

# Tissue Cholinesterases. A Comparative Study of Their Kinetic Properties

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The substrate saturation and temperature-dependent kinetic properties of soluble and membrane-bound forms of acetylcholinesterase (AChE) from brain and butyrylcholinesterase (BChE) from heart and liver were examined. In simultaneous studies these parameters were also measured for AChE in erythrocyte membranes and for BChE in the serum from rat and humans. For both soluble and membrane-bound forms of the enzyme from the three tissues, two components were discernible. In the brain,  $K_m$  of component I (high affinity) and component II (low affinity) was somewhat higher in membrane-bound form than that of the soluble form components, while the  $V_{max}$  values were significantly higher by about five fold. In the heart,  $K_m$  of component II was lower in membrane-bound form than in the soluble form, while  $V_{max}$  for both the components was about four to six fold higher in the membrane-bound form. In the liver,  $V_{max}$  was marginally higher for the two components of the membrane-bound enzyme; the  $K_m$  only of component I was higher by a factor of 2. In the rat erythrocyte membranes three components of AChE were present showing increasing values of  $K_m$  and  $V_{max}$ . In contrast, in the human erythrocyte membranes only two components could be detected; the one corresponding to component II of rat erythrocyte membranes was absent. In the rat serum two components of BChE were present while the human serum was found to possess three components. Component I of the human serum was missing in the rat serum. Temperature kinetics studies revealed that the Arrhenius plots were bi-phasic for most of the systems except for human serum. Membrane binding of the enzyme resulted in decreased energy of activation with shift in phase transition temperature ( $T_i$ ) to near physiological temperature.

## Introduction

In the vertebrates two different enzymes hydrolyze acetylcholine. Acetylcholinesterase (AChE: E.C. 3.1.1.7) terminates the action of acetylcholine at the post-synaptic membrane on neuromuscular junction. The second enzyme butyrylcholinesterase (BChE: E.C. 3.1.1.8), which is also called cholinesterase, pseudocholinesterase (PChE) or non-specific cholinesterase can also act on acetylcholine. The two enzymes differ with respect to their substrate specificity as well as their reactivity with various selective inhibitors. Thus AChE hydrolyzes acetylcholine faster than any other cholinesterase and is much less active on butyrylcholine. AChE also typically shows substrate inhibition at high substrate concentrations. BChE, by contrast, preferentially acts on butyrylcholine, but also hydrolyzes acetylcholine. However BChE is generally more active on synthetic substrates, propionylcholine or butyrylcholine. AChE is in-

hibited selectively by 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW 284C51), while BChE is selectively inhibited by 10-[2-diethylaminopropyl]-phenothiazine (ethopropazine) and isotetra monoisopropyl pyrophosphate tetramide (iso-OMPA). The two enzymes are widely distributed in all the vertebrate phyla and in a given organism the distribution of AChE and BChE is tissue-specific (Chatonnet and Lockridge, 1989; Massoulie *et al.*, 1993).

Earlier researchers have reported on the  $K_m$  and  $V_{max}$  values of the cholinesterases from Torpedo and mammalian tissues (Jagota, 1992; Lenz *et al.*, 1984; Shuttleworth *et al.*, 1990; Silver, 1974; Suhail and Rizvi, 1989). However in view of the more recent information on the existence of molecular isoforms, which are present in the soluble and the membrane-bound forms (Bisso *et al.*, 1991; Massoulie *et al.*, 1993), and their tissue-specific distribution (Bisso *et al.*, 1991; Chatonnet and Lockridge, 1989; Massoulie *et al.*, 1993), it is desirable

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to find out the kinetic properties of molecular isoforms themselves.

We have therefore carried out studies to illustrate the kinetic properties of soluble and membrane-bound forms of AChE from brain and BChE from heart and liver. In simultaneous studies we also measured these parameters for AChE in erythrocyte membrane preparations from rat and humans and for BChE in the serum of the two species. The results of these experiments are summarized in the present communication.

## Materials and Methods

### Chemicals

Acetylthiocholine iodide (ACTI), butyrylthiocholine iodide (BCTI) and ethopropazine hydrochloride (ETPZ.HCl) were purchased from Sigma Chemical Co., USA. 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) was purchased from SRL, India. All other chemicals were of analytical reagent grade and were purchased locally.

### Animal and human studies

For animal studies, adult male rats of Charles-Foster strain were used. The animals had free access to food and water.

In human studies the blood was collected from normal healthy volunteers (both males and females; 25–45 year old) by informed consent.

### Isolation of soluble and membrane-bound forms of cholinesterases

This was achieved essentially by following the procedure of Bisso *et al.* (1991) as described earlier (Khandkar *et al.*, 1995).

Briefly, the tissues (brain, heart and liver) from male adult rats were quickly removed after decapitation and placed in beakers containing chilled (0–4 °C) 38 mM tris(hydroxymethyl)aminomethane (Tris)-HCl buffer pH 8.5. The tissues were repeatedly washed with the same buffer and 10% (w/v) homogenates were prepared using a Potter-Elvehjem type glass-teflon homogenizer. The homogenates were centrifuged at 100,000×g for 1 h and the supernatant was carefully decanted. The supernatant served as the source of the soluble form of the enzyme. The pellet was resuspended

by gentle homogenization in the same volume of 38 mM Tris-HCl buffer, pH 8.5 containing 0.25% Triton X 100 and subjected to a further centrifugation at 100,000×g for 1 h. The second supernatant thus obtained was used as the source of the membrane-bound enzyme. All the operations were carried out at 0–4 °C.

### Preparation of erythrocyte membranes

Preparation of erythrocyte membranes was essentially according to the method of Hanahan and Ekholm (1978).

Briefly, blood was collected in the presence of sodium citrate (final concentration 10 mM) and was centrifuged at 475×g for 8 min. Supernatant and the buffy coat overlying the red blood cell (RBC) pellet was discarded and the pellet was washed twice with 0.9% NaCl. The washed RBCs were then subjected to hypotonic lysis in 14 mM Tris-HCl buffer pH 7.4 (Kumthekar and Katyare, 1992) and after centrifugation at 30,000×g for 35 min the supernatant was discarded. The pellet was washed repeatedly with the same buffer to obtain the hemoglobin free membranes. The pellet was resuspended in the same buffer (ca. 1 mg protein/ml) and was used as the source of the enzyme.

### Serum

The rat or human blood was allowed to aggregate at room temperature and sera were collected after centrifugation in a clinical centrifuge.

### Assay of AChE and BChE activities

The AChE/BChE activities were determined essentially according to the procedure of Ellman *et al.* (1961) as described previously with some modifications (Khandkar *et al.*, 1995).

Thus for AChE activity determination, the assay system contained in a total volume of 1 ml: 100 mM potassium phosphate buffer pH 8.0, 0.32 mM DTNB, 0.1 mM ETPZ.HCl, and 10 to 50 µg protein of the soluble form or the membrane-bound form of the brain enzyme or erythrocyte membranes. For substrate kinetics studies the concentration of substrate *i.e.* ACTI was varied from 0.05 to 10 mM. Linear rate of reaction recorded over a period of 60 to 90 seconds at 37 °C was used for calculation of the rate of the reaction. ETPZ.HCl was in-

cluded in the assay medium as the inhibitor of BChE.

For determination of BChE activity the assay system was the same as above except that 0.05 M Tris-HCl buffer pH 8.0 replaced the potassium phosphate buffer, ETPZ.HCl was omitted and BCTI was the substrate. 10 to 50 µg of protein served as the source of tissue enzymes, while for serum, 10 µl of rat serum and 20 µl of 1:10 diluted human serum were used as the source of enzyme.

In separate experiments we have ascertained that for the BChE activity, better results are obtained in 0.05 M Tris-HCl buffer pH 8.0, than in 0.1 M potassium phosphate buffer pH 8.0.

DTNB solution was prepared by dissolving 13 mg DTNB + 5 mg NaHCO<sub>3</sub> in 10 ml potassium phosphate or in Tris-HCl buffer for AChE and BChE assays, respectively (Ellman *et al.*, 1961).

The data were analyzed by the Lineweaver Burk, Eadie-Hofstee and Eisenthal and Cornish-Bowden methods for the determination of  $K_m$  and  $V_{max}$  (Dixon and Webb, 1979). The values of  $K_m$  and  $V_{max}$  obtained by the three methods of analysis were in close agreement and were averaged. The results are given as mean ± S.E. of the averaged values. The data were computer-analyzed employing Sigma Plot version 5.0.

### Temperature kinetics studies

The enzyme activities were determined over a temperature range of 5 to 53 °C with optimum substrate concentration (5 mM). The analysis of the data for determination of energies of activation for the high and the low temperature ranges (E1 and E2, respectively) and phase transition temperature ( $T_i$ ) was according to Raison (1972). All analyses were performed on computer employing Sigma Plot version 5.0 as described above.

Protein estimation was according to the procedure of Lowry *et al.* (1951).

Statistical evaluation of the data was by Students' t-test.

## Results and Discussion

The typical Eadie-Hofstee plots for the soluble and membrane-bound enzymes from the three rat tissues are shown in Fig. 1. It is evident that for all the samples tested there are two components of the enzymes; component I has low  $K_m$  and low  $V_{max}$  and component II has high  $K_m$  and high  $V_{max}$ . The values of  $K_m$  and  $V_{max}$  for the two components of the soluble and the membrane-bound forms of the enzyme are given in Table I.

Table I. Substrate kinetics properties of soluble and membrane-bound cholinesterases from rat brain, heart and liver.

Tissue	Enzyme	Component I		Component II	
		$K_m$	$V_{max}$	$K_m$	$V_{max}$
Brain	soluble(4)	0.036 ± 0.003	126.9 ± 9.7	0.30 ± 0.024	172.4 ± 13.5
	membrane-bound(6)	0.050 ± 0.008	608.3 ± 18.7****	0.55 ± 0.198	955.3 ± 56.8****
Heart	soluble(6)	0.180 ± 0.031	15.6 ± 1.2	1.49 ± 0.165	36.0 ± 2.3
	membrane-bound(6)	0.240 ± 0.069	97.6 ± 8.0****	0.92 ± 0.067***	152.3 ± 8.5****
Liver	soluble(3)	0.030 ± 0.005	10.2 ± 1.5	1.46 ± 0.242	25.9 ± 1.0
	membrane-bound(3)	0.068 ± 0.009*	16.4 ± 1.5*	0.86 ± 0.102	34.1 ± 1.7**

The results are given as mean ± S.E. of the numbers of independent observations indicated in the parentheses. The experimental details are as given in the text. Substrate kinetics measurement of brain AChE were carried out using ACTI as the substrate over the concentration range of 0.05 to 10 mM. The substrate used for BChE substrate kinetics determination of the heart and liver enzyme was BCTI over the concentration range of 0.2 to 10 mM.

Units:  $K_m$  = mM,  $V_{max}$  = nmol min<sup>-1</sup> mg protein<sup>-1</sup>.

\* p < 0.05, \*\* p < 0.02, \*\*\* p < 0.005, \*\*\*\* p < 0.001 as compared with corresponding soluble enzyme.

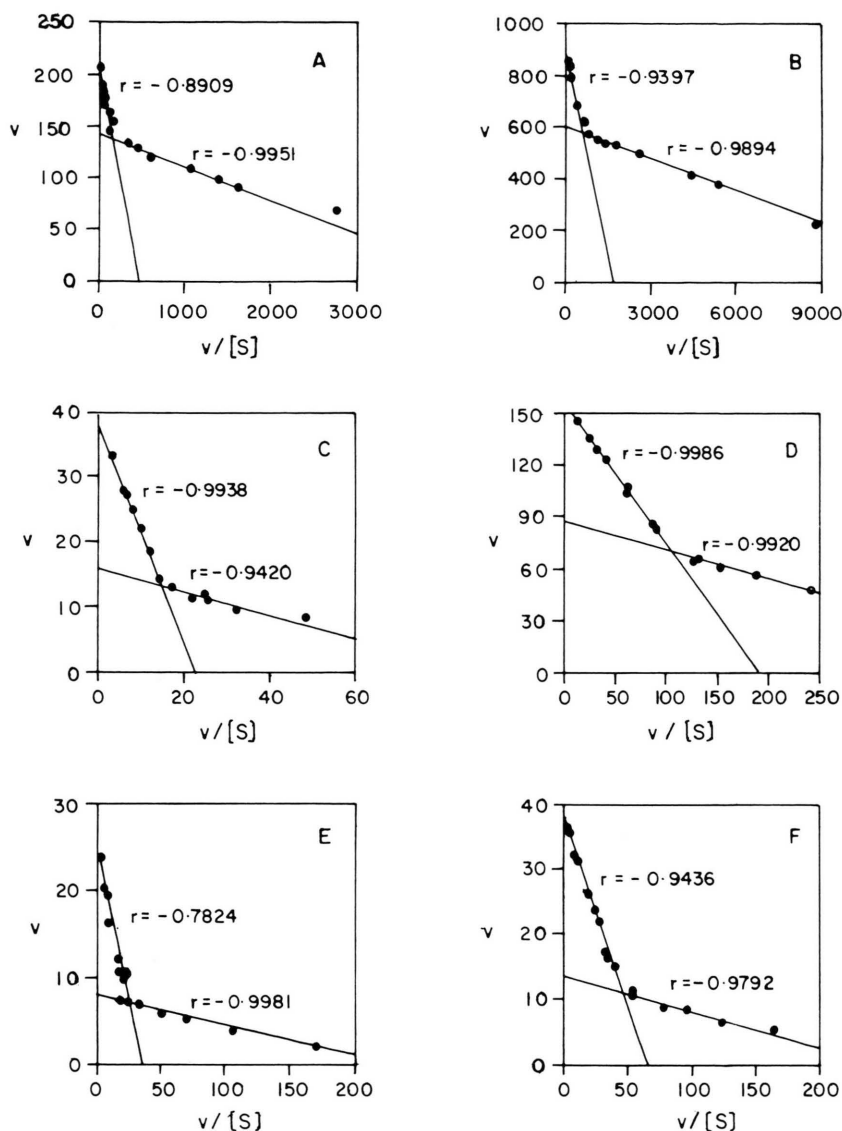


Fig. 1. Typical Eadie-Hofstee plots for rat tissue cholinesterases. (A) Brain:soluble, (B) brain:membrane-bound, (C) heart:soluble, (D) heart:membrane-bound, (E) liver:soluble, and (F) liver:membrane-bound fraction. Experimental details are given in text. For determination of substrate kinetics of AChE, in the brain, ACTI was used as the substrate over a concentration range of 0.05 to 10 mM. For the determination of substrate kinetics of BChE activity in the heart and liver, BCTI was used as the substrate over the concentration range of 0.2 to 10 mM. The abscissa represents the reaction velocity  $v$ , while the ordinate represents the  $v/[S]$  ratios. Reaction velocity =  $\text{nmol min}^{-1} \text{mg protein}^{-1}$ .  $v/[S]$  = reaction velocity divided by the corresponding substrate concentration.

Thus from the data in Table I it can be assumed that in the brain both the components of the membrane-bound form of AChE are characterized by higher values of  $K_m$  and  $V_{\max}$  compared to their counterparts in the soluble fraction. Although the  $K_m$  values were somewhat higher, the differences were not statistically significant. However, the  $V_{\max}$  values for both the components were about 5 times higher and the differences were statistically highly significant. The higher  $V_{\max}$  values for both the components of the membrane-bound form of AChE in the brain would be consistent

with its role in hydrolysis of the neurotransmitter acetylcholine at the synapse (Massoulie *et al.*, 1993). It may, however, be mentioned here that in our earlier studies (Khandkar *et al.*, 1995) we were unable to delineate two components of the enzyme in the brain.

The predominant form of AChE in the brain is G4 which is preferentially presynaptic and mostly exposed on the external surface of the cell, although the G2 and G1 forms are also present as minor components (Marquis and Fishman, 1985; Bon *et al.*, 1991). The G4 and G2 forms are known

to be present in the membrane-bound as well as in the soluble form (Bisso *et al.*, 1991). The results of our present studies on the kinetic properties are consistent with and substantiate the above findings. Firstly, we were able to show the presence of two components differing in their kinetic properties. Secondly, the higher values of  $V_{\max}$  for the membrane-bound form of the enzymes may suggest that the concentration of membrane-bound form is substantially high, which emphasizes its physiological role. Besides, the results also suggest that membrane binding can significantly influence the kinetic properties of the enzyme. (e.g. also see Fig. 3 and Table III).

The information on distribution of molecular isoforms of BChE in the soluble and membrane-bound forms in the heart and liver is not available (Chatonnet and Lockridge, 1989; Massoulie *et al.*, 1993). However, our data on the  $K_m$  and  $V_{\max}$  (Fig. 1 and Table I) would suggest the multiplicity of molecular isoforms in both the soluble and membrane-bound fraction also in these two tissues *i.e.* heart and liver.

The typical Eadie-Hofstee plots for erythrocyte membrane AChE and serum BChE from rat and humans are shown in Fig. 2. It can be noted that the RBC membranes from rat display the presence of 3 components of AChE with increasing values of  $K_m$  and  $V_{\max}$ . In contrast, the human RBC membranes show presence of only two components which correspond, to component I and component III of the rat erythrocyte membranes respectively (Table II). The rat serum includes two BChE components (Fig. 2). In contrast to this, the human serum contains three BChE components with increasing values of  $K_m$  and  $V_{\max}$ ; compared with the human serum, in the rat serum component I is absent (*viz.* see also Table II). It is also of interest to note that for any given component, the value of  $V_{\max}$  for both erythrocyte membrane AChE or serum BChE were always higher in the human samples.

It is likely that the presence of multiple component of AChE which we observe in the erythrocyte membranes may be attributed to the differential membrane locations and differential glycosylation

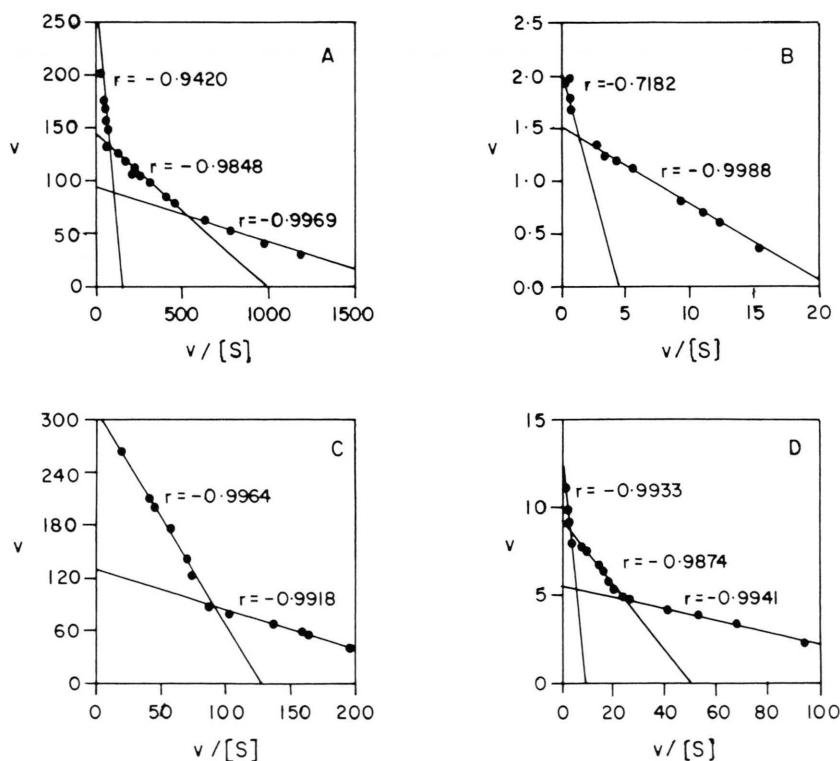


Fig. 2. Typical Eadie-Hofstee plots for cholinesterases from (A) Rat RBC membranes, (B) human RBC membranes, (D) rat serum and (E) human serum. Experimental details are described in text. For determination of substrate kinetics of erythrocyte AChE activity, ACTI was used as the substrate over a concentration range of 0.05 to 10 mM. Substrate kinetics of serum BChE were carried out with BCTI as the substrate over the concentration range of 0.2 to 10 mM. The abscissa represents the reaction velocity  $v$ , while the ordinate represents the  $v/[S]$  ratios. Reaction velocity =  $\text{nmol min}^{-1} \text{ mg protein}^{-1}$ .  $v/[S]$  = reaction velocity divided by the corresponding substrate concentration.



Table II. Substrate kinetics properties of RBC membrane and serum cholinesterases of rats and humans.

	Species	Component I		Component II		Component III	
		$K_m$	$V_{max}$	$K_m$	$V_{max}$	$K_m$	$V_{max}$
RBC membranes	rat(6)	$0.063 \pm 0.012$	$0.099 \pm 0.010$	$0.26 \pm 0.034$	$0.189 \pm 0.015$	$1.535 \pm 0.188$	$0.307 \pm 0.017$
	human(6)	$0.055 \pm 0.006$	$1.225 \pm 0.147$	---	---	$0.983 \pm 0.159$	$1.924 \pm 0.229$
Serum	rat(7)	---	---	$0.53 \pm 0.061$	$0.136 \pm 0.015$	$2.38 \pm 0.143$	$0.261 \pm 0.016$
	human(7)	$0.057 \pm 0.007$	$6.410 \pm 0.156$	$0.27 \pm 0.022$	$10.03 \pm 0.299$	$1.476 \pm 0.169$	$13.35 \pm 0.402$

The results are given as mean  $\pm$  S.E. of the numbers of independent observations indicated in the parentheses. The experimental details are as given in the text. Substrate kinetics measurement of RBC membrane AChE were carried out using ACTI as the substrate over the concentration range of 0.05 to 10 mM. The substrate used for BChE substrate kinetics determination of the serum enzyme was BCTI and concentration was over the range of 0.2 to 10 mM.

Units:  $K_m$  = mM,  $V_{max}$  =  $\mu\text{mol min}^{-1} \text{mg protein}^{-1}$ , for RBC membranes, and  $V_{max}$  =  $\mu\text{mol min}^{-1} \text{ml serum}^{-1}$ , for serum.

of the enzyme and/or membrane components referred to above (Massoulie, 1993). It also is reasonable to expect that the RBC membranes from rats and humans will differ in their lipid/phospholipid composition; the phospholipid composition of the RBC membrane in the two species is known to be widely different (Katewa, 1997; Labrousche *et al.*, 1996; Zubay, 1993). The observed differences in the kinetic properties of AChE in the erythrocyte membranes from human and rats may be thus attributed to these factors.

The serum BChE is believed to originate in the liver (Khoury *et al.*, 1987) and in human plasma presence of monomers, dimers and tetramers called C1, C3 and C4 respectively has been reported (Masson, 1979). Our data which show presence of three components in human serum (Table

II) are consistent with the above observation (Masson, 1979). However, our data point out that, in the rat serum the pattern is different in that there are only two components of BChE. The function of BChE in the tissues and serum is not clearly understood. From study of natural allelic variants, BChE seems to be dispensable, while the circulating AChE activity is clearly vital (Massoulie, 1993; Tunek and Svensson, 1988).

Nevertheless, our present studies have brought forth species-specific differences in AChE and BChE distribution and kinetic properties, respectively, in the erythrocyte membranes and serum.

The typical Arrhenius plots for the soluble and membrane-bound enzymes from rat tissues are shown in Fig. 3 from which it is apparent that in all the cases the Arrhenius plots are biphasic. The

Table III. Temperature kinetics of cholinesterases from rat brain, heart and liver.

Tissue	Fraction	Energy of activation (kJ/mol)		Phase transition temperature $T_i$ [ $^{\circ}\text{C}$ ]
		E1	E2	
Brain	soluble(3)	$47.5 \pm 3.72$	$92.9 \pm 5.10$	$13.9 \pm 1.15$
	membrane-bound (3)	$25.3 \pm 1.62^{**}$	$59.9 \pm 0.55^{***}$	$31.4 \pm 2.06^{****}$
Heart	soluble(3)	$26.3 \pm 1.20$	$37.1 \pm 0.95$	$33.7 \pm 2.61$
	membrane-bound(3)	$22.0 \pm 0.43^{*}$	$36.1 \pm 1.86$	$26.4 \pm 1.58$
Liver	soluble(3)	$106.3 \pm 3.05$	$40.2 \pm 1.55$	$42.8 \pm 0.46$
	membrane-bound(3)	$27.9 \pm 1.83^{*****}$	$35.9 \pm 1.57$	$25.8 \pm 1.49^{*****}$

The results are given as mean  $\pm$  S.E. of the number of independent observations indicated in the parentheses. Enzyme activities were determined with optimum substrate concentration (5 mM). ACTI was the substrate for the brain AChE while for heart and liver BChE, BCTI was the substrate.

\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.005$ , \*\*\*\*  $p < 0.002$  and \*\*\*\*\*  $p < 0.001$  as compared with the corresponding soluble enzyme.

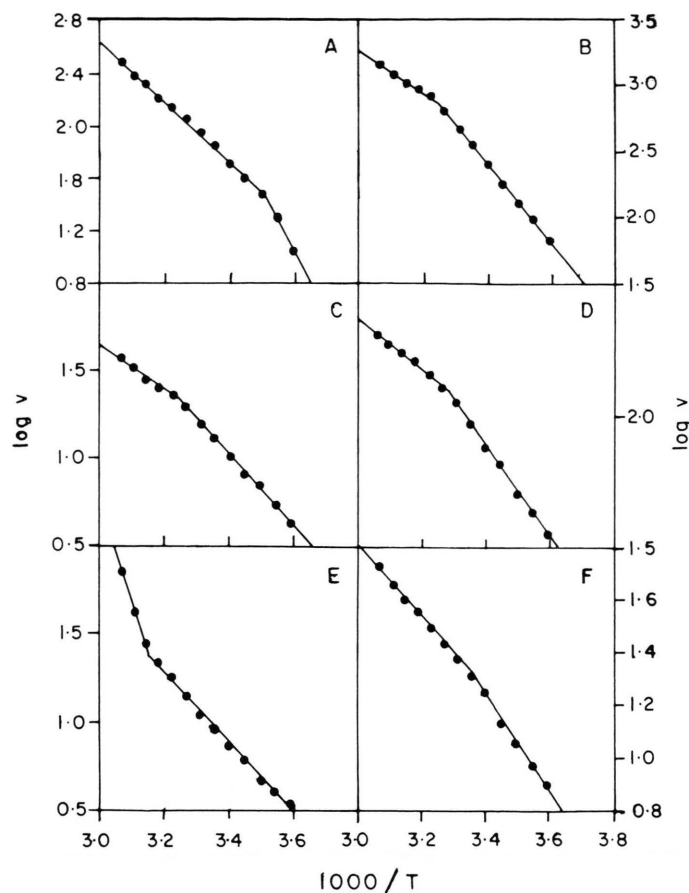


Fig. 3. Typical Arrhenius plots for rat tissue cholinesterases. (A) Brain:soluble, (B) brain:membrane-bound, (C) heart:soluble, (D) heart:membrane-bound, (E) liver:soluble and (F) liver membrane-bound fraction. Enzyme activities were monitored over the temperature range of 5 to 53 °C. Experimental details are described in text and in legends to Fig. 1 and Fig. 2. AChE activity in the brain enzyme was measured with optimum concentration of ACTI *i.e.* 5 mM; for measurement of BChE activity in the heart and liver BCTI concentration was 5 mM. The abscissa represents the log of reaction velocity  $v$ , while the ordinate represents reciprocal of absolute temperature TX1000. Reaction velocity =  $\text{nmol min}^{-1} \text{mg protein}^{-1}$ . Absolute temperature  $T = ^\circ\text{Kelvin}$ .

values of  $E_1$ ,  $E_2$  and  $T_t$  are given in Table III, from which it can be concluded that the membrane-bound form of AChE in the brain has about 40–50% lower values for  $E_1$  and  $E_2$  with the  $T_t$  being close to physiological temperature; in the soluble enzyme the value of  $T_t$  was lowered by about 18 °C. Significance of this latter observation remains obscure. In the heart, the value of  $E_2$  was comparable for the membrane-bound and soluble forms of the enzymes, but the value of  $E_1$  in the latter was somewhat low. The  $T_t$  was lowered by 7 °C in the membrane-bound form although the difference was not statistically significant.

For the soluble enzyme in the liver the value of  $E_1$  was higher than  $E_2$ . The picture was reversed for membrane-bound form which also displayed a 17 °C decrease in the  $T_t$  which was statistically highly significant.

The typical Arrhenius plots for erythrocyte membrane AChE and serum BChE in the rat and

humans are shown in Fig. 4, from which it is evident that these plots were biphasic except for the human serum for which a monophasic plot was obtained. The values of  $E_1$ ,  $E_2$  and  $T_t$  as applicable are given in Table IV from which it can be noted that for erythrocyte membrane AChE from both the sources the values for  $E_1$  are about 2 times higher than those for  $E_2$  as was found earlier for the liver soluble enzyme (Table III), and that the  $T_t$  was near physiological temperature. For the rat serum BChE, the value of  $E_2$  was about double that of  $E_1$  with  $T_t$  occurring around 21 °C; for human serum the energy of activation was comparable with  $E_1$  for rat serum with no phase transition being evident.

The Arrhenius plot analyses have brought into focus differences in the temperature kinetics of the soluble and membrane-bound forms of the enzymes from the tissues and erythrocyte membranes. Seemingly the energies of activation are

lower in the membrane-bound form of the enzymes (Table III) and the phase transition temperature for the membrane-bound enzymes is near

physiological temperature. The results suggest that possibly membrane binding results in increased efficiency.

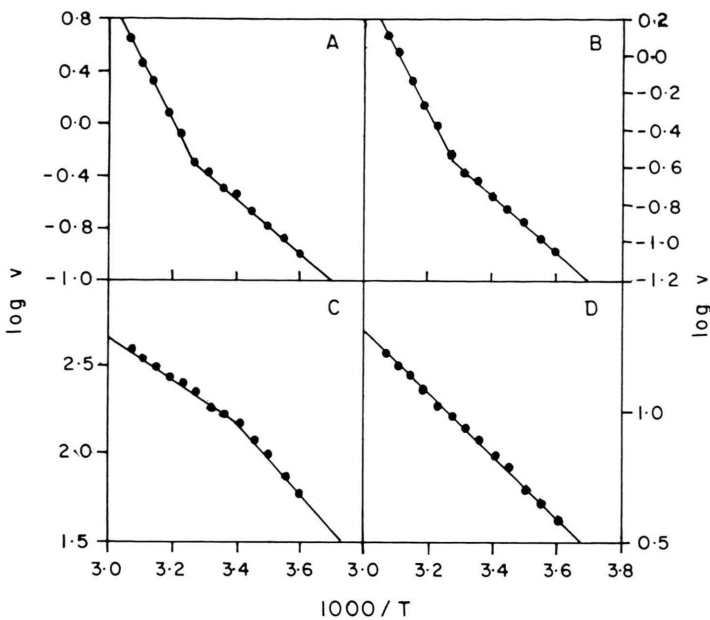


Fig. 4. Typical Arrhenius plots for cholinesterases from (A) Rat RBC membranes (B) human RBC membranes (C) rat serum and (D) human serum. Enzyme activities were monitored over the temperature range of 5 to 53 °C. The experimental details are described in the text. The AChE activity in RBC membranes was determined with optimum concentration of ACTI *i.e.* 5 mM while BChE activity in the serum was measured with optimum (5 mM) concentration of BCTI. The abscissa represents the log of reaction velocity *v*, while the ordinate represents reciprocal of absolute temperature TX1000. Reaction velocity = nmol min<sup>-1</sup> mg protein<sup>-1</sup>. Absolute temperature *T* = °Kelvin.

Table IV. Temperature kinetics of cholinesterases in RBC membrane and serum from rats and humans.

		Energy of activation (kJ/mol)		Phase transition temperature <i>T<sub>t</sub></i> [°C]
		E1	E2	
RBC membranes	rat(6)	88.8 ± 2.53	40.6 ± 0.99	38.5 ± 1.00
	human(6)	55.1 ± 5.00	30.8 ± 1.85	34.8 ± 1.29
Serum	rat(8)	23.5 ± 1.26	46.6 ± 3.51	21.2 ± 1.52
	human(7)	24.4 ± 0.55	---	---

The results are given as mean ± S.E. of the number of independent observations indicated in the parentheses. Enzyme activities were determined with optimum substrate concentration (5 mM). ACTI was the substrate for the RBC membranes AChE while for serum BChE, BCTI was the substrate.



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