Inhibitory Properties of Aminoalkylsuccinimides on Isolated Horse Serum Butyrylcholinesterase: *N*-Methyl-2-phenyl-2-(*tert*-aminoalkyl)succinimides

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Abstract □ The butyrylcholinesterase inhibitory activities of several *N*-methyl-2-phenyl-2-(*tert*-aminoalkyl)succinimides were evaluated. The derivatives, all of which showed a mixed type of inhibition, were more potent as competitive than noncompetitive inhibitors. The results are discussed in terms of a possible mechanism for the formation of enzyme-inhibitor complexes.

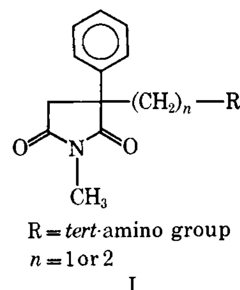
Keyphrases □ *N*-Methyl-2-phenyl-2-(*tert*-aminoalkyl)succinimides—inhibition, butyrylcholinesterase □ Butyrylcholinesterase inhibition—aminoalkylsuccinimides □ TLC—analysis

The effectiveness of compounds containing the functional moiety N—C—C—CO—N (aminopropionamide) as inhibitors of serum cholinesterase (acylcholine acylhydrolase, E.C. 3.1.1.8) has been demonstrated (1–6). Several structural parameters have been investigated for possible correlations with potency of enzyme inhibition. For example, Lasslo *et al.* (3) found a direct relationship between the length of the alkyl chain in 3-(1-alkylpiperidyl)carboxamides and their ability to inhibit plasma cholinesterase (PChE), and Beasley and Williford (5) observed that the introduction of aralkyl groups between the two ring nitrogen atoms in bis(3-piperidinecarboxamides) increases the inhibitory potency of these compounds.

The influence of amino substituents on the rates of hydrolysis of certain pseudocholinesterase substrates has also been investigated. Holmstedt and Sjoqvist (7) reported that the introduction of an amino group in butyrylcholine, the optimum substrate for pseudocholinesterase, to give γ-aminobutyrylcholine strongly inhibits its hydrolysis by this enzyme. Beckett *et al.* (8) reported that benzoylcholine has a greater affinity than butyrylcholine for PChE, even though it is hydrolyzed at only 15% of the rate of butyrylcholine. Prompted by the reported effect of the amino group in γ-aminobutyrylcholine, they evaluated *p*-aminobenzoylcholine and found that it not only has a greater affinity for the enzyme than does benzoylcholine, but it is a more potent inhibitor of the hydrolysis of butyrylcholine and acetylcholine.

Recently, the preparation of several *tert*-aminoalkylsuccinimides (Structure I) was reported (9). These compounds embody structural features resembling not only those in the 3-piperidinecarboxamides but also those in γ-aminobutyrylcholine and *p*-aminobenzoylcholine.

Interest in the influence of amino substituents on cholinesterase-inhibitor interactions prompted the authors to investigate the cholinesterase-inhibitory properties of these succinimides. In the present report, some preliminary data are presented and discussed.



R = *tert*-amino group
n = 1 or 2

I

MATERIALS AND METHODS

All reagents and derivatives used in this study were of analytically pure grade or equivalent. These include acetylcholine chloride[1] and horse serum butyrylcholinesterase (acylcholine acylhydrolase, E.C. 3.1.1.8) in the form of a lyophilized powder.[2] The succinimide derivatives were synthesized according to the procedures of Clemson *et al.* (9).

All the inhibitors were shown to be pure by TLC on silica gel, using various concentrations of absolute ethanol in benzene as the developing solvents (Table I).

Enzyme rate measurements were carried out potentiometrically with butyrylcholinesterase using a Radiometer automatic titrator (Type TTTlc) equipped with a recorder (SBR2c) and a syringe buret unit (SBU1a). Enzyme initial-velocity measurements were recorded at pH 7.40 ± 0.05 in a 25-ml. thermostated vessel at 27.0 ± 0.10°. All reactions were run under a nitrogen atmosphere using a combination glass–calomel electrode (GK2026c), mechanical stirring, and 0.01 *N* sodium hydroxide as the titrant. The titration procedure is essentially the method developed by Stein and Laidler (10) in their studies on the kinetics of α-chymotrypsin. The reaction mixtures were 0.04 *M* in magnesium chloride and 0.01 *M* in sodium chloride and contained substrate, inhibitor, and 0.11 mg. of enzyme.[3] The reaction mixtures, both control and with inhibitors, were preincubated for 5 min. at 27° prior to the initiation of the reaction by the addition of substrate. This preincubation time did not affect the initial rate measurement under the experimental conditions. Six substrate concentrations ranging from 7.7×10^{-3} *M* to 2.2×10^{-3} *M* (each differing by 1.1×10^{-3} *M*) were employed. The total reaction volume was 15 ml. At least two or more inhibitor concentrations were used, and the resulting data were plotted according to the method of Lineweaver and Burk (11). Data on the inhibitor dissociation constants were calculated from these plots (Fig. 1) according to a method described by Krupka (12). The final competitive (K_I) and noncompetitive (K_I') inhibitor dissociation constants are averages of at least two corresponding and independent determinations.

RESULTS AND DISCUSSION

The cholinesterase inhibitory potencies of the title compounds are shown in Table I. A typical plot used in determining the competitive (K_I) and noncompetitive (K_I') inhibitor dissociation constants for one of the inhibitors appears in Fig. 1. All of the com-

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Worthington Biochemical Corp., Harrison, N. J.
[3] The specific activity of the butyrylcholinesterase was found to be approximately 6.3 units/mg., where the activity is expressed as μmole of acetylcholine hydrolyzed/min./mg. of protein at 27° and pH 7.4.

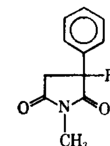


Table I—Inhibition of Isolated Horse Serum Cholinesterase by Succinimides

Compound	R	Absolute EtOH in Benzene[a]	Competitive Inhibitor Dissociation Constant (K_I)	Noncompetitive Inhibitor Dissociation Constant (K_I')
1	$—CH_2—N(CH_3)_2 \cdot HCl$	2%	1.57×10^{-4}	8.53×10^{-4}
2	$—CH_2—N(C_2H_5)_2 \cdot HCl$	2%	1.66×10^{-4}	7.31×10^{-4}
3	$—CH_2—N$(pyrrolidinyl) . HCl	2%	2.41×10^{-4}	1.25×10^{-3}
4	$—CH_2—N$(piperidinyl) . HCl	1%	3.00×10^{-5}	2.01×10^{-4}
5	$—CH_2—N$(piperazinyl)$N—CH_3$. 2HCl	25%	1.11×10^{-4}	7.05×10^{-4}
6	$—CH_2—CH_2—N(CH_3)_2 \cdot HCl$	25%	4.44×10^{-4}	2.40×10^{-3}
7	$—CH_2—CH_2—N(C_2H_5)_2 \cdot HCl$	50%	2.44×10^{-5}	6.77×10^{-4}
8	$—CH_2—CH_2—N$(pyrrolidinyl) . HCl	25%	1.69×10^{-4}	6.36×10^{-4}
9	$—CH_2—CH_2—N$(piperidinyl) . HCl	10%	1.35×10^{-4}	7.97×10^{-4}
10	$—CH_2—CH_2—N$(piperazinyl)$N—CH_3$. 2HCl	50%	1.07×10^{-4}	6.32×10^{-4}
11	—H	50%	1.45×10^{-3}	5.43×10^{-3}

[a] As an index of purity, all compounds were chromatographed on Eastman Silica Gel Chromagram Sheets (6061), using solutions of absolute ethanol in benzene as developing solvents.

pounds in this study displayed a mixed type of inhibition (13), thus indicating the presence of a competitive and a noncompetitive component. This suggests that the inhibitors are binding both at the active site (competitive) and at a peripheral site (noncompetitive).

The aminoalkyl derivatives of *N*-methyl-2-phenylsuccinimide are significantly more potent inhibitors of butyrylcholinesterase than is the parent compound (Table I). This is reflected by their lower competitive and noncompetitive inhibitor dissociation constants. However, it should be noted that these amino derivatives are more effective as competitive than as noncompetitive inhibitors. Discussion of the data will be limited to the competitive component, since it is this component that directly interferes with the binding of substrate.

It is apparent from Table I that there is no correlation between potency of inhibition and length of alkyl chain between the carbonyl carbon and the tertiary nitrogen. Indeed, the lengths of the chains in Compounds 4 and 7, the most potent inhibitors of the series, differ by one methylene group. This is in contrast to the observed effect in the piperidinecarboxamide series. The 4-piperidinecarboxamides, which contain three carbon atoms between the carbonyl carbon and the nitrogen atom, are less potent inhibitors than their 3-isomers, which contain only two (4). In the succinimide series, the aminoalkyl side chain is flexible and may, therefore, assume various conformations to accommodate the steric requirements of the active site on the enzyme. However, this is not possible in the piperidinecarboxamides since the aminoalkyl group, being part of the piperidine ring, is held in a rigid conformation.

From the results reported by Holmstedt and Sjoqvist (7), it would appear that the introduction of an amino group in butyrylcholine to give γ-aminobutyrylcholine induces a change in its mode of binding to butyrylcholinesterase. This modification of the substrate molecule may result in an improper orientation of the ester group at the esteratic site and, thereby, produce a reduction in the rate of hydrolysis. To illustrate this hypothesis, one may assume that the conformation of butyrylcholine in Fig. 2a is required for maximum hydrolysis. Then, as a consequence of an interaction between the amino group and a complementary group at Site A (A′, Fig. 2b), γ-aminobutyrylcholine may assume a slightly different conformation. The resulting conformational change of the ester moiety at the esteratic site (Site B) may be unfavorable for maximum hydrolysis. This effect is analogous to the apparent disturbance of an "ideal fit" between the enzyme and substrate by α- and β-methyl groups in butyrylcholine (8).

From all indications, binding of benzoylcholine and *p*-aminobenzoylcholine to the enzyme is similar to that for butyrylcholine and γ-aminobutyrylcholine (8). Benzoylcholine has a greater affinity but a lower hydrolytic rate than does butyrylcholine. The hydrophobic interactions of the phenyl ring of benzoylcholine with Area A (Fig. 2) would explain this increase in binding energy and would also tend to explain the decreased rate of hydrolysis due to an accompanying change of conformation of the ester group at Site B (esteratic site). The additional binding energy reported by Beckett *et al.* (8) in *p*-aminobenzoylcholine strongly suggests that the amino substituent further contributes to the affinity of the molecule for the enzyme by bonding with a complementary group at Site A.

It would seem probable that the interaction between the aromatic amino substituent in *p*-aminobenzoylcholine and a complementary

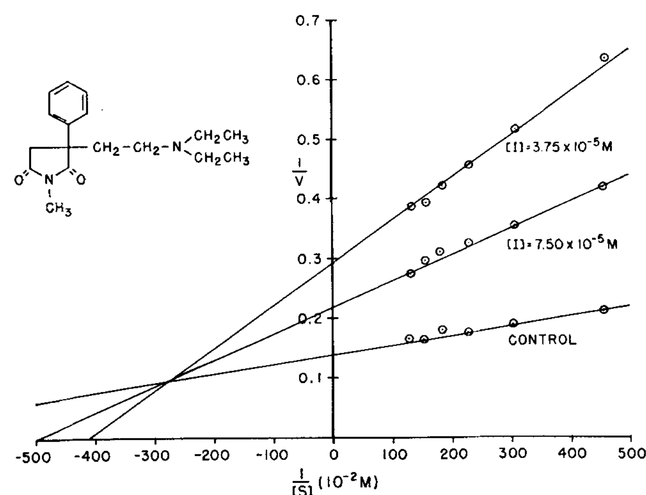


Figure 1—*Mixed inhibition of horse serum cholinesterase (butyrylcholinesterase) by* N*-methyl-2-phenyl-2-(2-diethylaminoethyl)succinimide (Compound 7, Table I). Abscissa, reciprocal molar acetylcholine chloride concentration; ordinate, reciprocal enzyme initial velocity. Velocity is expressed in μmoles of acetylcholine hydrolyzed/min./mg. of protein.*

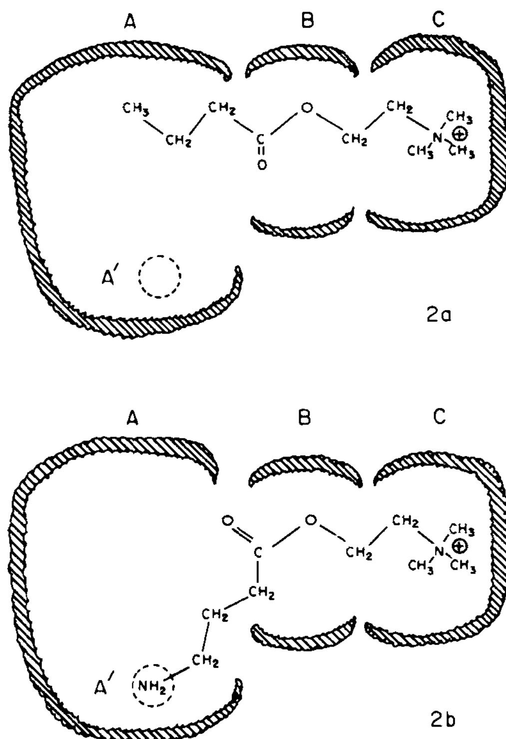


Figure 2—*Concept of differences in conformations of butyrylcholine (2a) and γ-aminobutyrylcholine (2b) at the substrate binding site of butyrylcholinesterase. Key: A = nonpolar area; A′ = complementary area in A which interacts with amino group; B = esteratic site; and C = "anionic" site.*

group on the enzyme is not ionic in nature. Esters of *p*-aminobenzoic acid are weakly basic, *e.g.*, pK_b of ethyl *p*-aminobenzoate is 10.84 (14) and, consequently, the existence of the conjugate acid of *p*-aminobenzoylcholine at physiological pH would be negligible. Thus, it would appear that hydrogen bonding or dipolar interactions are more likely involved. However, in the case of γ-aminobutyrylcholine, its conjugate acid would be quite significant at physiological pH and could, therefore, enter into H-bond or ion dipole formation at Site A.

The mode of competitive binding of *N*-methyl-2-phenylsuccinimide (Compound 11, Table I) and its derivatives may be visualized as being analogous to those for butyrylcholine, benzoylcholine, and their amino derivatives. In this case, however, the imide function is pictured as complexing with the esteratic site. The interaction of amide moieties with esteratic sites of cholinesterases has already been proposed by several investigators (4, 6, 15). The aromatic ring, like that in benzoylcholine, may be involved in hydrophobic interactions with Site A and may, thereby, exert a directing influence in the formation of the enzyme-inhibitor complex. The increase in binding energies of the aminoalkylsuccinimide derivatives may be attributable to an additional interaction between the amino substituents and an appropriate group at Site A.

The data presented in this report on the inhibitory properties of some *tert*-aminoalkylsuccinimides are consistent with the hypothesis advanced to explain the interactions of γ-aminobutyrylcholine and *p*-aminobenzoylcholine with butyrylcholinesterase. From a study on the inhibitory properties of a series of 1-substituted 3-piperidinecarboxamides, Beasley *et al.* (6) offered an alternate explanation for the interaction of amino groups with the active site of butyrylcholinesterase. They suggested that the amide function binds at the esteratic site while the nitrogen atom of the piperidine ring binds at the "anionic" site (Site C, Fig. 2). There is no definitive evidence at the present time that discounts either of these hypotheses. Perhaps both modes of binding are operative. The real significance of these proposed mechanisms must await further experimentation.

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