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HUMAN TISSUE CHOLINESTERASES: RATES OF RECOVERY AFTER INHIBITION BY NEOSTIGMINE; MICHAELIS–MENTEN CONSTANTS*

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Abstract—The kinetics of inhibition of acetylcholinesterase by neostigmine is briefly reviewed. The acid-transferring mechanism of inhibition which is similar to that of diisopropylfluorophosphate and tetraethylpyrophosphate has recently been shown to be also appropriate for neostigmine. Both solutions and suspensions of cholinesterases were obtained from human brain, skeletal muscle, and red cells. Using *in vitro* techniques, it was demonstrated that the half-time for the recovery of catalytic ability after inhibition by neostigmine was similar in both the suspensions and the solutions for the three tissues and averaged 34 min.

The Michaelis-Menten constants for the tissue acetylcholinesterases were also shown to have similar values, about 2×10^{-4} M. These constants had not previously been reported for human brain or skeletal muscle enzymes. Both the recovery times and the Michaelis-Menten constants are of the same order as reported by Wilson for eel acetylcholinesterase.

These studies indicate the kinetic similarity of human and eel acetylcholinesterase with respect to carbamate inhibition. The relationship of these investigations to clinical observations of patients treated with neostigmine is discussed.

NEOSTIGMINE, a potent anticholinesterase compound, is principally used in the treatment of myasthenia gravis.[1–3] It is also therapeutically effective as an antagonist to curare types of myoneural blockade, as a stimulant to peristalsis and micturition in patients with adynamic ileus or atonic bladder, and as a depressant of cardiac rate in certain arrythmias.[4,5] The time interval between intravenous injection and clinically discernible onset of action has been reported as 1 to 2 min.[6,7] The duration of inhibition produced by 15 mg taken orally or 0·5 mg given intravenously is variable, but has been reported from 1 to 12 hr and averaging 3 to 6 hr.[7–11] These data are based mainly on observations in patients with myasthenia gravis. The cholinesterase activity in this disease is reported to be at normal levels.[12]

Determinations *in vitro* of cholinesterase inhibition prior to 1960 were mostly based on the assumption that physostigmine and neostigmine function by forming a reversible complex with the enzyme at its active site.[13] The duration of inhibition is, therefore, directly dependent on the concentration of the inhibitor and will persist as long as the concentration remains at appropriate levels. Values reported for 50 per cent inhibition range from $2{\cdot}3 \times 10^{-7}$ M[14] to $3{\cdot}5 \times 10^{-8}$ M[15] for acetylcholinesterase

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[acetylcholine acetyl-hydrolase (3.1.1.7)[16] derived from human red cells, 3×10^{-6} M[17] for electric eel acetylcholinesterase (3.1.1.7), and $6{\cdot}9 \times 10^{-7}$ M[18] to $5{\cdot}6 \times 10^{-8}$ M[14] for human plasma cholinesterase [acylcholine acyl-hydrolase (3.1.1.8)][16].

The meaning of these values may be questioned, however, since Wilson has recently demonstrated that the mechanism of inhibition is similar to that produced by the organophosphates, e.g. DFP (diisopropylfluorophosphate), TEPP (tetraethylpyrophosphate).[19,20] This involves the transfer of the acidic portion of the inhibitor molecule to the enzymes' active site in the formation of a stable intermediate compound with the enzyme. This new compound hydrolyzes slowly, allowing regeneration of the enzymes' catalytic abilities. This mechanism is quite different from the simple adsorption complex originally postulated and on which the previous work was based.

Using *in-vitro* methods and acetylcholinesterase partially purified from the electric organ of the electric eel, *Electrophorus electricus*, Wilson allowed the enzyme inhibited with neostigmine to recover its hydrolytic properties. The half-time of recovery was reported as about 30 min.[20] It is difficult to compare this figure, obtained under well-controlled conditions *in vitro*, to the 3 to 6 hr duration of action estimated in the human being. The possibility existed that the recovery rates of neostigmine-inhibited human cholinesterases are prolonged, indicating a difference in enzyme characteristics.

The accurate determination of the Michaelis-Menten constant for acetylcholinesterase extracted from various human tissues would also be of value. Apparently it has not been attempted except for red blood cells.

It seemed of interest, then, to determine (1) whether the cholinesterases in human tissues have similar Michaelis-Menten (K_m) constants, (2) whether human cholinesterases when inhibited by neostigmine recover activity at rates similar to those reported by Wilson for highly purified eel cholinesterase and (3) whether these rates were different for cholinesterases extracted from different human tissues.

MATERIALS AND METHODS

Tissue preparation

The human tissues used for study were skeletal muscle, brain, and red cells selected from patients who either were not ill or suffering from diseases not usually associated with an alternation of cholinesterase function. The concentration and solubilization techniques are outlined below.

Assay technique

All kinetic measurements were made at 37°, pH 7·4, in a Beckman type K titrator modified to deliver 0·2μl aliquots of 0·005 M to 0·02 M standardized base as required. Thorough mixing, constant temperature, and CO_2-free techniques were employed.

The reaction medium consisted of 99 ml of a solution of 0·1 M NaCl, 0·02 M $MgCl_2$ and 5×10^{-5} M EDTA, to which were added appropriate amounts of substrate and enzyme. All determinations were done in duplicate and in some cases triplicate.

For the determination of cholinesterase activity an amount of acetylcholine solution sufficient to make the final concentration 3×10^{-3} M was added to the reaction medium. An accurately measured amount of tissue extract containing enzymes was then added to start the reaction. The acetic acid produced during the hydrolysis of the

substrate by the enzyme was automatically titrated with appropriate base. The activity was calculated from the amount of base titrated per minute to maintain a constant pH.

Muscle. Portions of pectoralis major muscle were obtained at operations from three patients undergoing radical mastectomy for carcinoma. The muscle was immediately cut into small sections, washed in copious quantities of 0·9% NaCl until free of blood. Fifty g of the "squeezed-dry" muscle was homogenized with 200 ml of 5% ammonium sulfate for 2 min in a Waring blender. The crude homogenate was then subjected to 15 min processing in a tissue homogenizer followed by centrifugation at 30,000 × *g*, 10°, for 1 hr. The pellet was discarded, and solid ammonium sulfate was added to the supernatant until a 36% solution was obtained. It has been shown that the enzyme and other proteins are precipitated from solution at this concentration. The mixture was allowed to stand for 2 hr at 4° and subjected to high speed centrifugation for 1 hr. The supernatant was discarded and the pellet (about 10 ml) resuspended volume for volume with 5% ammonium sulfate, homogenized for 15 min in a tissue homogenizer, and then dialyzed against 6,000 ml of 0·1 M NaCl in 0·001 M phosphate buffer at pH 7·4, 10°, for 12 hr. The dialyzed material was then centrifuged for 15 min at 30,000 × *g*, 10°; both the supernatant and resuspended (v/v with fresh dialyzing solution) pellet were used for determinations.

The activity expressed as micromoles of substrate hydrolyzed per mililiter-minute was 0·15 for the supernatant and 0·72 for the pellet.

Red blood cells. Whole blood was centrifuged as above, the buffy coat discarded, and the packed cells treated by two different methods. To "solubilize" the enzyme, a portion of the cells was subjected to the butanol freeze-drying extraction technique described by Cohen and Warringa.[21] The final solution contained enzymes in 0·1 M NaCl and 0·001 M phosphate buffer at pH 7·4.

The remaining packed cells were lysed with glass-distilled water and centrifuged as before. The stromata were rewashed and centrifuged until relatively free of hemoglobin. The pellet was then suspended v/v with saline–buffer solution when ready for use.

The activity expressed as micromoles of substrate hydrolyzed per mililiter-minute was 5·05 for the supernatant and 8·96 for the pellet.

Brain. The caudate nucleus, as one of the brain's richest sources of acetylcholinesterase,[22] was obtained from three patients who died from diseases not usually affecting the central nervous system. The tissue, obtained from the neuropathologist several hours after death, was washed free of blood with 0·9% NaCl. Fifteen g of the tissue was homogenized with 60 ml of 0·1% NaCl in 0·001 M phosphate buffer, pH 7·4, in a Waring blender for 1 min, and in a tissue homogenizer for 15 min. It was then centrifuged at 30,000 × *g*, 10°, for 1 hr. Both the supernatant and pellet (resuspended v/v with saline–buffer solution) were used in the determinations.

The activity expressed as micromoles of substrate hydrolyzed per mililiter-minute was 0·89 for the supernatant and 12·1 for the pellet.

The amount of butyrylcholinesterase (plasma cholinesterase) present in the extract was obtained by substituting 2×10^{-3} M butyrylcholine (final concentration) for acetylcholine and the rate of hydrolysis assayed as described.

To determine the Michaelis-Menten constant an appropriate amount of tissue extract was added to the medium and the reaction started by the addition of substrate solution calculated to make a final concentration of 1×10^{-4} M. After 5 min, during

which a small amount of the substrate was hydrolyzed, another portion of acetylcholine solution was added and a new substrate concentration was reached. Five increments of substrate were added at 5 min intervals and the hydrolysis rate obtained for each substrate concentration. The optimum concentration of 3×10^{-3} M was not exceeded because of substantial substrate inhibition. The data were plotted in the double reciprocal manner of Lineweaver and Burk and the value for the K_m obtained (see Discussion).

To determine the rates at which inhibited enzymes spontaneously recover activity, appropriate amounts of tissue extract were incubated with varying concentrations ($1{\cdot}9 \times 10^{-7}$ M to $2{\cdot}5 \times 10^{-8}$ M) of neostigmine for 1 hr at 37°, pH 7·4. The inhibited enzyme was then allowed to recover its activity by extensive dilution (500 to 5,000 fold) in the reaction medium containing 3×10^{-3} M substrate. The log fractional inhibition was plotted as a function of time in accordance with the equations developed in the Discussion, and the time for 50 per cent recovery obtained. The rate of recovery was followed by automatic titration.

DISCUSSION

Kinetics

The chemistry of acetylcholine hydrolysis and anticholinesterase activity has recently been reviewed in detail.[23–25] Briefly, the kinetics of acetylcholinesterase action is best understood by postulating the formation of an intermediate compound, an acyl enzyme derivative, during substrate hydroysis. The mechanism may be simply diagramed:

$$1.\ E + S \rightleftharpoons E{\cdot}S \rightarrow E' + P_1$$
$$2.\ E' + HOH \rightarrow E + P_2$$

where E, S and E·S have their usual meanings, E′ is the acetyl enzyme intermediate and P_1, P_2 are the products of the reaction, choline and acetic acid.

While the group of prosthetic inhibitors (edrophonium, tetramethylammonium ion) act principally by forming a readily reversible complex with the enzyme,[24] the organophosphates (TEPP, DFP), the carbamates (neostigmine) and the methanesulfonates (methanesulfonyl fluoride) react with the enzyme by transferring the acid portion of the inhibitor molecule to the active site in the formation of an intermediate compound (E′).[26] These are the oxydiaphoric or acid-transferring inhibitors.[27] The intermediate compounds (E′) have varying degrees of stability measured in microseconds for the acetyl enzyme but lasting for minutes to months for the compounds derived from the inhibitors.[23]

$$3.\ E + I \underset{}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I$$
$$\qquad\qquad k_2 \downarrow k_3$$
$$\qquad k'_3 \searrow \downarrow$$
$$4.\qquad\qquad E' + P_1$$
$$\qquad\qquad\quad k^4$$
$$5.\ E' + HOH \longrightarrow E + P_2$$

The scheme proposed for simple prosthetic inhibition is given by equation 3. The reaction sequence for oxydiaphoric inhibition progresses from equations 3 through 5.

The mathematical consequences that may be developed from this inhibition scheme are formally the same as for substrate hydrolysis; oxydiaphoric inhibition merely proceeds at a much slower rate. The system may be simplified *in vitro* if the concentration of I, inhibitor, is much less than the K_i; under such circumstances recognition of E·I and k_3 is not necessary, and the step characterized by k'_3 may be substituted. If the concentration of I is kept much greater than the enzyme concentration, E, the concentration of I remains relatively constant during the reaction, and a steady state is reached where the rate of inhibition (k'_3) is equal to the rate of spontaneous hydrolysis of the carbamyl enzyme (k_4). This steady state is achieved in the 60 min incubation time allowed in these experiments. These relationships may be expressed as $(E'/E)ss = k'_3\ I/k_4$; [20] k_4/k'_3 is equal to the concentration of inhibitor which produces 50 per cent inhibition. This interpretation of I_{50} is quite different from that usually [14,27] used for the prosthetic inhibitors where $K_i = I_{50} \times K_m/K_m + S$ or $K_i = 0{\cdot}09 \times I_{50}$ under the ordinary conditions of measurement. Because these compounds are not prosthetic inhibitors, the I_{50} should not be interpreted in terms of a reversible binding constant.

The psuedo first-order rate constant for the reaction of the inhibited enzyme with water, $k_{4'}$, can be evaluated by greatly diluting the inhibited enzyme and following its rate of recovery. This may be expressed as $\ln E'/E'_0 = -k_4t$ [19] where E'_0 is the initial concentration of inhibited enzyme; E′ is the concentration indirectly measured (by determining free enzyme activity) as a function of time. The experimental data are plotted in accordance with this equation.

A value of $2{\cdot}7 \times 10^{-8}$ M for 50 per cent inhibition of eel cholinesterase in the steady state has recently been reported.[20] Thus neostigmine ranks as a very potent inhibitor and possesses a remarkable degree of molecular complementarity, i.e. "fit" to the enzymes' active surface. We have made the assumption, then, that if the concentration of this inhibitor is kept low, minimal binding to other enzymes and proteins in our crude suspensions and solutions will result. The data may therefore be expected to reflect principally the relationships between this enzyme and inhibitor system rather than multiple protein–enzyme–inhibitor reactions.

RESULTS

Acetylcholinesterase has yet to be crystallized in a pure form from any tissue. Preparations with high specific activities have been obtained from the electric organ of *Electrophorus electricus*;[28] those extracted from human tissues are very crude by comparison.

The data for K_m determinations are plotted in the double reciprocal manner of Lineweaver and Burk and the values for the constant obtained. The K_ms for the various tissues tested are found in Table 1. They average 2×10^{-4} M for skeletal muscle, 2×10^{-4} M for red blood cells, and $1{\cdot}9 \times 10^{-4}$ M for the caudate nucleus of brain. Wilson and Bergman [29] have reported a value of $2{\cdot}6 \times 10^{-4}$ M and Wilson and Alexander[30] recently published a value of $1{\cdot}4 \times 10^{-4}$ M for eel acetylcholinesterase at 25°, pH 7·0. Foldes *et al.*[14] have reported a K_m of $3{\cdot}4 \times 10^{-4}$ M for human red cell cholinesterase. No values were previously available for human brain or skeletal muscle enzyme.

Other choline ester-splitting enzymes are present in these tissues, but plasma cholinesterase (butyrylcholinesterase) is probably the only one in a concentration possibly sufficient to influence our results. Therefore the activity of butyrylcholinesterase in the

tissues was assessed by determining the rates of butyrylcholine hydrolysis. Butyrylcholinesterase accounted for less than 2 per cent of the activity in muscle solution and brain suspension; in all other tissue preparations it was negligible.

It seems reasonable to conclude, then, that acetylcholinesterase having very similar K_ms are present in both the solutions and suspensions of the human tissues tested. In terms of activity they are quite possibly the same enzyme.

TABLE 1. MICHAELIS-MENTEN CONSTANTS (K_m) FOR ACETYLCHOLINESTERASE EXTRACTED FROM HUMAN TISSUES

	Skeletal muscle	Red cells	Caudate nucleus
Suspension	$2{\cdot}2 \times 10^{-4}$ M (3)*	$1{\cdot}8 \times 10^{-4}$ M (2)	$1{\cdot}8 \times 10^{-4}$ M (3)
Solution	$1{\cdot}8 \times 10^{-4}$ M (3)	$2{\cdot}1 \times 10^{-4}$ M (2)	$1{\cdot}9 \times 10^{-4}$ M (3)
Average	$2{\cdot}0 \pm 0{\cdot}2 \times 10^{-4}$ M	$2{\cdot}0 \pm 0{\cdot}15 \times 10^{-4}$ M	$1{\cdot}9 \pm 0{\cdot}1 \times 10^{-4}$ M

* The figure in parentheses is the number of determinations performed at pH 7·4, 37°, using automatic titration. The final substrate concentration was 3×10^{-3} M acetylcholine bromide, and the reaction medium was 0·1 M NaCl, 0·02 M $MgCl_2$, and 5×10^{-5} M EDTA.

The inhibited enzyme (carbamyl enzyme, E′) formed during the incubation period should be identical if the enzyme species derived from these tissues are the same or kinetically similar. Therefore, when the steady-state system is disrupted by extensive dilution, the rates of recovery of the enzymes' activity should also be the same. A typical recovery curve is shown in Fig. 1, and the results for all of the tissue solutions and suspensions are tabulated in Table 2. Control values were obtained by similarly diluting uninhibited enzyme aliquots and assaying the activity. No change was found when the activity was followed for periods up to 5 hr.

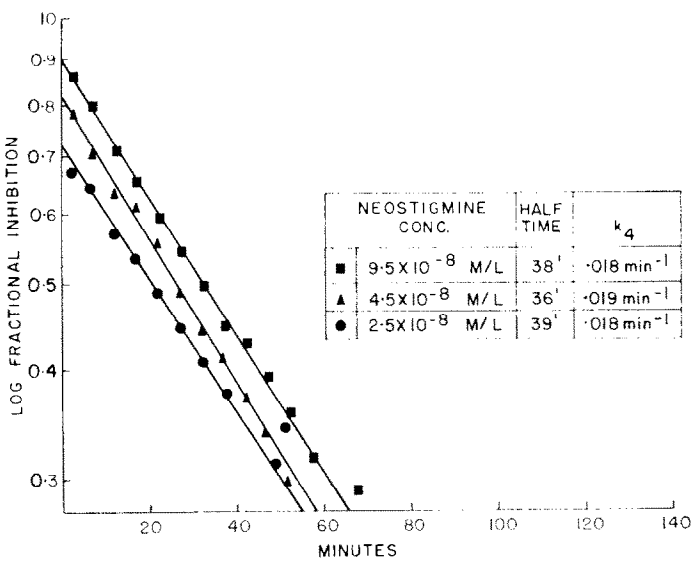

FIG. 1. The recovery of activity of neostigmine-inhibited human red cell acetylcholinesterase is plotted as a function of time. The recovery was followed by automatic titration at pH 7·4, 37°.

The recovery times averaged 34 min. They are not identical but indicate that the enzymes are at least kinetically similar. Because of the crudeness of the preparations, we do not rule out the possibility that they are identical.

Wilson reported the half-life of the dimethylcarbamyl enzyme derived from neostigmine and eel acetylcholinesterase as 25 to 30 min, assayed at 25°, pH 7·0.[20] These measurements indicate the kinetic similarity of eel and human acetylcholinesterases with respect to carbamate inhibition.

TABLE 2. RECOVERY TIMES AND RATE CONSTANTS FOR NEOSTIGMINE-INHIBITED HUMAN TISSUE ACETYLCHOLINESTERASES

	Skeletal muscle		Red cells		Caudate nucleus	
	$T_{1/2}$ (min)	k_4 (min^{-1})	$T_{1/2}$ (min)	k_4 (min^{-1})	$T_{1/2}$ (min)	k_4 (min^{-1})
Suspension	28·5 ± 3·5 (6)*	0·027	40·0 ± 0 (2)	0·017	29·2 ± 2·7 (6)	0·23
Solution	36·5 ± 0·5 (6)	0·019	37·2 ± 1·2 (4)	0·019	35·5 ± 3·5 (6)	0·022
Average	32·5	0·023	38·6	0·018	32·3	0·023

* The figure in parentheses refers to the number of determinations performed at pH 7·4, 37°. $T_{1/2}$ is related to k_4 by $T_{1/2} \times k_4 = 0{\cdot}693$. The inhibitor and enzyme were incubated for 1 h and then extensively diluted in the reaction medium consisting of 0·1 M NaCl, 0·02 M $MgCl_2$, 5×10^{-5} M EDTA, and 3×10^{-3} M acetylcholine bromide. The rate of recovery of enzyme activity was followed by automatic titration.

Under the conditions of the present study the recovery of neostigmine-inhibited human acetylcholinesterase is about 75 per cent complete in 1 hr. These results seem to indicate then that the rate of recovery is not responsible for the 3 to 6 hr duration of action *in vivo*. The concentration of neostigmine probably remains at appropriate levels to account for sustained action. Although the exact fate of neostigmine in the human organism is unknown, there is presumptive evidence that it is partially destroyed in the liver,[7,31,32] excreted in the urine, and hydrolyzed by blood and tissue esterases.[31,32] It seems apparent that these processes are relatively slow and that the conditions necessary for the rapid recovery of acetylcholinesterase activity may not be operant *in vivo*.

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