The Effect of Piperidinecarboxamide Derivatives on Isolated Cholinesterase Systems. Substituted N¹-Benzyl-3-(N,N-diethylcarbamoyl)piperidines[1]

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A series of substituted N¹-benzyl-3-(N,N-diethylcarbamoyl)piperidine hydrobromides containing methyl, methoxyl, chloro, and nitro substituents in the *meta* and *para* positions were evaluated for inhibitory activity in isolated human plasma cholinesterase and horse serum cholinesterase systems. The derivatives were found to inhibit competitively both enzyme preparations. For each isomeric pair of inhibitors, the *meta*-substituted analog was always more potent than the corresponding *para*-substituted isomer. N¹-(*m*-Methylbenzyl)-3-(N,N-diethylcarbamoyl)piperidine hydrobromide was the most potent of the inhibitors studied and N¹-(*p*-nitrobenzyl)-3-(N,N-diethylcarbamoyl)piperidine hydrobromide the least potent in both enzyme systems, although the order of inhibitory potency for the other derivatives was not identical. The data suggest that differences in the specificities of human plasma cholinesterase and horse serum cholinesterase may be attributed to variations in structure at or near the anionic site.

The presence of an anionic site[2] in cholinesterases appears to be the distinguishing feature between this class of enzymes[3] and other esterases. Differences in the restrictive nature of the anionic sites of serum cholinesterase[4] (acylcholine acylhydrolase, E.C. 3.1.1.8) and of acetylcholinesterase[5] (acetylcholine hydrolase, E.C. 3.1.1.7) may be responsible for specificity and activity differences observed for these two enzyme systems. Current indications are that an additional binding site may be present in BuChE.[6]

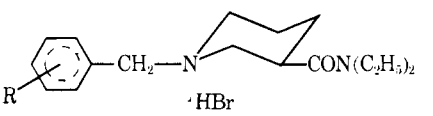
Wilson and Quan,[7] in a study on the complementariness of AChE[8] inhibitors, observed that the position and nature of substituent groups in substituted phenyltrimethylammonium ions were important in determining their effectiveness as AChE inhibitors. In an earlier investigation[9] we also noted that the position of the carbamoyl constituent in piperidinecarboxamide inhibitors of PChE affected their potencies.

Recently, Quintana and Smithfield[10] prepared a series of *meta*- and *para*-substituted N¹-benzyl-3-(N,N-diethylcarbamoyl)piperidine hydrobromides (Table I) to study the effect, on the electron density around the heterocyclic nitrogen, which substituent variation would induce. They were also interested in determining the influence which such structural variations would exert on the lipophilic–lipophobic characteristics of the subject moieties.

In accord with our interests concerning the influence of physicochemical parameters[11] on cholinesterase–inhibitor interactions, it seemed appropriate to investigate the cholinesterase-inhibitory properties of this series of derivatives (Tables I and II).

TABLE I

INHIBITION OF ISOLATED HORSE SERUM CHOLINESTERASE BY *meta*- AND *para*-SUBSTITUTED N¹-BENZYL-3-(N,N-DIETHYLCARBAMOYL)PIPERIDINE HYDROBROMIDES[a]



Compd	R	$-K_1 \times 10^5$ [b,c] — *m*	*p*	π[d]
I	H		2.16	0
IV	CH_3		2.11	0.52
V	CH_3	1.02		0.57
II	OCH_3		7.81	0.18
III	OCH_3	1.42		0.31
VI	Cl		1.56	0.54
VII	Cl	1.52		0.61
VIII	NO_2		9.52	−0.39
IX	NO_2	5.14		−0.36

[a] Although the subject molecules have not been specifically analyzed conformationally, sufficient evidence is available on the relative stability of the various conformers of piperidine and carbamoylpiperidine analogs [*cf.* W. Barbieri and L. Bernardi, *Tetrahedron*, **21**, 2453 (1965); E. L. Eliel, "Stereochemistry of Carbon Compounds," McGraw-Hill Book Co., Inc., New York, N. Y., 1962, p 246; W. P. Purcell and J. A. Singer, *J. Chem. Soc.*, 1431 (1966)] to justify showing them in the chair conformation. [b] Inhibition studies were conducted titrimetrically at 26° using the procedure described in the text. [c] A K_1 value of 2.16×10^{-9} *M* was found for physostigmine sulfate. This agrees well with previously published values (J. P. Long in "Handbuch der Experimentellen Pharmakologie," G. B. Koelle, Subed., Springer-Verlag, Berlin, 1963, p 377). [d] π values from partition coefficients (octanol–water) of substituted nitrobenzenes.[22]

Experimental Section

All reagents and derivatives used in this study were of analytically pure grade or the equivalent. AChCl, physostigmine sulfate, and PChE (Type II, pseudo)[12] were obtained from

(1) This investigation was supported by Grants GB-2381 and GB-4453 from the National Science Foundation and a grant from Marion Laboratories, Inc., Kansas City, Mo. Computer facilities were provided through U. S. Public Health Service Grant H.E. 09495.

(2) K. B. Augustinsson in "Handbuch der Experimentellen Pharmakologie," Vol. XV, G. B. Koelle, Subed., Springer-Verlag, Berlin, 1963, p 101.

(3) R. A. Oosterbaan in "Comprehensive Biochemistry," Vol. XVI, M. Florkin and E. H. Stotz, Ed., Elsevier Publishing Co., New York, N. Y., 1965, Chapter 5.

(4) I. B. Wilson, *J. Biol. Chem.*, **208**, 123 (1954).

(5) J. C. Kellett and C. W. Hite, *J. Pharm. Sci.*, **54**, 883 (1965).

(6) K. B. Augustinsson, *Biochim. Biophys. Acta*, **128**, 35 (1966).

(7) I. B. Wilson and C. Quan, *Arch. Biochem. Biophys.*, **73**, 131 (1958).

(8) Abbreviations used in this paper are: AChE, acetylcholinesterase; PChE, human plasma cholinesterase; BuChE, butyrylcholinesterase; ACh, acetylcholine.

(9) J. G. Beasley, R. P. Quintana, and G. G. Nelms, *J. Med. Chem.*, **7**, 698 (1964).

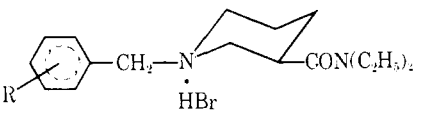
(10) R. P. Quintana and W. R. Smithfield, *ibid.*, **10**, 1178 (1967).

(11) W. P. Purcell, J. G. Beasley, R. P. Quintana, and J. A. Singer, *ibid.*, **9**, 297 (1966).

(12) The specific activity of this preparation was found to be approximately 3 units/mg where activity is expressed as μmole of ACh hydrolyzed/min per mg of protein at 26° and pH 7.4.

TABLE II

INHIBITION OF ISOLATED HUMAN PLASMA CHOLINESTERASE BY *meta*- AND *para*-SUBSTITUTED N¹-BENZYL-3-(N,N-DIETHYLCARBAMOYL)PIPERIDINE HYDROBROMIDES



Compd	R	I_{50} ± SE) × 10^3 M[a,b] — *m*	— *p*	K_i × 10^3 M[a,c] — *m*	*p*
I	H		1.47 ± 0.01		1.87
IV	CH_3		0.38 ± 0.02		0.48
V	CH_3	0.16 ± 0.00		0.20	
II	OCH_3		2.45 ± 0.15		3.11
III	OCH_3	0.22 ± 0.01		0.28	
VI	Cl		1.10 ± 0.02		1.40
VII	Cl	0.46 ± 0.15		0.58	
VIII	NO_2		10.90 ± 0.25		13.8
IX	NO_2	2.91 ± 0.01		3.70	

[a] Molarity of compound effecting 50% inhibition. [b] The inhibitory activities were determined manometrically at 37° using the method described in ref 9. [c] Inhibition studies on selected members of this series showed them to be completely competitive inhibitors and allowed us to calculate K_i values using the equation of F. Bergmann and R. Segal [*Biochem. J.*, **58**, 692 (1954)], $v_0/v_i = 1 + IK_m/K_i(K_m + S)$, where v_0 is the uninhibited rate, v_i the inhibited rate, I the inhibitor concentration, S the substrate concentration, and K_m and K_i represent, respectively, the Michaelis and inhibitor dissociation constants. For competitive inhibition, $I = I_{50}$ since $v_0 = 2v_i$ and the equation reduces to $K_i = I_{50}\left(\frac{K_m}{K_m + S}\right)$ or $K_i = I_{50} \times 0.127$. [d] The I_{50} value for physostigmine sulfate was found to be $5.24 \pm 0.19 \times 10^{-8}$ M, which is in good agreement with previous reports (*cf.* footnote *c*, Table I).

Sigma Chemical Co. Butyrylcholinesterase[13] was obtained as a stable lyophilized powder from Worthington Biochemical Corp. The inhibitors used in this study (Table I) were prepared by one of us (W. R. S.) and their synthesis and properties have been reported.[10]

Enzymatic rate measurements were carried out manometrically at 37° in isolated PChE using a procedure described previously[9] and potentiometrically in isolated BuChE using a Radiometer automatic titrator (Type TTTlc) equipped with a recorder (SBR2c) and syringe buret (SBUla). Reactions were conducted at pH 7.40 ± 0.05 in a 25-ml jacketed vessel at 26.0 ± 0.05° under N_2[14] using calomel and glass electrodes, mechanical stirring, and standard alkali (0.01 N NaOH) as the titrant.[15] The titration procedure used here is a modification of the method developed by Stein and Laidler[16] in their studies on the kinetics of α-chymotrypsin.

Our reaction mixtures were 0.04 M in $MgCl_2$, 0.01 M in NaCl, and contained substrate, inhibitor, and approximately 0.1 mg (about 0.5 unit) of enzyme. At least seven substrate concentration ranging from 7.7×10^{-3} to 1.1×10^{-3} M were used. Two or more inhibitor concentrations were used and final K_i values represent an average of no less than two independent determinations. A stock enzyme solution (2 mg of BuChE/ml) was prepared in 0.15 M NaCl; it was stored in the refrigerator when not in use and kept at 0° in an ice bucket during rate measurements. No change in catalytic activity could be detected when the stock enzyme solution was stored for periods up to 2 weeks under these conditions.

(13) The specific activity of this preparation was found to be approximately 6 units/mg, where activity is expressed as μmole of ACh hydrolyzed/min per mg of protein at 26° and pH 7.4.

(14) Prepurified grade nitrogen (The Matheson Co.), bubbled sequentially through 0.02 N KOH, concentrated H_2SO_4, and distilled water, was passed continuously over the surface of the reaction mixture.

(15) K. Jorgensen, *Scand. J. Clin. Lab. Invest.*, **11**, 282 (1959).

(16) B. R. Stein and K. J. Laidler, *Can. J. Chem.*, **37**, 1272 (1959).

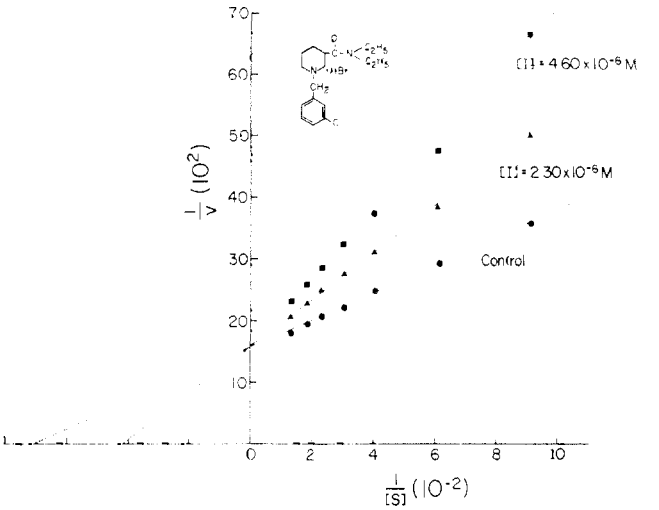


Figure 1.—Competitive inhibition of horse serum cholinesterase by two concentrations of N¹-(*m*-chlorobenzyl)-3-(N,N-diethylcarbamoyl)piperidine hydrobromide (VII). Reactions were initiated by the addition of 0.1 mg of enzyme. Abscissa, reciprocal molar acetylcholine chloride concentration; ordinate, reciprocal enzyme initial velocity which is expressed in μmoles/min per mg of protein.

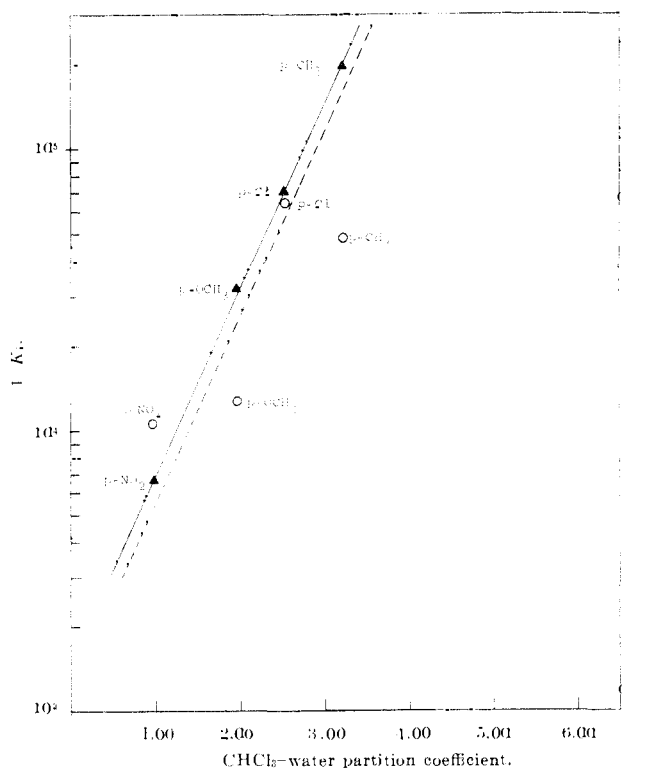


Figure 2.—The relationship of the logarithm of the reciprocals of the inhibitor dissociation constants to the $CHCl_3$–water partition coefficients (from ref 10) of *para*-substituted N¹-benzyl-3-(N,N-diethylcarbamoyl)piperidine hydrobromides: O– – –O, BuChE; ▲———▲, PChE.

Reaction mixtures (less substrate) were preincubated[17] for 20 min at room temperature prior to initiation of the hydrolytic reaction by adding substrate. Rates were determined from a plot of volume of titrant added *vs.* time and directly reflect the catalytic activity of the enzyme. Autohydrolysis of the substrate under the reaction conditions was insignificant during the period required for rate determinations. Initial rates were recorded

(17) J. H. Fellman and T. S. Fujita, *Biochim. Biophys. Acta*, **89**, 360 (1964).

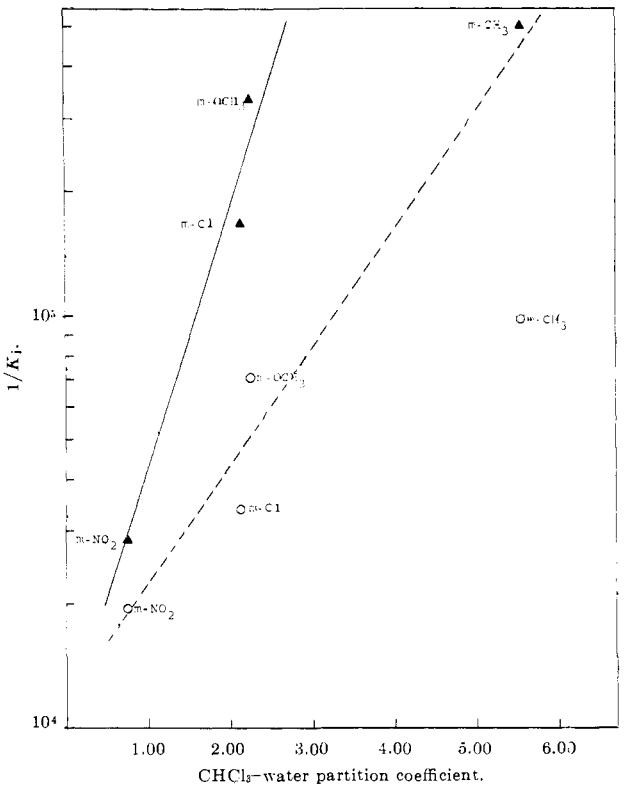


Figure 3.—The relationship of the logarithm of the reciprocals of the inhibitor dissociation constants to the $CHCl_3$–water partition coefficients (from ref 10) of *meta*-substituted N^1-benzyl-3-(N,N-diethylcarbamoyl)piperidine hydrobromides: O– – –O, BuChE; ▲——▲, PChE.

for a period of not less than 5 min after initial pH adjustment and were linear for at least 10 min or longer in all instances. Identical procedures were used for control and inhibited reactions.

Results and Discussion

The cholinesterase inhibitory potencies of the subject derivatives shown in Table I were obtained from Lineweaver–Burk[18] plots and the inhibitor dissociation constants (K_i) were calculated according to Dixon and Webb[19] from eq 1 where I is molarity of inhibitor,

$$K_i = I/(K_p/K_m - 1) \quad (1)$$

K_p is the effective Michaelis constant in the presence of inhibitor at concentration I, and K_m is the Michaelis constant. K_i values were reproducible within 10%. I_{50} values were determined as described previously.[9] A typical plot used in evaluating K_i for one of our inhibitors is shown in Figure 1. Inhibition was competitive with substrate for all compounds evaluated.

Similar determinations conducted with PChE at 37° for representative members of our inhibitor series also showed them to be competitive inhibitors (Table II) in this system.

The resemblance of our inhibitors to ACh and the competitive nature of the inhibition are consistent with the assumption that the amide function binds at the esteratic site and the nitrogen of the piperidine ring at the anionic site.

(18) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

(19) M. Dixon and E. C. Webb, "Enzymes," Longmans, Green and Co., Ltd., London, 1964, p 328.

The most potent and least potent inhibitors for both enzyme preparations were N^1-(*m*-methylbenzyl)-3-(N,N-diethylcarbamoyl)piperidine hydrobromide (V) and N^1-(*p*-nitrobenzyl)-3-(N,N-diethylcarbamoyl)piperidine hydrobromide (VIII), respectively. However, the order of inhibitory potencies for the remaining subject molecules was not identical for both BuChE and PChE.[20]

In both systems the *meta*-substituted benzyl derivatives are always more powerful inhibitors than their *para* analogs. Similarly, the K_a' values[10] for the *meta*-substituted inhibitors are larger than for the corresponding *para* compounds. Quintana and Smithfield[10] have demonstrated a linear relationship between pK_a' and Hammett σ constants for this series of derivatives and, in addition, have determined their apparent partition coefficients. Comparison of ChE inhibition with pK_a' values for this series suggests that inhibitor–enzyme interactions are more complex than those previously observed for our N^1-alkylpiperidinecarboxamide derivatives.[11]

The π values for these substituents as determined by Fujita, *et al.*[22] (Table I), from partition coefficients (octanol–water) of substituted nitrobenzenes are larger for the *meta* function in each instance. Plots of $1/K_i$ *vs.* partition coefficient (Figures 2 and 3) for both *meta* and *para* series show a general but not strictly linear relationship, again indicating a complex system. The most potent inhibitor, V, is less potent than might be anticipated from the partition coefficient data.

All derivatives containing *ortho,para*-directing substituents (III–VII), with the exception of the *p*-methoxy compound, II,[23] are better ChE inhibitors than the unsubstituted analog, I, while those containing *meta*-directing substituents are poorer.

The facts presented implicate the involvement of an electronic factor[11] in ChE–inhibitor interactions for the compounds studied. They further indicate that differences in PChE and BuChE may be due to differences in inhibitor binding at or near the anionic site of the enzyme.

Acknowledgment.—The authors wish to thank Dr. W. P. Purcell and Miss J. A. Singer for their helpful comments, Mrs. S. M. Litchford and Mrs. M. O. Clark for technical assistance, and the National Science Foundation and Marion Laboratories, Inc., of Kansas City, Mo., for support of the research.

(20) Although there is some evidence[21] that a low concentration of a second active enzyme component may be present in PChE, at the concentrations of enzyme, substrate, and inhibitor used in our experiments, the measured reaction rates followed classical Michaelis–Menten kinetics. Thus, it appears that the observed differences in specificity reflect differences in binding at or near the anionic site and not the contribution of a second active component.

(21) (a) I. B. Wilson, *J. Biol. Chem.*, **208**, 123 (1954); (b) W. K. Berry, *Biochim. Biophys. Acta*, **39**, 348 (1960); (c) J. G. Beasley and S. T. Christian, unpublished data.

(22) T. Fujita, J. Iwasa, and C. Hansch, *J. Am. Chem. Soc.*, **86**, 5175 (1964).

(23) Hammett substituent constants[24] for the benzyl substituent R groups might lead one to suspect that the *p*-methoxy compound II might behave in an anomalous manner.

(24) CH_3 (σ_m, −0.069; σ_p, −0.170), OCH_3 (σ_m, 0.115; σ_p, −0.268), Cl (σ_m, 0.373; σ_p, 0.227), NO_2 (σ_m, 0.710; σ_p, 0.778).[25]

(25) D. H. McDaniel and H. C. Brown, *J. Org. Chem.*, **23**, 420 (1958).