estimations of strength approximating that found for the more potent inhibitor eserine. Further study of the inhibition produced by the bifunctional diamine N-(β-dimethylamino)-ethylpiperidine (II) in the 10^{-4}–10^{-7} M concentration range has revealed a decrease in effectiveness on incubation in glass which appears to depend largely on reaction volume and incubation time. This has been interpreted in terms of sorption on protein and on glass at these low levels, and is in accord with previous observations that appreciable amounts of II readily can be absorbed from solution by such proteins as gelatin and serum components. A further test of the steric requirements of the enzymatic surface between those loci capable of adsorbing the polar functions of inhibitors or substrates has been made with the compound 1,4-diazabicyclo[2,2,2]octane. Kinetic tests indicate that the size of the cage structure of this molecule exceeds the sterically acceptable limits of the surface.

Introduction

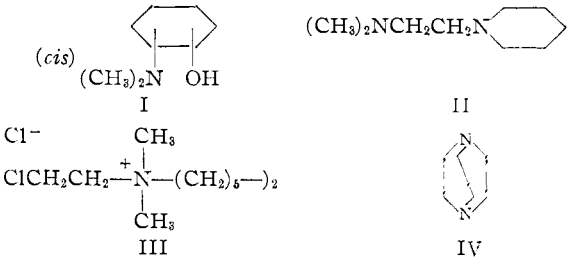
In continuation of previous studies designed to probe into chemical and stereochemical aspects of the catalytic regions on the surface of acetylcholinesterase (AChE), with aminoalcohols[2] and diamines[3] being used as bifunctional inhibitors to reveal ele-

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

(2) (a) H. D. Baldridge, W. J. McCarville and S. L. Friess, THIS JOURNAL, **77**, 739 (1955); (b) S. L. Friess and H. D. Baldridge, *ibid.*, **78**, 2482 (1956).

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

ments of surface detail, the present work deals with certain interesting features of the inhibition produced by the tertiary aminoalcohol I, the substituted ethylenediamines II and IV, and the bis-quaternary salt III. Work with compound I was



stimulated by the possibility that its tertiary amino structure would lead to greater membrane permeability than that possible for quaternary structures, in connection with biophysical studies of conduction in nerve,[4] while that with II was prompted by enzymatic indications[5] that its inhibitory effect at very low concentration levels depended on the time sequence of operations involved in the activity measurements. Experiments with compound III were undertaken to expand the picture of non-competitive inhibition developed with I, while tests of compound IV were made to continue the process of probing the nature of the steric requirements of the intersite surface of AChE.

Results

Inhibition with the Aminoalcohol I.—In the previous studies[2] of the AChE surface using stereospecific inhibitors as test agents it was observed that *cis*-derivatives in the 2-aminocyclohexanol series are in general more potent than the *trans*-isomers. A key intermediate in the preparation of these *cis*-compounds was the aminoalcohol I, and it was of considerable interest to determine whether at *p*H 7.4 compound I possessed any residual inhibitory activity so characteristic of its methiodide.[2] The results of these tests were surprising in several respects.

First, the compound was more active as an inhibitor than might have been expected from its tertiary aminoalcohol structure, with effective inhibition in the concentration range 10^{-4} to 10^{-5} M indicating a strength better by at least one order of magnitude than that displayed[2a] by its methiodide. But of greater interest was the observation that although its inhibition obeys the linear, competitive plot[6] (eq. 1) of v/v_I *vs.* [Inhibitor] at a

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

v_I = inhibited velocity
K_m = Michaelis–Menten constant
K_I = enzyme-inhibitor dissociation constant

fixed initial substrate concentration, the slope of this plot appears to be independent of substrate in its concentration range below 4×10^{-3} M. This kind of behavior has been taken[6] as one of the means of distinguishing between competitive and non-competitive inhibition of the reversible type, with the latter obeying an inhibition equation in a form independent of substrate concentration

$$v/v_I = 1 + \frac{[I]}{K_I} \qquad (2)$$

The results of v/v_I *vs.* [I] plots using eq. 1 and 2 for inhibition of the AChE–acetylcholine system at two levels of substrate concentration are summarized in Table I below. It is seen that over a twofold substrate range, the competitive K_I values vary by more than a factor of two, but the non-competitive K_I value of $6.0 \pm 0.4 \times 10^{-5}$ is roughly constant as predicted by eq. 2.

TABLE I

COMPETITIVE *vs.* NON-COMPETITIVE INHIBITION PLOTS
*p*H 7.4, 25.14°

Inhibitor	[Substrate]$_0$, $M \times 10^3$	Slope $\times 10^{-4}$ of v/v_I *vs.* [I] Plot	$K_I \times 10^6$ by eq. 1	$K_I \times 10^5$ by eq. 2
I	1.68	1.588 ± 0.027	8.4 ± 0.2	6.3
I	3.41	1.787 ± 0.027	4.0 ± 0.1	5.6
III	1.70	36.34	0.37	0.28
III	3.41	41.88	0.17	0.24

This result is of more than cursory interest in the field of AChE kinetics, in view of the generalization developed by Morales[7] that non-competitive inhibition for an enzyme system requires that the Michaelis–Menten constant K_m be a true equilibrium constant under the given conditions, and hence be a direct reflection of the strength of binding between enzyme and substrate.

Further support is added to this indication of the existence of non-competitive inhibitors by the observations of Jenden and Baldridge[8] on the anticholinesterase activity found in a series of bis-quaternary salts. In particular, the decamethylene derivative III was shown to be non-competitive in its inhibitory action, with the degree of inhibition over a given inhibitor range independent of substrate concentration, on the low side of the substrate concentration *vs.* activity profile. These results have been confirmed in the present study, with the data for compound III inhibition summarized in Table I. It is seen that the non-competitive K_I values again are essentially constant on changing initial substrate concentration, in accordance with eq. 2, whereas the competitive K_I values change with substrate level. This finding of non-competitive AChE-inhibitory characteristics in compounds as widely different in structure as I and III would appear to make the general theorem that K_m directly measures AChE–substrate binding, under these conditions, most attractive.

A further interesting comparison was afforded by the experimental evaluation[9] of the AChE-inhibitor dissociation constant for I on a frog *rectus*

(4) J. W. Moore, S. L. Friess and R. H. McCoy, *Federation Proc.*, **13**, Part I, 101 (1954).

(5) Private communications from Dr. D. J. Jenden of these laboratories, and Dr. I. B. Wilson.

(6) P. W. Wilson, "Respiratory Enzymes," H. A. Lardy, ed., Burgess Publishing Co., Minneapolis, Minn., 1949, p. 23.

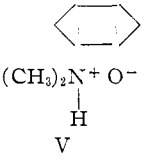
(7) M. F. Morales, THIS JOURNAL, **77**, 4169 (1955).

(8) D. J. Jenden and H. D. Baldridge, "Dose–Response Relationships in a Series of Bis-quaternary Anticholinesterases," paper presented before the British Pharmacological Society, Edinburgh, July, 1956.

(9) Kindly performed by Dr. D. J. Jenden of these laboratories using the technique described in the paper: D. J. Jenden, *J. Comp. Cell Physiol.*, in press (1956).

abdominis preparation, in which the effect of the inhibitor on potentiation of the action of acetylcholine through its blockade of tissue-localized enzyme is employed. This determination is somewhat clouded by neuromuscular block which sets in at the higher inhibitor concentrations, but readings of contracture at the lower levels led to a K_I value of about 2.1×10^{-3} at 37°. The ratio of K_I (frog rectus) to K_I (non-competitive) is therefore about 35, which approximates both in magnitude and in direction the discrepancy factor of about 20 found[9] for the competitive inhibitor eserine on comparing the kinetic and frog rectus values. It would appear then that the order of magnitude difference in K_I values afforded by these two techniques does not rest primarily on whether the AChE is inhibited competitively or non-competitively (eserine *vs.* I) or even on whether the inhibitor is comparatively strong (eserine) or moderate (I) in its action. A portion of this difference undoubtedly depends on the temperature difference between the two determinations.

On the point of the absolute inhibitory strength of I, its unexpected power against both isolated enzyme and tissue-localized enzyme has led to scrutiny of its tertiary structure for a clue as to its source of activity. One fruitful suggestion[10] draws upon the special geometry of I in respect to the relatively small (2.5 to 2.9 Å.) separation distance[11] between N and O atoms of the polar functions of I, leading to the possibility of prototropy and a stabilized zwitterion V as the effective inhibitory



V

species. Such a species would fulfill the previous postulated[3a] requirements (a polymethylated, charged N atom and a properly situated locus of high electron density) for strong binding at the bifunctional[12] catalytic unit of the enzymatic surface and account for the observed order of activity of the inhibitor.

Inhibition with the Diamine II.—In continued study of the enzymatic and physical properties of the diamine II, a mild detergency action in dilute solution was noted, leading to some speculation as to the concentration stability with time of the very dilute (10^{-6} to 10^{-7} M) solutions used in the inhibition work. Now, inhibition measurements have pointed out some peculiar features of the retention of power of dilute aqueous solutions of II, including an effectiveness varying with the volume of solution in contact with the Pyrex glass of the titration apparatus and with the contact time.

Initial enzymatic indications pointed[5] to a disappearance of effective inhibitory amounts of II in the 5×10^{-7} M range and upward on incubation with AChE in dilute buffer for periods of 5 minutes and more, when the reaction volume was of the order of 5 ml. or greater. Since these same concentrations of inhibitor were highly effective[3] in a smaller reaction volume of 3.20 ml., with constancy of action over a relatively long (10–15 minute) time interval, it was of considerable interest to investigate the influence of solution volume and incubation time, with or without enzyme, on the subsequent degree of inhibition of an initially fixed concentration of II.

The water-jacketed, cylindrical titration cell (27 mm. in diameter, 25 mm. deep) fitted with a pair of two-inch Beckman *p*H electrodes, capillary stirrer and microburet could accommodate reaction volumes up to 10 ml., so that for the present comparative study three sets of inhibition determinations at 3.20, 6.40 and 9.60 ml. (1×, 2×, and 3×) volumes were employed to furnish the volume variants. For certain comparisons at fixed enzyme concentration but varying volume it was also necessary to use one-, two- and threefold aliquots (1×, 2× and 3×) of enzyme solution. The initial inhibitor concentration was fixed at 1.1×10^{-6} M for the bulk of the determinations, all of which were carried out at *p*H 7.4 and 25.14°. Experiments to determine effects on inhibition were controlled with respect to the aging period in the cell of II dissolved in buffer, before the addition of enzyme (preincubation) and after enzyme addition (postincubation). The results of the total sequence of kinetic determinations are summarized in Table II. Figure 1 presents a representative

TABLE II

AChE-DIAMINE INCUBATION *vs.* INHIBITORY ACTIVITY

Phosphate buffer, *p*H 7.4, 25.14°, $[II]_0 = 1.1 \times 10^{-6}$ M, $[Substrate]_0 = 3.36 \times 10^{-3}$ M

Run	Units of enzyme	Total units of reaction vol.	Incubation period (min.) Pre-enzyme	Incubation period (min.) Post enzyme	Inhibition results after substrate addn. Initial inh., %	Inhibition results after substrate addn. Duration, min.	Inhibition results after substrate addn. Subsequent inh., %
1	1X	1X	0	5	54	3	38
2	1X	1X	5	5	46	5	52
3	1X	1X	5	1	58	>10	
4	1X	1X	30	1	6	>10	
5	1X	2X	0	1	47	4;	
					then 32	10	0
6	1X	2X	0	5	51	2	4
7	1X	2X	5	1	Slight	1	0
8	2X	2X	0	1	58	2	37
9	2X	2X	0	6	57	3	31
10	2X	2X	5	1	16	5	..
11	2X	2X	5	5	2	>10	..
12	1X	3X	0	1	52	5	5
13	1X	3X	5	1	49	4–5	~40
14	1X	3X	15	1	4	>10	..
15	1X	3X	30	1	0	..	..
16	1X	3X	5	5	44	6	0
17	3X	3X	0	1	65	~10	8
18	3X	3X	0	5	<1	..	..
19[a]	1X	2X	0	2	48	~4	0
20[b]	1X	2X	24 hrs.[b]	2	2	..	..

[a] Initial concentration of II, 1.29×10^{-6} M. [b] Buffer containing an initial 8.23×10^{-6} M concentration of II aged in Pyrex at 4° for 24 hours; an aliquot then placed in the titration cell followed by enzyme and substrate as indicated. Final [II] if no loss to glass, 6.68×10^{-6} M.

(10) By Dr. S. A. Bernhard of these laboratories.

(11) G. E. McCasland and D. A. Smith, THIS JOURNAL, **72**, 2190 (1950).

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

comparison of inhibition results at the three volume levels with initial enzyme (1X), substrate and inhibitor fixed, and total incubation times of the order of 5–10 minutes as in runs 3, 6 and 16.

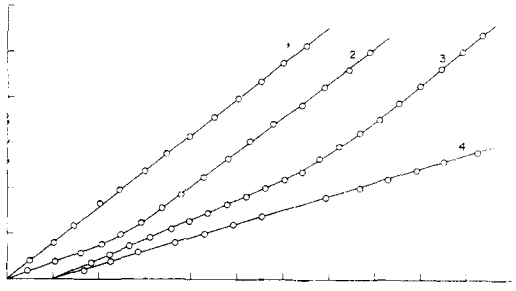


Fig. 1.—Rates of acetylcholine cleavage for: (1) uninhibited hydrolysis at 1× enzyme and 2× volume; (2) inhibited run 6 at 2× volume; (3) inhibited run 16 at 3× volume; (4) inhibited run 4 at 1× volume.

The results of Table II as illustrated in Fig. 1 seem to fit a pattern in which the time sequence of disappearance of inhibitory activity on incubation of a fixed inhibitor concentration depends on the volume of solution involved during the aging process, and also to some extent on the presence of enzyme in solution during this period. For example, keeping all variables constant except volume and time during incubation of inhibitor with buffer, runs 2 and 3 at the 1× volume level show relatively little difference created in the 50% inhibition mark by an additional period of 5 minutes of incubation after enzyme is added; at the 2× volume level (runs 6 and 7) essentially equal 5–6 minute periods of incubation *before or after* enzyme addition lead to the loss of virtually all inhibitory activity; and for the 3× volume (runs 13 and 16) further incubation of 5 minutes after enzyme addition causes a rapid drop to zero inhibition as contrasted with the smaller drop in inhibition when substrate is added 1 minute after enzyme. At both high and low volumes (runs 4, 14 and 15) periods of incubation in buffer ranging from 15 to 30 minutes prior to addition of enzyme are sufficient to lower the percentage inhibition to essentially zero.

The kinetic results at higher (2×, 3×) total amounts of enzyme for the most part show similar decreases in inhibition resulting from short-time incubation periods before or after addition of enzyme. Runs 8, 9, 11, 17 and 18 all show the effects of disappearing inhibition that seems to run to completion within about 15 minutes of total contact time. No order of magnitude differences in speed of disappearance as a function of total amount of enzyme can be detected, although runs 17 and 18 at 3× enzyme appear to show loss of inhibition somewhat more rapidly than runs 8 or 9 at 2× enzyme.

That this loss of inhibition can be completely independent of the presence of the enzyme during the aging process and also free of dependence on the geometry of the cell plus electrode system as incubating space is indicated by the results of run 20, in which aging in buffer for the extended period of 24 hours at 4° of an eightfold higher concentration of II than that in the rest of the series was permitted to occur in a Pyrex volumetric flask. On pipetting of a sample into the titration cell, followed by the rapid addition of enzyme and substrate, only minimal inhibition was observed.

These results indicate that constancy of inhibition at the 10^{-6} to 10^{-7} M concentration levels generally employed in the *in vitro* enzymatic work with compound II is only to be found at the low volumes previously employed.[3] Further, severe limitations are also necessarily imposed on the preparation and storage of very dilute solutions of II in glass, considering the loss of inhibitory activity resulting from such storage in buffered medium as shown in run 20.

This disappearance of inhibitory activity by II has also been followed at inhibitor concentrations several orders of magnitude higher, with conclusive results following the pattern of those at the lower concentration levels. For example, in a representative set of experiments, a 2× concentration of enzyme in a final reaction volume of 6.40 ml. was subjected to the action of high concentrations of II in a controlled sequence. First, the incubation of enzyme at 25.14° in *p*H 7.4 buffer was allowed to occur in the presence of a 1.20×10^{-4} M concentration of II, for a 30-minute period. Following the addition of acetylcholine substrate (3.36×10^{-3} M) and kinetic assay for activity, it was found that only 42.7% inhibition of the standard activity remained. This value corresponds to the loss of 99.4% of the inhibitory power of II that might have been expected, based on the effectiveness[3a] of the thousand-fold lower concentrations of II in smaller reaction volume. That this effect is essentially independent of any direct denaturing action of II on the enzyme during this extended incubation time was shown in an accompanying experiment in which the incubation of a 1.31×10^{-4} M concentration of II in *p*H 7.4 buffer for 30 minutes prior to the addition of 2× enzyme, followed by addition of enzyme and only 5 minutes of further incubation before assay with substrate, produced virtually the same result; *i.e.*, a 39.4% inhibition of AChE activity which corresponds to a loss of 99.5% of the inhibitory power which the given aliquot of II would possess under high dilution–low volume conditions.

The striking inability of II to cause irreversible damage to the enzyme protein or in turn be irreversibly absorbed by the enzyme with subsequent loss of activity was illustrated by the following, high concentration level experiment. A buffered solution containing 15.6 mg. of enzyme protein (dry weight) and a 1.07×10^{-3} M concentration of II was incubated at 4° for a period of 2.78 hours. It was then placed in a cellophane dialysis sack and dialyzed against the standard *p*H 7.4 buffer in the cold for a period of 15 hours, with frequent changes of buffer to remove II. The residual solution in the sack was then diluted and assayed for total enzymatic activity, with a resulting small deviation (+ 2.7%) from the original standard activity well within the estimated uncertainty of the determination. Accordingly, incubation of the enzyme with even this relatively high concentration of the inhibitory diamine II results in no significant ir-

reversible loss of catalytic activity of the recovered protein.

It would appear from these data that the phenomenon of disappearance of inhibition recorded in the low concentration experiments of Table II is not unique to these low levels. It seems likely that it is related to a sorption process of II on both protein and glass, although the latter's ability in this respect is clouded by the observation that the smallest loss of activity occurs at 1× volume, where the surface/volume ratio is greatest.

The inference with respect to possible sorption of II on protein (both catalytic and non-catalytic portions) is supported by previous observations[13] that such polyelectrolyte solutions as diluted cat serum (containing its own serum esterase) or 0.10% gelatin in buffer can cause the disappearance of esterase inhibition to be expected from relatively large concentrations of II, on incubation with the inhibitor prior to kinetic analysis, and the related observation[13] that inhibition of AChE by the monomethiodide of I can be lowered by a factor of 50% after preincubation in the presence of 0.5% bovine serum albumin.

Inhibition with the Diamine IV.—An interesting type of interference with AChE surface binding of diamines of the type II was previously shown[14] in the inhibitory action of the α- and β-methyl substituted derivatives of II. It was observed that substitution of a methyl group on either of the methylene carbons of II seriously lowers the inhibitory activity, with a slight differentiation in effect of α *vs.* β-substitution reflecting a corresponding difference in steric requirements of the enzyme surface lying under these portions of a given adsorbed molecule. In an attempt to probe further into this region of protein surface between the dual sites[12] responsible for binding of the two polar functions of inhibitor or substrate, the compound IV has been prepared[15] as its dihydrochloride and tested for its inhibitory activity on the AChE–acetylcholine system at *p*H 7.4.

With the two nitrogen functions of IV serving for adsorption at the anionic and esteratic sites of AChE (one of the N atoms in all probability being monoprotonated at *p*H 7.4 in view of the nearly fixed pairs of pK_a' values observed[16] for these ethylenediamines) it was thought that the cage or barrel configuration lent to IV by the three N-linking $-CH_2CH_2-$ bridges would lead to a serious bulk problem on attempted adsorption at the protein surface. Models indicated that interference with a surface by the barrel structure of IV might be more serious than with either methylated derivative of II.

In terms of the resulting inhibition produced by adsorption at the catalytic region, this expectation has been fully borne out. It was observed that at *p*H 7.4 and 25.14°, compound IV is completely inactive as an inhibitor of AChE at concentrations ranging up to the high value of 1.1×10^{-2} *M*. Part of this lack of inhibitory activity may perhaps be due to a lack of methyl groups *per se* on the positively charged N atom, to lend additional binding strength *via* van der Waals interaction with the anionic site,[12] but the completeness of the phenomenon indicates that bifunctional adsorption to the surface over a region where chain-linked methyl groups interfere is entirely obviated by the steric requirements of the cage in IV.

Experimental

Kinetic determinations of enzymatic activity at 25.14 ± 0.03° and *p*H 7.4 were carried out by the constant-*p*H titration technique previously described.[3a] The enzyme preparations from electric eel tissue[17] assayed at 1.02×10^4 and 6.97×10^3 μmoles acetylcholine hydrolyzed/hr./mg. of dry weight protein at the substrate optimum of 3.3×10^{-3} *M*. Doubly recrystallized acetylcholine chloride, triply distilled water and a freshly distilled sample of II were used throughout.• The aminoalcohol I was a freshly distilled sample prepared as in the previous work,[2] with m.p. 34–35°. The poorly poised buffer employed was 0.015 *M* in phosphate, 0.10 *M* in NaCl and 0.01 *M* in $MgCl_2$, and adjusted to *p*H 7.4. Compounds III and IV dihydrochloride were freshly recrystallized before use.

Incubations at any given volume level were carried out in the thermostated cell with constant stirring. Following completion of the final incubation after addition of the desired aliquot of enzyme, an aliquot of freshly prepared substrate solution was added and kinetic measurements started within one minute's time. Non-inhibited reference runs at the higher total amounts of enzyme (especially 3×) showed a pronounced tendency to decelerate slightly after some 5–10 minutes of reaction time, whereas hydrolysis rates at the 1× and 2× levels by and large remained quite constant with time over the 10–20% extent of reaction studied. The unit of enzyme designated as IX amounted to 6.2×10^{-4} mg. of protein on the dry weight basis in runs with II.

Rate runs with and without inhibitor were in general reproducible to within ±5%. K_I values were obtained from least-squares evaluation of the slopes of v/v_I *vs.* [I] plots, with a K_m value of 2.6×10^{-4} for these experimental conditions being employed in the slope calculations from eq. 1. The values tabulated were for a fixed temperature of 25.14 ± 0.03°.

Acknowledgment.—We are indebted to Dr. H. S. Polin for his generous aid in procurement of electric eel tissue and its initial extracts, to Dr. D. J. Jenden for the frog rectus assay, and to Mr. P. A. French for technical assistance.

BETHESDA, MD.

(13) H. D. Baldridge, E. B. Cook, S. L. Friess, D. J. Jenden and J. R. Tureman, Naval Medical Research Report, NM 000 018.12.06, 1956.

(14) S. L. Friess and H. D. Baldridge, THIS JOURNAL, **78**, 966 (1956).

(15) According to the improved procedure of O. Hromatka and O. Kraupp, *Monatsh.*, **82**, 880 (1951), as based on earlier methods developed by Hromatka and co-workers, and by F. G. Mann and D. P. Mukherjee, *J. Chem. Soc.*, 2298 (1949).

(16) See ref. 3b, and also A. Gero, THIS JOURNAL, **76**, 5158 (1954).

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