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EVALUATION OF KINETIC CONSTANTS FOR MIXED INHIBITORS OF CHOLINESTERASE*

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Abstract—A quantitative method has been developed to determine the kinetic constants for inhibitors showing mixed inhibition, i.e. which can bind to both free enzyme and an enzyme substrate complex. Both K_I, the competitive inhibition constant, and $K_I' = \alpha K_I$, the noncompetitive inhibition constant, can be evaluated, provided the two do not differ by much more than tenfold. A wide variety of amines, many of which are pharmacologically active, are shown to be mixed inhibitors of bovine erythrocyte cholinesterase, and their kinetic constants have been evaluated. For a series of benzomorphans no adequate correlation could be made between inhibition behavior and either structure or pharmacological activity. For two compounds, trimethylamine and psilocin, it was observed that $K_I' < K_I$. This result, which demonstrates that inhibitor is bound more tightly to an enzyme–substrate complex than to free enzyme, is used as evidence for the "induced fit" theory of enzyme structure.

THE RECOGNITION of the physiological significance of acetylcholine has resulted in the evaluation of thousands of synthetic compounds as inhibitors of acetylcholinesterase[1] and the elucidation of the mechanism of action of the enzyme.[2] The latter studies have revealed that the enzyme most probably follows a multistep path involving an initial enzyme substrate complex, and another complex which may be an acyl enzyme intermediate, followed by formation of product and regeneration of active enzyme. Consistent with this picture, several inhibitors have been shown to act in a "mixed" manner.[3, 4] Experimentally this means that they affect the observed Michaelis constant and also modify the specific rate of the enzyme-catalyzed reaction. This behavior has been interpreted theoretically as reflecting the ability of the inhibitor to interfere not only with the free enzyme but also with one of the subsequent intermediates.[5, 6]

The recognition of complex inhibition patterns is significant for pharmacological investigations. Barlow[7] has recently emphasized the relationship between studies of enzyme–inhibitor interaction and drug action. However, he considers only the limit situations of competitive and noncompetitive interaction. A more detailed discussion by Ariens and his co-workers[8] has related types of drug action to various limit cases of enzyme–agent interaction. The interaction of small molecules with receptors may not always be interpretable in terms of simple, limit situations. For the specific case

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of cholinesterase, "mixed" inhibition has been reported for pharmacologically significant compounds.[8] A recent molecular theory of cholinesterase action[9] makes use of the observed complex inhibition.

Although the evaluation of precise binding constants for an enzyme–inhibitor system that exhibits mixed inhibition presents no serious theoretical difficulties, it requires a large amount of data and an efficient and reproducible system. These requirements have seldom been met. We have developed such a system and have evaluated inhibition constants for a variety of compounds. This information may increase our knowledge about the active surface of acetylcholinesterase.

MATERIALS AND METHODS

Acetylcholine bromide (Eastman) was recrystallized from ethanol, dried in a vacuum oven at 75°, and stored in a desiccator over phosphorus pentoxide. Bovine acetylcholinesterase was obtained from Special Chemicals Division, Winthrop Laboratories. The enzyme and enzyme solutions were stored frozen when not in use. Inhibitors either were commercial products of highest purity or were supplied by Dr. Louis Harris and Dr. Frank Rosenberg, Sterling-Winthrop Research Laboratories.

Kinetic studies were carried out with a Radiometer pH-stat and recorder. Solutions were prepared in doubly distilled, carbon dioxide-free water. A stream of nitrogen was passed over the thermostated reaction cell. All inhibition studies were carried out at 25·0°, pH 7·0, in 0·10 M potassium chloride. Any given inhibitor was evaluated at three substrate concentrations, in the range between $1{\cdot}00 \times 10^{-4}$ and $9{\cdot}00 \times 10^{-4}$ M. The uninhibited rate was determined daily as the average of five determinations. K_m values for acetylcholine were determined as the average of more than twenty Lineweaver-Burk plots from "blank" runs performed over several weeks. The final value, $K_m = 1{\cdot}500 \pm 0{\cdot}003 \times 10^{-4}$, is in good agreement with other reports in the literature.[10]

Base and enzyme concentrations were adjusted so that initial reaction velocities could be conveniently measured for each run. Recorder traces were linear for at least the first 4 min of reaction. Kinetic data were obtained under conditions in which inhibitor and enzyme were not preincubated for more than a few minutes. Control experiments with the benzomorphans reported here demonstrated that incubation of inhibitor and enzyme for periods up to 100 min resulted in no change in observed inhibition.

Data analysis

Since recorder traces obtained under the reaction conditions were uniformly linear, slopes were determined from them by hand. The data were plotted as 1/rate vs. $[I]$ to give three straight lines for each inhibitor. The point of intersection of these lines can be used to evaluate the desired constants. Mixed inhibition can be described[11] in terms of two constants, $K_I = [E][I]/[EI]$ and $K_I' = [ES][I]/[ESI]$. The quantities in brackets refer to the concentrations of free enzyme, $[E]$; free inhibitor, $[I]$; enzyme inhibitor complex, $[EI]$; enzyme substrate complex, $[ES]$; and enzyme–substrate–inhibitor complex, $[ESI]$. The first constant, K_I, is the binding constant for inhibitor with free enzyme. It is the competitive inhibition constant, equal to the negative value of the abscissa coordinate, X_X, of the intersection point.[12] The ordinate of the intersection

point, Y_X, is related to the intersection of each of the lines on the ordinate axis, Y_0, by equation (1).

$$K_I'/K_I = \alpha = 1/1 - (1 + K_S/[S]_t)(Y_X/Y_0). \quad (1)$$

The two coordinates of the intersection point, (X_X, Y_X), together with known values of K_S and substrate concentration, $[S]_t$, for each line, provide all the necessary data for calculation of both inhibitor constants.

A typical graph is presented in Fig. 1. In principle, data obtained at two substrate concentrations would be sufficient. However, the use of three concentrations proved much more accurate. The data for each inhibitor, which usually consisted of a total

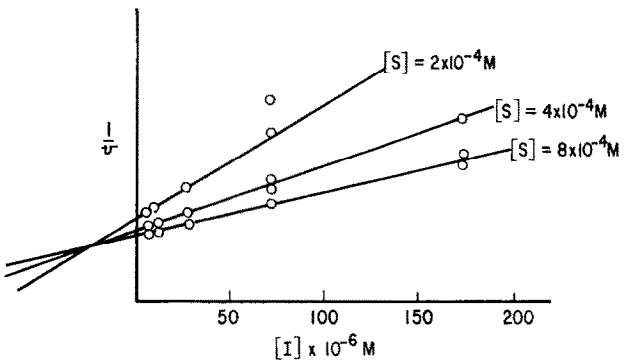


FIG. 1. Plot of reciprocal rate vs. inhibitor concentration for a typical benzomorphan that exhibits mixed inhibition. The intersection points of the three lines and of each line with the Y axis are used to calculate K_I and α, as indicated in the text.

of 25–30 points, were analyzed by a computer program which evaluated, by a reiterative least squares method, the best three lines that would pass through a common point. The results are reported in terms of K_I and α. The errors reported are maximum and minimum limits of equation (1) when the experimental error in K_0 plus the computed errors in Y_0 and Y_X are introduced.

RESULTS

Table 1 lists competitive inhibition constants and α-values for several cholinesterase inhibitors, including compounds that exhibit central nervous system activity. Large values of α indicate that there is no measurable binding of inhibitor to any *ES* complex; that the inhibitor acts competitively. Noncompetitive inhibition would be indicated by $\alpha = 1$; that is, $K_I = K_I'$. Intermediate values of α represent mixed inhibition. The observation of $\alpha < 1$ demonstrates that the inhibitor is bound *more tightly* to an *ES* complex than to free enzyme. This observation is discussed more fully below.

For comparison purposes "K_I" values for some common inhibitors of cholinesterase are also listed in Table 1. These numbers are not strictly comparable, because they were obtained under different experimental conditions and, more important, the carbamate inhibitors neostigmine and eserine inhibit cholinesterase by a different mechanism.[13]

Table 2 compares a few of the present results with literature data. The values reported by Foldes *et al.*[14] and Young *et al.*[15] are essentially in agreement with the present

results, when one takes into account the different methods of expressing inhibition. However, the present study indicates that whatever the relative magnitudes of inhibition of cholinesterase by these three compounds, only levallorphan shows significant noncompetitive inhibition. It acts *qualitatively* differently from morphine and nalorphine. This type of information may permit a new empirical correlation of

TABLE 1. COMPETITIVE INHIBITION CONSTANTS AND NONCOMPETITIVE COMPONENT FOR INHIBITORS OF BOVINE ERYTHROCYTE CHOLINESTERASE*

Compound	$K_I \times 10^6 M$†	$\alpha_{average}$‡	α_1	α_2	α_3
Phenol	6820 ± 3720	large			
Trimethylamine	4130	0·87	0·87 ± 0·06	0·88 ± 0·08	0·87 ± 0·12
Tetramethylammonium iodide	1280 ± 90	large			
β-Phenylethylamine	1160 ± 70	5·3			
Morphine	53·7 ± 5·9	large			
Psilocin	46·3 ± 3·5	0·70	0·70 ± 0·04	0·70 ± 0·04	0·69 ± 0·03
Nalorphine	40·5 ± 4·0	large			
p-Hydroxyphenyl-trimethylammonium iodide	32·3 ± 2·5	large			
LSD	16·6 ± 2·7	6·5	5·2 ± 5·0	6·0 ± 50	8·2
Phenazocine	10·7 ± 0·56	2·3	2·2 ± 0·1	2·4 ± 0·1	2·2 ± 0·1
2-Br LSD	7·12 ± 0·65	19	12·0 ± 74	16 ± 145	27
Levallorphan	2·21 ± 0·19	5·5	5·0 ± 0·3	5·9 ± 0·7	5·7 ± 0·5
Tensilon	0·33§				
Eserine	0·061¶				
Neostigmine	0·16¶; 0·028‖				

* Determined at 25·0 ± 0·1°; pH = 7·00 ± 0·05, 0·1 M KCl with acetylcholine concentrations ranging from 1·00–9·00 × 10^{-4} M.

† Competitive inhibition constant; see text for method of evaluation.

‡ Ratio of K'_I/K_I, where K'_I = noncompetitive inhibition constant.

See text for method of evaluation. Errors in α-values increase with the magnitude of α, and range from approximately 2 to 20 per cent.

§ Ref. 21.

¶ Ref. 22.

‖ Ref. 23.

TABLE 2. INHIBITION OF CHOLINESTERASE BY SELECTED MORPHINE DERIVATIVES

Compound	This study		Foldes[14]	Young[15]
	$K_I \times 10^5$	$\alpha_{average}$	$I_{50} \times 10^5$	
Morphine	5·37 ± 0·59	large	98	27% at 10^{-3} M
Nalorphine	4·5 ± 0·40	large	98	36% at 10^{-3} M
Levallorphan	0·221 ± 0·019	5·5	4·0	76% at 10^{-3} M

pharmacological measurement with chemical structure. In order to establish such a structure–activity correlation, it is necessary to examine a large number of compounds of a single type. We included in this study a selection of benzomorphans, with the results summarized in Table 3. These results permit two types of correlation between structure and enzyme inhibition: the effect of structure on the *magnitude* of the inhibition and the effect of structure on the *nature* of the inhibition.

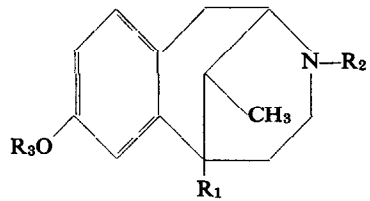
A free hydroxyl group in the benzomorphan appears to be necessary for effective inhibition. Etherification raises K_I approximately tenfold. Since the pentacyclic compounds morphine and nalorphine also have relatively high K_I values, effective binding

TABLE 3. CHOLINESTERASE INHIBITION CONSTANTS FOR BENZOMORPHANS*

Compound†							
R_1	R_2	R_3	$K_I \times 10^6$M‡	$\alpha_{average}$‡	α_1	α_2	α_3
CH_3	i-C_5H_{11}	CH_3	84·0 ± 3·6	9·8	8·8 ± 1·2	9·1 ± 1·1	11·6 ± 2·3
CH_3	n-C_3H_7	$CH_2CH{=}CH_2$	79·3 ± 5·1	19	15·6 ± 4·0	21·3 ± 12·0	19·6 ± 9·8
C_2H_5	$CH_2CH{=}C(CH_3)_2$	H	10·7 ± 2·9	3·6	3·2 ± 0·1	4·0 ± 0·2	3·7 ± 0·1
CH_3	$CH_2CH_2C_6H_5$	H	10·7 ±	2·3	2·2 ± 0·1	2·4 ± 0·1	2·2 ± 0·1
C_2H_5	$CH_2CH_2CH_3$	H	9·56 ± 0·51	3·4	3·4 ± 0·1	3·4 ± 0·1	3·3 ± 0·2
C_2H_5	$CH_2CH{=}CH_2$	H	7·61 ± 1·55	5·3	4·5 ± 0·2	6·6 ± 0·7	4·8 ± 0·4
CH_3	$CH_2CH{=}C(CH_3)_2$	H	5·87 ± 0·80	13	7·4 ± 0·7	12·3 ± 2·4	20·0 ± 9·0
CH_3	$CH_2CH(CH_2)_2$	H	4·89 ± 0·18	13	10·4 ± 7·1	11·1 ± 7·6	18·1 ± 30·1
CH_3	$CH_2C{\equiv}CH$	H	4·07 ± 0·99	1·74	1·76 ± 0·03	1·79 ± 0·04	1·75 ± 0·04
CH_3	$CH_2CH{=}CHCl$	H	3·74 ± 0·27	6·0	5·8 ± 0·4	5·5 ± 0·5	6·9 ± 1·0
CH_3	$CH_2CH{=}CH_2$	H	2·59	large	14·1 ± 2·3	37·0 ± 25	
C_2H_5	$CH_2C{\equiv}CH$	H	1·65 ± 0·20	large			
CH_3	$CH_2CH_2CH_3$	H	0·93 ± 0·31	4·5		4·76	4·55

* Determined at 25·0 ± 0·1°, pH = 7·00 ± 0·05, 0·1 M KCl with acetylcholine concentrations ranging from 1·00–9·00 × 10^{-4} M.

† R_1, R_2, and R_3 refer to substituents on the general formula



‡ See Table 1 for definitions.

to the cholinesterase surface apparently requires access to an -OH and to an adjacent unobstructed hydrocarbon residue. The significance of the nitrogen substituents on the tricyclic benzomorphan skeleton is emphasized by the variation in K_I values with changes in the structure of these groups. There is no apparent correlation of K_I with the size, electronic properties, stereochemical arrangements of these groups, or with the reported[16] analgesic or psychotomimetic properties of the benzomorphans.

The present results indicate that the interaction of many inhibitors with cholinesterase is complex. The drastic changes in both the magnitude and nature of the inhibition with small changes in structure can be taken as suggestive evidence that the inhibitor modifies the structure of the enzyme. It was this type of evidence that prompted Koshland[17] to propose the concept of "induced fit"; that enzymes are endowed with a high degree of conformational adaptability toward substrates and inhibitors. This concept has been applied to molecular theories of drug action[7, 18, 19] with particular reference to cholinesterase.[7, 19] Most of the evidence favoring the view that enzyme–substrate or enzyme–inhibitor complexes represent a modified structure in comparison to free enzyme is indirect, although the arguments presented for the case of cholinesterase[19, 20] are appealing.

We believe that our observation of α-values less than one for trimethylamine and psilocin have a direct bearing on this point. Any picture involving a rigid enzyme which is not perturbed by substrate can account for a range of inhibition from strictly non-competitive through completely competitive. One molecule of substrate on the enzyme surface might offer no resistance to the binding of a molecule of inhibitor ($\alpha = 1$, $K_I = K_I'$); it might prevent such binding completely ($\alpha = \infty$; $K_I' = \infty$) or any case in between. However, the observation that inhibitor is bound more tightly to an *ES* complex than to free enzyme demonstrates that *the interaction of enzyme, substrate, and inhibitor cannot be interpreted by means of a model which considers only the relative disposition of the species.* In this case, the addition of substrate to inhibitor makes a *positive* contribution to the further binding capacity of the enzyme. The exact nature of this contribution will have to be determined through future studies.

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