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NONCOMPETITIVE INHIBITION OF ACETYLCHOLINESTERASE BY ESERINE

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Abstract—The inhibition of acetylcholinesterase by eserine was shown to be noncompetitive if the enzyme and inhibitor were allowed to preincubate for as short a time as 6 min, in the absence of substrate; when substrate and inhibitor are added simultaneously the inhibition is competitive.

K_i values of $3{\cdot}1 \times 10^{-6}$, $1{\cdot}9 \times 10^{-7}$ and $1{\cdot}0 \times 10^{-7}$ M were obtained at preincubation times of zero, 6·8 and 18·9 min respectively. The data are compatible with the concept that a reversible inhibitor–enzyme complex is initially formed and that carbamylation of the enzyme then proceeds slowly as the preincubation time is increased.

AN EXAMINATION of the literature concerned with the inhibition of acetylcholinesterase (AChE, EC 3.1.1.7) by eserine (physostigmine) reveals that some conflicting concepts exist with respect to the inhibitory mechanism. While some authors have alluded to the noncompetitive nature of the inhibition,[1, 2] it is generally accepted that the inhibition is of the simple competitive type,[3] and it is so stated in many modern biochemistry and pharmacology textbooks.

The currently accepted mechanism for the hydrolytic action of acetylcholinesterase, as outlined by Wilson *et al.*,[4] postulates an initial attraction of the substrate to protein by electrostatic forces and the formation of an acylated enzyme prior to the final hydrolytic step. In keeping with the concept that eserine is a competitive inhibitor, it could be theorized that a protonated nitrogen group on the eserine molecule competes with acetylcholine for the anionic site, the binding to which is presumably a necessary step prior to the hydrolytic reaction.

The kinetics of the reaction are not simple, however, since it has been reported that eserine becomes a better inhibitor if it is allowed to preincubate with the enzyme in the absence of substrate, i.e. K_i apparently decreases as a function of preincubation time.[3, 5] This observation is not in keeping with the concept of simple competitive inhibition, but is indicative rather of a slow reaction which in some way causes a decisive change in the enzyme.

This report is concerned with the inhibitory effects of different eserine levels on erythrocytic AChE at each of three enzyme–eserine preincubation times; acetylcholine iodide (AChI) concentrations ranging from $2{\cdot}28 \times 10^{-4}$ to $4{\cdot}57 \times 10^{-3}$ M were employed. The data reveal that there is indeed a noncompetitive contribution to the inhibition of AChE by eserine.

EXPERIMENTAL

An automated system, based upon the principle of continuous flow dialysis in a closed system, was used to obtain the pertinent data.[6] In this investigation, however,

acetic acid concentrations as determined from a standard curve, rather than absorbance values, were used for the calculation of inhibition constants; the enzyme-substrate incubation time was decreased from 4 to 2 min.

The enzymic reaction buffer media contained 0·30 M NaCl, 00·10 M $MgCl_2$ and 0·0015 M KH_2PO_4, adjusted to pH 8·2 with HCl. Erythrocytic AChE (Nutritional Biochemicals Corp., Cleveland, Ohio) and AChI (Eastman Organic Chemicals, Rochester, N.Y.) solutions were prepared in the buffer. The stock enzyme concentration was 1 mg per ml, equivalent to 32 units per ml; the stock substrate concentrations varied from $6{\cdot}94 \times 10^{-4}$ to $1{\cdot}84 \times 10^{-2}$ M.

The indicator solution contained 65 mg of phenol-red sodium salt (Allied Chemical Corp., New York, N.Y.) per liter of buffer. The indicator diluent in the analytical system contained 0·30 M NaCl and 0·5 ml per liter of a 10% Brij 35 (Atlas Chemical Industries, Wilmington, Del.) surfactant solution, adjusted to pH 8·2 with NaOH.

The reagent solutions were protected from atmospheric CO_2 by storing and aspirating them from bottles fitted with Ascarite tubes. All of the reactions were carried out at 23·5°. The individual solutions were maintained at this temperature with the exception of enzyme and substrate which were kept in an ice bath.

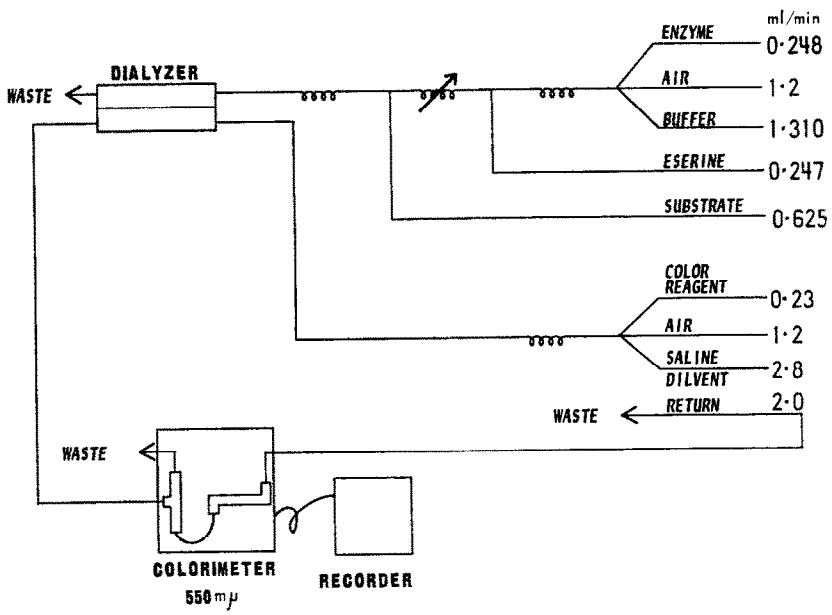

FIG. 1. AutoAnalyzer flow diagram for inhibition studies of acetylcholinesterase with eserine.

The flow system utilized is shown in Fig. 1. Operationally, a series of eserine solutions were aspirated from the sampler turntable programmed at 40 per hr, and added to the enzyme which had been diluted with buffer. Then, depending upon the desired preincubation time, a solution containing a given substrate concentration was added immediately, or 6·8 or 18·9 min later. After a 2-min incubation period the stream was fed into a dialyzer where the acetic acid formed in the hydrolytic reaction was picked up in a phenol-red indicator stream and ultimately analyzed colorimetrically at 550 mμ.

The eserine, enzyme and substrate solutions were diluted approximately $\frac{1}{10}$, $\frac{1}{10}$ and $\frac{1}{4}$, respectively, in the flow system.[6] Thus, the enzyme concentration in the incubation medium was 3·2 units/ml, an activity equivalent to the hydrolysis of 23 μg ACh per minute at pH 7·4 at 37·5°.

When data were obtained at one substrate level, the substrate line was placed into a vessel containing the next concentration. After a 10-min period necessary to establish a baseline, the eserine solutions were resampled. This procedure was repeated until data were obtained at all of the desired AChI levels.

Duplicate eserine samples were taken at every concentration. The standard deviation of the duplicates was 0·005 absorbance units.

RESULTS AND DISCUSSION

The results obtained at zero eserine–enzyme preincubation time are shown in Table 1. As can be seen at each of the eserine concentrations, the per cent inhibition decreases as substrate concentration increases, as would be expected for simple, competitive inhibition. When the data are plotted as the quotient of 1 minus the fractional inhibition and the fractional inhibition, multiplied by the eserine concentration, versus substrate concentration, $(1 - i)/i\,[I]$ vs. $[S]$, a curve is obtained which is consistent for competitive inhibition of a system exhibiting substrate inhibition.[7] A K_i value of $3{\cdot}1 \times 10^{-6}$ M is obtained from the intercept on the ordinate.

TABLE 1. INHIBITION AT ZERO PREINCUBATION TIME

	Eserine concn, M × 10^6						
	0	1·60		2·40		4·80	
AChI M × 10^3	V*	V	% inhibition	V	% inhibition	V	% inhibition
0·228	0·598	0·393	34·3	0·341	43·0	0·233	61·0
0·457	0·924	0·635	31·3	0·524	43·3	0·360	60·4
0·914	1·153	0·894	22·5	0·773	33·0	0·555	51·9
1·827	1·287	1·086	15·6	0·996	22·6	0·829	35·6
3·197	1·258	1·107	12·0	1·038	17·5	0·943	25·0
4·568	1·147			1·029	10·3	0·926	19·3

* Expressed as molarity of acetic acid standard × 10^3; no correction was made for dilution in the incubation system, which was approximately 1:4.

The results obtained at 6·8 min eserine–enzyme preincubation time are shown in Table 2. These data reveal that even at this short time the kinetics of inhibition have changed. It is apparent that the per cent inhibition does not decrease at a given eserine level as substrate concentration increases; within experimental error, the per cent inhibition is essentially independent of substrate concentration. This condition is typical for noncompetitive inhibition. When the data are plotted as indicated previously, a K_i value of $1{\cdot}9 \times 10^{-7}$ M is yielded, approximately $\frac{1}{15}$ of that obtained at zero preincubation.

Table 3 lists the results obtained at 18·9 min preincubation times. Again, it is obvious that increasing concentrations of substrate do not protect the enzyme from

TABLE 2. INHIBITION AT 6·8 min PREINCUBATION TIME

	Eserine concn, M × 10^7						
	0	0·70		1·40		2·33	
AChI M × 10^3	V*	V	% inhibition	V	% inhibition	V	% inhibition
0·228	0·632	0·500	20·9	0·362	42·7	0·252	60·1
0·685	1·031	0·814	21·0	0·624	39·5	0·495	52·0
0·914	1·094	0·829	24·2	0·630	42·4	0·470	57·0
1·827	1·200	0·903	24·8	0·682	43·2	0·502	58·2
3·198	1·176	0·902	23·3	0·666	43·4	0·490	58·3
4·568	1·151	0·834	27 5	0·632	45·1	0·462	59·9

* Expressed as molarity of acetic acid standard × 10^3; no correction was made for dilution in the incubation system, which was approximately 1:4.

TABLE 3. INHIBITION AT 18·9 min PREINCUBATION TIME

	Eserine concn, M × 10^7						
	0	0·27		0·54		1·08	
AChI M × 10^3	V*	V	% inhibition	V	% inhibition	V	% inhibition
0·457	0·904	0·737	18·5	0·639	29·3	0·478	47·1
0·685	1·002	0·801	20·1	0·639	36·2	0·455	54·6
0·914	1·128	0·886	21·5	0·753	33·2	0·488	56·7
1·827	1·152	0·971	15·7	0·778	32·5	0·469	59·3
3·197	1·136	0·945	16·8	0·720	36·6	0·467	58·9
4·568	1·077	0·847	21·4	0·655	39·2	0·416	61·4

* Expressed as molarity of acetic acid standard × 10^3; no correction was made for dilution in the incubation system which was approximately 1:4.

inhibition at a given eserine level. There is actually a moderate increase in inhibition as the substrate concentration is increased, but the significance of the trend is questionable. A K_i value of $1{\cdot}0 \times 10^{-7}$ M was obtained from these data.

A graphical representation of the data presented in Tables 1–3, suitable for the determination of the K_i values cited, is shown in Fig. 2. The points are the average of values obtained with the different eserine levels at each of the substrate concentrations; the range of the values is indicated by the lines between the bars. It is obvious that the nature of the inhibition changes when the preincubation time is greater than zero. The bottom two straight lines are to be expected with noncompetitive inhibition, whereas the top diagonal curve is typical for competitive inhibition.[7]

These findings are in general agreement with the conclusion that carbamylation of the enzyme by the inhibitor takes place when the two are preincubated. This proposal, first made by Myers and Kemp[8, 9] has been postulated by Wilson *et al.* to occur via the following reaction scheme:[2, 10]

$$I + E \rightleftarrows E{\cdot}I \rightarrow E' \rightarrow E + \text{carbamic acid} \qquad (1)$$

where I and E are the carbamate inhibitor and enzyme, respectively, $E{\cdot}I$ is a reversible enzyme–inhibitor complex, and E' is the carbamylated enzyme. At zero time pre-

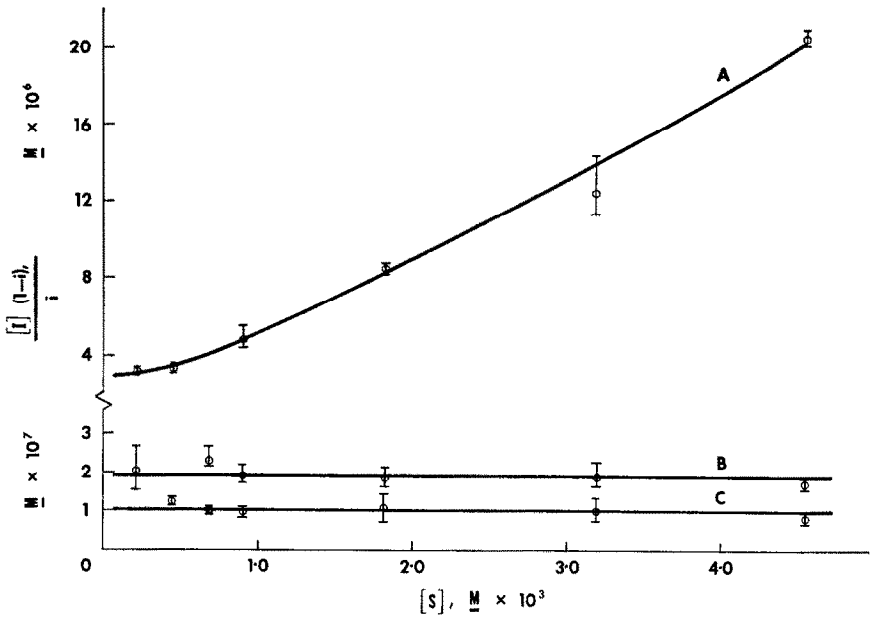

FIG. 2. Kinetics of inhibition of acetylcholinesterase by eserine at three different enzyme–inhibitor preincubation times. K_i is estimated from the intercept on the ordinate at $S = 0$. A, zero; B, 6·8 min; C, 18·9 min.

incubation the inhibition will be competitive, but at longer times carbamylation will occur and the inhibition will become noncompetitive; the apparent K_i value will decrease as carbamylation proceeds (preincubation time increases) until a steady state value is attained.

Additional evidence to support this overall concept has been published.[11–14] Main and Hastings[13] found K_i, the equilibrium affinity constant, for eserine and erythrocytic acetylcholinesterase to be $3{\cdot}33 \times 10^{-6}$ M; the competitive K_i of $3{\cdot}1 \times 10^{-6}$ M reported here is in good agreement with this value. The steady state noncompetitive K_i in the media employed in this work is close to 1×10^{-7} M.

It has once more been demonstrated that the mode of inhibition of AChE by eserine changes as the enzyme and inhibitor are incubated prior to the addition of substrate. Therefore, statements referring to the competitive nature of the inhibitor are only partially correct, and should be qualified so as to reflect a possible noncompetitive component.

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