Succinylcholine Analogs as Substrates and Inhibitors of Pseudocholinesterase

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The preparation of the *meso* forms of succinyl-α- and succinyl-β-methylcholine iodide is described, and the inhibitory properties of succinylcholine iodide and the optically active diester forms of 1-dimethylamino-2-propyl and 2-dimethylamino-1-propyl succinate methiodide toward the hydrolysis of butyrylcholine iodide by the pseudocholinesterase present in both horse and human sera were investigated; all the compounds were shown to be competitive inhibitors of the enzyme. Hydrolysis of the inhibitors by horse and human sera was investigated, and, significantly, the phenomenon of excess substrate inhibition was observed in all cases except for that of the L-α isomer.

Cholinesterases have been reviewed extensively[1-4] and their classification has been discussed.[5,6] The term pseudocholinesterase (PChE) is used here to describe that group of enzymes designated by the International Union of Biochemistry as acylcholine acylhydrolase (EC 3.1.1.8).

Direct chemical analyses, such as have been used to show the involvement of the imidazole group of a histidine residue,[7] a serine hydroxyl group,[8] and a free carboxyl group adjacent to the serine residue[9] at the esteratic site of PChE and related enzymes, provide little information concerning the tertiary structure of the protein during its biologically active phase. The induced-fit theory of Koshland[10] suggests that conformational changes in a flexible active site may be induced by interaction with the substrate, and such induced changes have since been postulated for several systems[11-14] including the cholinesterases.[15,16] Stereochemical studies have shown that activity is closely related to the configuration of the substrate used,[17] and work showing that β-methyl substitution of butyrylcholine reduced the rate of hydrolysis by horse serum PChE more than did α-methyl substitution is interpreted using the induced-fit theory to indicate the involvement of a fold of limited dimenions in the region of the active site of PChE.[15] Augustinsson[18] too has demonstrated the importance of steric interference in interactions between derivatives of pyridine and cholinesterases. These results suggest differences between the esteratic sites of acetyl- and butyrylcholinesterase, the most important of which is the difference in the nature of the bonding forces responsible for orientation and interaction at the active sites.

Succinylcholine (SCh_2) is commonly employed as a short-acting curareform muscle relaxant during surgical anesthesia. It is hydrolyzed by PChE, and therefore the mechanism of hydrolysis and the structure of the active site of the detoxifying enzyme are of interest. The curareform action of SCh_2 is reduced by branching of the alkyl chain,[19] the amount of the reduction and the type of activity produced being dependent upon the position of the branch, *i.e.*, α- or β-methyl.

Inhibitory studies using SCh_2, which is a reversible inhibitor of PChE,[20] and the optically active isomers of α- and β-MeSCh_2 were carried out using diluted horse serum in an attempt to provide more information concerning both the configuration of the functional groups in the active site of PChE and also its mechanism of action. In view of the important clinical use of SCh_2 some of this work was repeated using diluted human serum; the hydrolysis of some of these compounds by undiluted horse and human sera was studied. Serum was used as the enzyme source during these investigations because it has been shown that the results obtained using a purified preparation do not significantly differ from those obtained using serum.[15]

Experimental Section

Chemistry.—The intermediate dimethylaminoalkyl monohydrogen succinate hydrochlorides used in the preparation of *meso*-succinyl-α- and *meso*-succinyl-β-methylcholine iodide were prepared by a modification of the method used by Phillips[21] for the preparation of the monocholine succinate ester. The intermediate half-ester was converted to its acid chloride with $SOCl_2$, and the acid chloride was then treated with the isomeric amino alcohol to give the *meso* diester.

***meso*-Succinyl-β-methylcholine Iodide (*meso*-β-MeSCh_2).**—Succinic anhydride (1.9 g) was added to D-(−)-dimethylamino-2-propanol (2.0 g) in ether and the mixture heated slowly for 30 min then at 100° for about 60 hr. The residual syrupy liquid containing the levorotatory half-ester of succinic acid was treated with freshly distilled $SOCl_2$ (25 ml), kept cold until all the syrupy liquid had dissolved, and then heated for 2 hr at 50–60°. Excess $SOCl_2$ was removed by evaporation under reduced pressure. L-(+)-Dimethylamino-2-propanol was added dropwise to the solid residue with constant stirring and cooling, and the mixture was then allowed to stand at room temperature for 2 hr. The mixture was dissolved in ice–water (2–3 ml), and made alkaline with saturated NaOH, excess anhydrous Na_2CO_3 was added, and the damp mass was extracted with ether (four 50-ml portions). The combined ethereal extracts were dried

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(Na_2SO_4) and the ether was removed by evaporation under reduced pressure. The basic residue was then dissolved in Me_2CO (10 ml), filtered, and treated with MeI (1 ml). The product was crystallized from MeOH–Me_2CO to give 0.85 g of a white crystalline solid, mp 242–243°, equiv wt 289. *Anal.* Calcd for $C_{16}H_{34}I_2N_2O_4$: C, 33.60; H, 5.94. Found: C, 33.56; H, 6.10.

meso-Succinyl-α-methylcholine iodide (*meso*-α-$MeSCh_2$) was prepared similarly from succinic anhydride (1.9 g) using the D and L isomers of 2-dimethylamino-1-propanol. The reaction was worked up as described above to yield 0.5 g of a crystalline solid, mp 233–234°, equiv wt 285. *Anal.* Calcd for $C_{16}H_{34}I_2N_2O_4$: C, 33.60; H, 5.94. Found: C, 33.70; H, 5.91.

Ir spectra of both *meso* diesters showed the presence of CO with no trace of OH. The specific rotations in MeOH were found to be zero, thus confirming that the products were the required *meso* diesters of succinyl-α- and succinyl-β-methylcholine iodides.

Enzyme Studies.—The hydrolysis and inhibitor studies were carried out using the Warburg manometric technique as previously described.[17] All solutions were prepared in a $NaHCO_3$ buffer (pH 7.4), which was freshly prepared, and gassed (30 min) with a mixture of CO_2 (5%) in N_2 before use as described by Augustinsson.[22] The rates of enzymic hydrolysis were measured in terms of microliters of CO_2 liberated at 38° in the initial 30 min of a 40-min period. During the inhibitor studies, the inhibitors were preincubated with the enzyme for a period of 30 min at 38° prior to mixing the contents of the flask. For each substrate concentration ([S] = 10^{-2}, 2×10^{-2}, and 3×10^{-2} *M*) investigated, four inhibitor concentrations ([I] = 0, 10^{-3}, 3×10^{-3}, and 5×10^{-3} *M*) were used.[23] The enzyme source was horse serum obtained from the Wellcome Research Laboratories and serum obtained from fresh human blood (CLV). Serum preparations were stored at 0°. The hydrolytic activity of a freshly prepared enzyme solution was required to be such that 1.5 ml would in 30 min hydrolyze approximately 2 mg of butyrylcholine iodide ([S] = 3×10^{-2} *M*) in a total volume of 3 ml. Dilutions of 0.6 in 20 and 0.5 in 20 for horse and human sera, respectively, before use were found to have a satisfactory activity. Butyrylcholine iodide[24] (BuCh) was used as the substrate in all inhibitor studies and the inhibition of its hydrolysis by PChE was studied using the following compounds: succinylcholine iodide,[25] mp 254–256° (SCh_2); succinyl-α,α-dimethylcholine iodide,[26] mp 233.5–234.5° (α-Me_2SCh_2); L-(−)-succinyl-α-methylcholine iodide,[17] mp 201–202°, $[\alpha]^{24}D$ −7.2° (*c* 5.0, MeOH) (L-(−)-α-$MeSCh_2$); D-(+)-succinyl-α-methylcholine iodide,[17] mp 200.5–201°, $[\alpha]^{20}D$ +7.74° (*c* 2.0, MeOH) (D-(+)-α-$MeSCh_2$); L-(+)-succinyl-β-methylcholine iodide,[17] mp 247°, $[\alpha]^{22}D$ +22.2° (*c* 2.0, 90% MeOH) (L-(+)-β-$MeSCh_2$); D-(−)-succinyl-β-methylcholine iodide,[17] mp 254°, $[\alpha]^{20.5}D$ −20.9° (*c* 2.0, 90% MeOH) (D-(−)-β-MeSCh); *meso*-β-$MeSCh_2$; and *meso*-α-$MeSCh_2$.

K_i values were determined by the method of Dixon[27] using the rearranged expression for competitive inhibition, *viz*

$$\frac{1}{V_i} = \frac{K_m[I]}{K_i V[S]} + \frac{1}{V}\left(1 + \frac{K_m}{[S]}\right)$$

Plots of $1/V$ *vs.* [I] for particular values of [S] give straight lines which intersect at a common point to the left of the ordinate. At the point of intersection the rate (V_i) is common to each [S] system, and the above expression reduces to $-[I] = -K_i$. Graphs were plotted from the experimental data; Figure 1 shows a typical example. All of the compounds were shown to be competitive inhibitors and the derived inhibitor characteristics are presented in Table I.

The rates of enzymic hydrolysis of the compounds studied were determined using diluted serum of the same activity as that used for the inhibition studies. In all cases, excepting only that of the hydrolysis of L-(−)-α-$MeSCh_2$ by horse serum, the rates of hydrolysis were found to be negligible (Table II). SCh_2, L-(−)-, and D-(+)-α-$MeSCh_2$ were all significantly hydrolyzed by undiluted horse and human serum. Although the effect on the K_i value was very small, the *b* values[22] (microliters of CO_2 per 1.5 ml of enzyme solution per 30 min) obtained during the L-(−)-α-$MeSCh_2$ inhibitor studies with horse serum were corrected by subtracting the values obtained for the hydrolysis of the inhibitor.

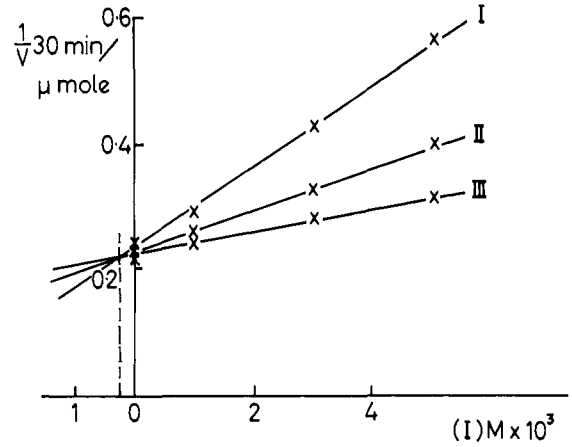

Figure 1.—Inhibition of the hydrolysis of BuCh ([S] = 10^{-2} (I), 2×10^{-2} (II), and 3×10^{-2} (III) *M*) at 38° by horse serum diluted 1:33 in the presence of SCh_2 using the Warburg manometric technique.

TABLE I
K_i VALUES OBTAINED USING DILUTED HORSE AND HUMAN SERA

Inhibitor	K_i, *M* × 10⁴ Horse serum	K_i, *M* × 10⁴ Human serum
SCh_2	2.5	6.0
L-(−)-α-$MeSCh_2$	6.5	17.5
D-(+)-α-$MeSCh_2$	3.5	8.5
meso-α-$MeSCh_2$	5.0	
α-Me_2SCh_2	32.5	
L-(+)-β-$MeSCh_2$	1.5	7.5
D-(−)-β-$MeSCh_2$	7.5	25.0
meso-β-$MeSCh_2$	3.5	

TABLE II
HYDROLYSIS OF SOME SUCCINYLCHOLINE ANALOGS BY DILUTED HORSE AND HUMAN SERA

Substrate, [S][a]	*b*, μl of CO_2[b] Horse	Human	Substrate, [S][a]	*b*, μl of CO_2[b] Horse	Human
SCh_2	1.5	1.6	α-Me_2SCh_2	1.0	...
L-(−)-α-$MeSCh_2$	4.6	1.8	L-(+)-β-$MeSCh_2$	0.6	0.4
D-(+)-α-$MeSCh_2$	1.2	0.7	D-(−)-β-$MeSCh_2$	0.6	0.4
meso-α-$MeSCh_2$	2.7	...	*meso*-β-$MeSCh_2$	0.5	...

[a] [S] = $[S]_{opt}$, *i.e.*, the values quoted are the maximum rates observed. [b] Per 1.5 ml of enzyme solution per 30 min.[22]

The results obtained in the hydrolysis studies using undiluted sera are presented in Table III and Figures 2 and 3. V_{max} and

TABLE III
HYDROLYSIS OF SOME SUCCINYLCHOLINE ANALOGS BY UNDILUTED HORSE AND HUMAN SERA[a]

Substrate conen, *M*	pS	*b*, μl of CO_2[c] Horse serum[b] b	d	Human serum[b] b	d	e	f	g
5×10^{-2}	1.30	21	...	52	38.8	...	...	...
3×10^{-2}	1.52	26	162	...	52.5	19.5	2.5	0.0
2.6×10^{-2}	1.59	...	...	...	56.0	...	...	...
10^{-2}	2.00	28	158	...	68.0	19.0	...	...
5×10^{-3}	2.30	28	152	57	65.0	...	...	...
3×10^{-3}	2.52	...	142	...	...	21.5	0.0	0.5
2.6×10^{-3}	2.59	...	...	...	61.0	...	...	...
10^{-3}	3.00	30	101	57	58.8	16.5	4.0	5.0
3×10^{-4}	3.52	10	...	...	40.0	...	...	...
10^{-4}	4.00	4	...	10	...	...	1.5	2.5

[a] See Figures 2 and 3. [b] b = SCh_2, d = L-(−)-α-$MeSCh_2$, e = D-(+)-α-$MeSCh_2$, f = L-(+)-β-$MeSCh_2$, g = D-(−)-β-$MeSCh_2$. [c] Per 1.5 ml of enzyme solution per 30 min.

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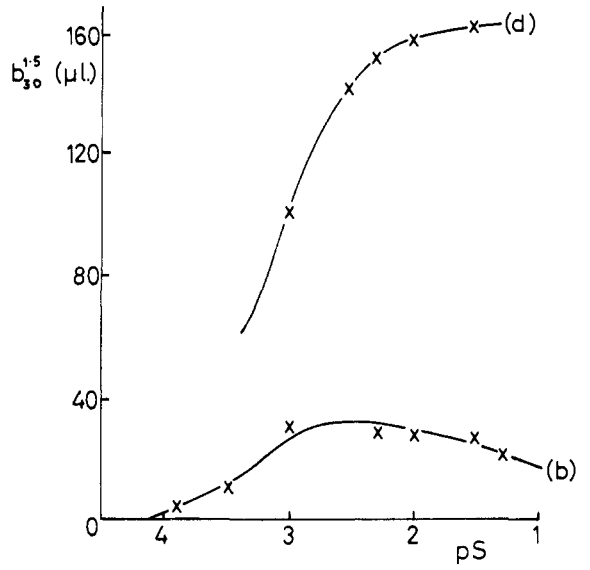

Figure 2.—The hydrolysis by undiluted horse serum of some succinylcholine analogs (b = SCh_2 and d = L-(−)-α-$MeSCh_2$) at 38° using the Warburg manometric technique (see Table III).

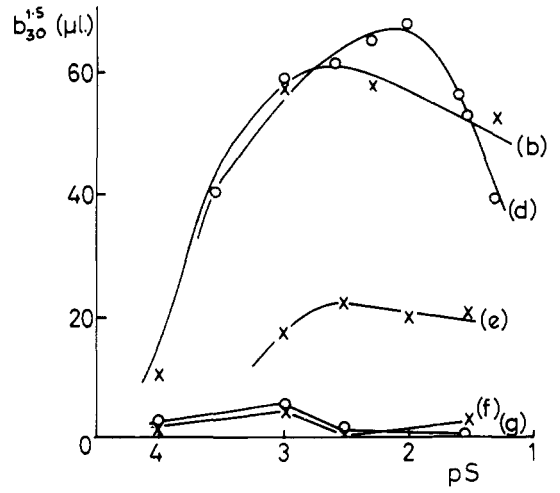

Figure 3.—The hydrolysis by undiluted human serum of some succinylcholine analogs (b = SCh_2, d = L-(−)-α-$MeSCh_2$, e = D-(+)-α-$MeSCh_2$, f = L-(+)-β-$MeSCh_2$, and g = D-(−)-β-$MeSCh_2$) at 38° using the Warburg manometric technique (see Table III).

$[S]_{opt}$ were obtained directly from these figures; the maximum rate of hydrolysis as observed was converted to μmole/30 min (μl/22.4 = μmole) and the values are given in Table IV. The rates of enzymic hydrolysis were found to be very low. In all cases, except that of the hydrolysis of L-(−)-α-$MeSCh_2$ by horse serum, the phenomenon of excess substrate inhibition was observed. As the accuracy of the manometric technique decreases significantly when such low rates are being recorded it was found impossible to make accurate determinations of the K_m values, except in the case of L-(−)-α-$MeSCh_2$ where the K_m value for its hydrolysis by horse serum was calculated from a Lineweaver–Burke plot and found to be 6.6×10^{-4} *M*. It is interesting to note that the K_i value for this compound with horse serum was 6.5×10^{-4} *M* (Table I).

Discussion

During the past few years the techniques of starch gel[28] and "two-system"[29] electrophoresis have been used to examine the components of enzyme systems, and variations in the properties of serum esterases from several species have been reported.[30] However, substrate specificity investigations involving the use of optically active compounds provide information not only about the difference in enzyme–substrate specificity between species but also provides information about the steric limitations and requirements in the region of the active site.

Similarities between horse and human serum esterase have already been reported[6,15] and the specificity patterns obtained during the present inhibitor and hydrolysis studies (Tables I–IV) confirm that essentially very similar enzyme systems are being studied. The higher K_i values obtained using human serum indicate that the affinities of all the compounds used are lower for human than horse serum PChE, although this is not paralleled, as might be expected, by higher rates of hydrolysis. Figures 2 and 3 clearly show the occurrence of excess substrate inhibition for all the compounds studied using human serum, and for SCh_2 using horse serum; the phenomenon was not apparent when L-(−)-α-$MeSCh_2$ was hydrolyzed by horse serum. In studies on the hydrolysis of SCh_2 by PChE, the only previous observation of excess substrate inhibition was made by Tammelin;[31] this appears to be the only example of a nonaromatic substrate inhibiting PChE activity. It is uncertain why use of the SCh_2 derivatives should tend to give ES_2 complexes, and then excess substrate inhibition, whereas use of BuCh does not. It could be that as [S] increases, substrate dimers form unreactive complexes with the enzyme, or alternatively, that the two quaternary groups combine with different parts of the enzyme molecule thus preventing the protein from achieving an active configuration. Why the same suppositions should not hold for L-(−)-α-$MeSCh_2$ is not clear.

In the present discussion, the term affinity is used to describe the attraction between E and S, and the term activity is used to define the ability of a substrate to initiate the hydrolytic process as discussed elsewhere.[15,32] K_i values are a direct measure of affinity ($1/K_i$ = the affinity constant of the enzyme–inhibitor complex), whereas K_m values include both affinity and activity terms.

The effect of β-methyl substitution was, in the work of Mitchard,[15] considerably greater than the effect of

Table IV

$[S]_{opt}$ and V_{max} Values Obtained for the Hydrolysis of Some Succinylcholine Analogs by Human and Horse Sera[a]

	Human serum		Horse serum	
Substrate	$[S]_{opt}$, *M*	V_{max}, μmole/30 min	$[S]_{opt}$, *M*	V_{max}, μmole/30 min
SCh_2	3×10^{-3}	2.7	3×10^{-3}	1.5
L-(−)-α-$MeSCh_2$	10^{-2}	3.0	10^{-2}	7.2
D-(+)-α-$MeSCh_2$	3×10^{-3}	1.0	...	...
L-(+)-β-$MeSCh_2$	10^{-3}	0.2	...	...
D-(−)-β-$MeSCh_2$	10^{-3}	0.2	...	...

[a] See Figures 2 and 3.

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α-methyl substitution, and it was concluded that this could be explained most satisfactorily in terms of an induced fold, as bulky substituents near the ester group would tend to hinder efficient fold formation. It can be seen from Table III that a similar pattern is observed in the present hydrolysis studies, a marked drop in the rate of hydrolysis occurring on β-methyl substitution of SCh_2, thus supporting the idea of induced-fold formation. Although low rates of hydrolysis are observed for both the L- and D-β-$MeSCh_2$ isomers, their affinities for the enzyme are different (Table I). The isomer with the higher affinity must also have a high activity, and *vice versa*, in order that similar rates of hydrolysis be observed. Conversely, when two compounds have the same affinities, differences in their rates of hydrolysis will reflect differences in relative activities. The high affinity of the L-β-methyl isomer, due possibly to an increase in charge on the ester oxygen caused by electron donation from the methyl group, coupled with its low rate of hydrolysis, indicates that fold formation is essential for activity but not for affinity. The electronic influence will be the same in both β enantiomers, and therefore the low affinity of the D-β-methyl isomer is probably due to steric interference from the methyl group which in this configuration prevents bond formation between the glutamic acid residue and the ester oxygen.

The most active substrate used in the present study was not SCh_2 but L-(−)-α-$MeSCh_2$. An analogous increase in the rate of hydrolysis upon L-α-methyl substitution of BuCh was not observed by Mitchard,[15] although in inhibitor studies using the α- and β-methyl analogs of *p*-aminobenzoylcholine, L-*p*-aminobenzoyl-α-methylcholine was shown to be a more potent competitive inhibitor of PChE than was *p*-aminobenzoylcholine. The D-α-methyl derivative has a high affinity and correspondingly low rate of hydrolysis, the L-α isomer has a lower affinity and is hydrolyzed rapidly, and SCh_2 has a high affiinity and a comparatively high rate of hydrolysis; the activity of the enzyme toward SCh_2 and L-α-$MeSCh_2$ must therefore be considerably higher than toward D-α-$MeSCh_2$. It can be seen that interpretation of structure–activity relationships must be undertaken with great care as the maximum velocity values observed are not necessarily indicative of the more fundamental affinity and activity values. The difference in the affinities of the α-methyl isomers will be due to (i) steric interference and (ii) differences in interaction with the hydrophobic site because the α-methyl group is too far from the ester group to exert any significant electronic influence.

It has been found[14] that β-methyl substitution reduces the pharmacological activity much more than does α-methyl substitution. The observation made during the present study that the rates of enzymic hydrolysis follow the same pattern as the pharmacological results indicate that similarities exist between the receptor site for these compounds and the active site of PChE. The K_i values, however, do not follow this pattern, and no definite conclusion can be reached concerning the nature of these similarities. Although the results obtained in the present study do not show the good correlation between inhibitor and hydrolytic studies which was observed by Mitchard,[15] they do appear to follow the same general pattern, indicating that application of the induced-fit theory to PChE is probably valid.

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Sympathetic Nervous System Blocking Agents. V. Derivatives of Isobutyl-, *t*-Butyl-, and Neopentylguanidine[1,2]

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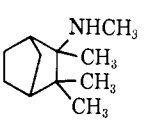
Guanidines prepared from simple *n*-alkylamines fail to show adrenergic neurone blocking activity, as determined by the effect on the cat nictitating membrane, while guanidines such as *t*-butylguanidine sulfate and neopentylguanidine sulfate are active. *t*-Octylguanidine hydrochloride (Table I, **5**) proved to be the most active member of the alkylguanidine series. It was subjected to extensive pharmacological evaluation and clinical trial. Substitution of one or two methyl groups on the α or β carbons of dialkylaminoalkylguanidines was also investigated. 2-Hexamethyleniminoisobutylguanidine sulfate (**20**) caused a remarkably long blockade of the sympathetic nervous system.

Mecamylamine[3] (I) and pempidine[4] (II) were the first substances found to possess ganglionic blocking activity which were not quaternary ammonium salts. Both are *t*-carbinamines, that is, the nitrogen atom of each is attached to a carbon atom containing three alkyl substituents. This hydrocarbon bulk which surrounds the nitrogen may be of great significance as far as the activity of I and II are concerned and sug-



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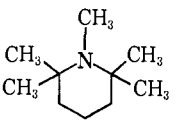

II

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