

## Subcellular Distribution and Characterization of Acetylcholinesterase Activities From Sheep Platelets: Relationships Between Temperature-Dependence and Environment

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Acetylcholinesterase is a key enzyme in cholinergic neurotransmission for hydrolyzing acetylcholine and has been shown to possess arylacylamidase activity in addition to esterase activity. The enzyme is found at various loci, where its functional significance remains to be clarified, and it exists in multiple molecular forms. Sheep platelets have been shown to exhibit acetylcholinesterase activity associated with plasma membrane (Bp), endoplasmic reticulum (Cp), mitochondria granules (Dp), and soluble (As) fractions. These activities show differences in some physicochemical and kinetic properties. The soluble acetylcholinesterase is the most thermostable, and the enzyme from the Cp fractions shows the lowest affinity for the acetylthiocholine substrate and the strongest inhibition by fluoride. In all cases a noncompetitive inhibition of the enzyme

by this ion is found. When membrane-bound acetylcholinesterases were assayed at temperatures between 12°C and 33°C, the Arrhenius plots of all activities exhibited a break point at about 17°C. This discontinuity was abolished by addition of detergent to the assay medium (0.02% Triton X-100, final concentration). Their Hill coefficients were calculated in the presence of fluoride, showing unitary values in all cases, which points to a noncooperative effect and nonallosteric behavior in the particulate enzyme. These results suggest that the sheep platelet acetylcholinesterase associated with membrane-bound systems is modulated by the physical state of its environment, despite the fact that the enzyme might be lipid- or nonlipid-dependent. © 1990 by The American Society of Hematology.

**T**HE PRINCIPAL ROLE of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7; AChE) is believed to be the termination of impulse transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine. In accordance with this requirement, the enzyme is associated primarily with cells involved in cholinergic synaptic transmission. Nevertheless, it is also found in a variety of other neuronal and a few non-neuronal cells such as erythrocytes, leukocytes, and platelets of different animal species.<sup>1,2</sup> In locations at which no acetylcholine is released, the presence of AChE is puzzling. The enzyme has been shown to possess other catalytic activities in addition to esterase activity.<sup>3,4</sup> AChE occurs in several molecular forms in vertebrate tissues, a major distinction being made between asymmetric (collagen tailed) and globular forms.<sup>1,2,5</sup> It appears that whereas asymmetric forms are anchored to extracellular structures, the globular forms include detergent-soluble species inserted into the membrane lipid bilayer by a hydrophobic domain.<sup>2,6</sup> In platelets, most of the AChE is membrane bound,<sup>7</sup> and during platelet activation a particular form of AChE is released.<sup>8</sup> The sheep platelet plasma membrane-bound AChE exists as a dimeric globular form (G<sub>2</sub>),<sup>9</sup> and it can be released in significant amounts by either *Staphylococcus aureus* phosphatidylinositol-specific phospholipase C (PIPLC) or deoxycholate-activated platelet PIPLC. Recent works suggest that the enzyme is anchored to the plasma membrane via a direct and specific interaction involving the head group of phosphatidylinositol.<sup>9-11</sup> However, the real contribution of this attachment and/or its lipidic environment to the catalytic activity of the platelet AChE has not been fully elucidated.

The modulation of red cell AChE by the membrane lipid environment has been investigated in depth. Beauregard and Roufogalis<sup>12</sup> have reported that the catalytic activity and temperature-dependence of bovine erythrocyte AChE is modulated by the physical state of a tightly bound lipid, probably cardiolipin, to the core of the protein structure by ionic interactions. However, Sihotang<sup>13</sup> has indicated that phosphatidylserine is required for full catalytic activity of human erythrocyte AChE. Bloj et al,<sup>14</sup> Farias et al,<sup>15</sup> and

Morero et al<sup>16</sup> have claimed that the fluidity of the membrane lipid phase affects fluoride inhibition of membrane-bound rat erythrocyte AChE and thus the allosteric properties of the enzyme. By contrast, Frenkel et al<sup>17</sup> did not observe these phenomena in purified and reconstituted human erythrocyte AChE, although their results show that the enzyme is strongly modulated by its hydrophobic environment. On the other hand, in temperature-dependence studies, Spinedi et al<sup>18</sup> support the view that human erythrocyte AChE cannot be regarded as a lipid-dependent enzyme in a strict sense.

G<sub>2</sub> AChE is also the predominant globular membrane-bound AChE from human erythrocytes (RBC AChE) and human skeletal muscle.<sup>19</sup> RBC AChE has been shown to have close structural relationships with several other mammalian AChEs according to immunochemical criteria.<sup>20</sup> Furthermore, pharmaceutical studies and enzyme kinetic analyses have suggested that all molecular species of globular AChE have identical, or nearly identical, enzymatic sites.<sup>20</sup>

The role of AChE in erythrocytes and platelets is not clear. It has been postulated that the enzyme might play a role in the aggregation of human platelets, similar to that thought to be involved in the acetylcholine-induced aggregation of canine platelets,<sup>21</sup> although it seems that this process is highly specific for canine species.<sup>22</sup> Abnormalities of erythrocyte AChE have been reported in some disease states such as

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*Submitted August 23, 1989; accepted April 24, 1990.*

*Supported by Grant No. PB 85-305 from the CICYT.*

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0006-4971/90/7604-0019\$3.00/0

paroxymal nocturnal hemoglobinuria, autoimmune hemolytic anemia of the warm antibody type, and ABO hemolytic disease of newborns,<sup>23</sup> with no single explanation for the findings.

The literature reviewed shows that the functions, subcellular location, and characteristics of platelet AChE from different sources, even from humans, have not been studied in detail. The influence of its lipidic environment upon this enzyme remains unknown.

In this article, we report base-line data on the AChE from sheep platelets, establishing its subcellular location, its physicochemical and kinetic properties, and the relationships between the environment and temperature dependence of AChE activities in relation to their subcellular distribution. The studies focused on sheep platelets because the quaternary structure and the nature of the attachment of the enzyme to the plasma membrane in this source is well known and probably have many similarities with human platelet AChE in accordance with the general properties of the globular form of AChE indicated above. Moreover, at our laboratory we have good experience in the procedure for the isolation of different subcellular organelles from sheep platelets,<sup>24</sup> which are very similar to human platelets in several biochemical and function parameters.

#### MATERIALS AND METHODS

Bovine serum albumin (BSA), Tris (hydroxymethyl) aminomethane, acetylthiocholine chloride, ethopropazine, 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), eserine (physostigmine), p-nitrophenol, the appropriate p-nitrophenyl glycoside and phosphate esters, sodium succinate acid, and p-iodonitrotetrazolium violet were from Sigma Chemical Co (St Louis, MO). D-sucrose was from Aldrich-Europe (Beerve, Belgium). 2,4,6-trinitrobenzene sulfonate (TNBS) was from Serva Feinbiochemical (Heidelberg, FRG). The thin-layer chromatography plates (Silica gel G, type 60) were from Merck (E. Merck, Darmstadt, FRG). The remaining products were from Probus (Barcelona, Spain). All organic solvents were glass-distilled before use.

**Preparation and labeling of platelets with TNBS.** Platelets were isolated from freshly collected blood of adult sheep (*Ovis aries* L. var. domestica) according to the procedure of Barber and Jamieson,<sup>25</sup> slightly modified. Blood was obtained from a local slaughterhouse and collected in 500 mL polythene vessels containing 80 mL 75 mmol/L trisodium citrate/40 mmol/L citric acid/125 mmol/L glucose as anticoagulant. The blood was spun at 300 g for 15 minutes and the platelet-rich supernatant plasma was freed of residual erythrocytes and leukocytes by three cycles of differential centrifugation at 1,500g for 3 minutes. Platelet preparations were precipitated at 3,000g for 30 minutes, then gently suspended in buffer solution 1 (5 mmol/L Na<sub>2</sub>PO<sub>4</sub>H/NaPO<sub>4</sub>H<sub>2</sub>, 0.154 mol/L NaCl, pH 7.4 to 7.5, containing 0.2% glucose), and washed twice in the same buffered solution. The labeling procedure was carried out according to Sánchez-Yagüe and Llanillo.<sup>26</sup> TNBS was added to whole platelets (5 to 6 μmol TNBS/mg protein) resuspended in solution 2 (50 mmol/L KCl/100 mmol/L NaHCO<sub>3</sub>, pH 8.5 to 8.6) at a final TNBS/lipid phosphorus molar ratio of 35. Labeled platelets were washed twice with solution 1 to remove free TNBS, sonicated (as indicated by Sánchez-Yagüe et al<sup>27</sup>), and centrifuged at 105,000g for 1 hour at 4°C, yielding the whole particulate fraction. Separation and identification of phospholipid classes, determination of their lipid phosphorus content, and calculation of labeled

phosphatidylserine (PS) and phosphatidylethanolamine (PE) were carried out as reported by Sánchez-Yagüe and Llanillo.<sup>26</sup>

**Isolation of subcellular fractions.** The platelets were resuspended in solution 3 (5 mmol/L Tris/HCl, pH 7.4 to 7.5, containing 0.3 mol/L sucrose) at a total volume of 2 mL/g of wet platelets. The suspension was homogenized with a Polytron operating at position 8 for 30 seconds intervals, followed by 30 seconds of recooling over a total of 5 minutes. Unbroken cells were precipitated at 4,300g for 10 minutes, resuspended in the same volume of solution 3, and homogenized again. This process was repeated once more. The supernatants of the three centrifugations were combined and designated as homogenate. The platelet homogenate was fractionated in a discontinuous sucrose gradient comprising the following layers of sucrose: 4 mL of 1.6 mol/L, 11 mL 1.1 mol/L, 6 mL 1.0 mol/L, and 4 mL 0.6 mol/L sucrose.<sup>25</sup> After centrifugation at 100,500g for 2 hours 15 minutes at 4°C in a IEC/B-60 ultracentrifuge (rotor SB-110), the gradient yielded one soluble fraction (band A) and three interface bands (B, C, and D). From these four bands one soluble (As) and three main particulate subcellular fractions (Bp, Cp and Dp) were obtained after dilution of the bands with 5 mmol/L Tris-HCl, pH 7.4 to 7.5, at a sucrose concentration between 0.2 mol/L and 0.3 mol/L and centrifugation at 105,000g<sub>av</sub> for 1 hour at 4°C in an ultracentrifuge IEC/B-60 (rotor A-110).

**Enzymatic characterization of subcellular fractions.** Bis (p-nitrophenyl)-phosphate phosphodiesterase (EC 3.1.16.1), 5'-dTMP-p-nitrophenyl ester phosphodiesterase (EC 3.1.1.35), 3'-dTMP-p-nitrophenyl ester phosphodiesterase (EC 3.1.3.34), β-N-acetylglucosaminidase (EC 1.3.99.1), acid phosphatase (EC 3.1.3.2), succinate dehydrogenase (EC 1.3.99.1), and glucose-6-phosphatase (EC 3.1.3.9) were measured and their activities expressed as indicated in a previous paper,<sup>24</sup> except succinate dehydrogenase-specific activities, which are expressed as micromoles of formazan formed per minute and milligrams of protein. The values were calculated from a measured value of 20.1 mmol/L<sup>-1</sup> · cm<sup>-1</sup> for the extinction coefficient of the formazan dissolved in ethyl acetate.<sup>28</sup> Lactate dehydrogenase (EC 1.1.1.27) was determined by the method of Wroblewski and La Due,<sup>29</sup> expressing specific activity as micromole of lactate formed/minute milligram of protein.

The protein content of different fractions was determined by the method of Lowry et al,<sup>30</sup> with BSA as standard.

**Assay of acetylcholinesterase activity.** AChE activity was determined by measuring the hydrolysis of acetylthiocholine by a modification of the method of Ellman et al.<sup>31</sup> The assay mixture (1 mL) contained 0.125 μmol DTNB in 80 mmoles sodium phosphate buffer, pH 7.5, 0.1 μmol ethopropazine, and 40 to 200 μg of sample proteins in the presence or absence of 0.02% Triton X-100. After temperature equilibration at 25°C in a thermostatically controlled bath, the reaction was started by addition of 1 μmol acetylthiocholine chloride and monitored at 412 nm using a double-beam spectrophotometer equipped with a recorder. One unit of enzyme (U) is defined as the amount of enzyme that forms 1 μmol of thiocholine per minute under the standard assay conditions. One mole of 2-nitro-5-thiobenzoate anion is formed per mole of thiocholine present in the medium. The nitromercaptobenzoate anion has an intense yellow color with a molar absorptivity of 13,600 mol/L<sup>-1</sup> · cm<sup>-1</sup> at 412 nm.

**AChE optimal pH.** The optimal pH was assayed in 0.8 mL incubation mixture containing 0.8 μmol of acetylthiocholine chloride, 0.02% Triton X-100, 0.08 μmol of ethopropazine, 40 to 200 μg of sample proteins, and buffer solutions with different pH values: 0.32 mmol citric acid/sodium phosphate (pH 3.5 to 6.0) or 0.32 mmol sodium phosphate (pH 6.0 to 8.0). After 5 minutes of incubation at 25°C, the reactions were stopped by addition of 0.01 nmol eserine. Then 0.2 mL of "color buffer" (0.625 mmol/L DTNB in appropriate buffer) were added at room temperature, and the reactions were read at 412 nm after 15 minutes. The buffers used for

preparation of the color buffers were: 0.3 to 0.5 mol/L Tris/HCl, pH 9.0, to assay the optimal pH between 3.5 to 5.0; 0.3 to 0.6 mol/L sodium carbonate/bicarbonate, pH 9.2, for assay mixtures with pH between 5.5 and 7.0; and 0.4 mol/L sodium phosphate, pH 7.5, for incubation mixtures with pH values between 7.5 to 8.0 to maintain the final pH in the mixture around 8.0.

**AChE thermal stability.** Samples were heated for varying periods of time (5 to 90 minutes) at several temperatures (35°C, 45°C or 55°C) in a thermostatically regulated bath with constant shaking. Control samples were kept at 4°C. All samples were then assayed for remaining enzyme activity at the same time, as previously described.

**AChE kinetic parameters.** The Michaelis constant ( $K_m$ ) and maximal velocity ( $V_{max}$ ) were calculated from Lineweaver-Burk plots<sup>32</sup> using acetylthiocholine concentrations of 0.05 to 2.8 mmol/L. Inhibition constants ( $K_i$ ) were determined by the Dixon plot,<sup>33</sup> using NaF as inhibitor at concentrations of 0.5, 1 and 3 mmol/L respectively in the standard assay mixture. Data were fitted to the plots by nonlinear regression analysis.

**AChE temperature-dependence assay.** The temperature-dependence of AChE activity was measured over a temperature range of 12°C to 33°C. Samples were assayed in triplicate at 1°C intervals in a thermostatically controlled water bath. Lines were fitted to the data points in Arrhenius and Hill plots by regression analysis using the least squares method, and  $\chi^2$  statistical analyses were used to choose the two-line fits.

## RESULTS

The transbilayer distribution of PE and PS was determined by the TNBS reaction<sup>26</sup> to test the degree of integrity of sheep platelets isolated. The intact platelets obtained according to the procedure of Barber and Jamieson<sup>25</sup> show 19% to 22% of plasma membrane PE located in the outer half of the plasma membrane, whereas PS appears to be nearly exclusively located on the cytoplasmic surface, in agreement with our previous work.<sup>26</sup> Nevertheless, using platelets obtained by the method of Llanillo and Cabezas,<sup>24</sup> the level of aminophospholipid modification was much higher. Ranges of 70% to 84% of plasma membrane PE and 27% to 44% of PS

were located in the outer bilayer. These latter results could point to some structural alterations in the plasma membrane due to the specific erythrocyte lysis solution used<sup>24</sup> and might in some way affect plasma membrane-bound enzymes, such as AChE. Accordingly, all studies described in this paper were made with sheep platelets isolated by the procedure of Barber and Jamieson.<sup>25</sup>

**Isolation and enzymatic characterization of subcellular fractions from sheep platelets.** Because of the small size of the blood platelets and their resistance to mechanical forces, the isolation of the platelet membranes is subject to certain particular difficulties. We have assayed several physicochemical methods for platelet disruption, obtaining the best results with the procedure described in "Materials and Methods." After discontinuous sucrose gradient centrifugation of sheep platelet homogenates, four main subcellular fractions were isolated: "As" fraction on the top of the gradient and the rest of the fractions in the interfaces of the following sucrose solutions: 0.6/1.0 mol/L (Bp), 1.0/1.1 mol/L (Cp), and 1.1/1.6 mol/L (Dp).

Table 1 shows the data of the enzymatic characterization of subcellular fractions. Plasma membranes (fraction Bp) had the highest specific activities of the plasma membrane marker bis-(p-nitrophenyl)phosphate phosphodiesterase (1.3-fold). The specific activities of contaminating organelle marker enzymes in the plasma membrane preparations were all less than unity compared to the crude homogenate, except for glucose-6-phosphatase, which was enriched 3.1-fold. Nevertheless, the highest glucose-6-phosphatase activity was found in the Cp fraction (14.6-fold), which may correspond to the presence of endoplasmic reticulum constituents. The highest  $\beta$ -N-acetylglucosaminidase, acid phosphatase, and succinate dehydrogenase activities were located, as expected, in the Dp fraction. After this enzymatic characterization, it can be concluded that the Bp fraction is enriched in plasma membranes. Cp contains mainly endoplasmic reticulum, and

**Table 1. Enzymatic Characterization of Subcellular Fractions From Sheep Platelets**

Enzyme	Fraction (%)				
	Homogenate	Bp	Cp	Dp	As
Bis (p-nitrophenyl) phosphate phosphodiesterase	5.2 ± 0.2 (100)	25.85 ± 0.5 (37.4)	20.4 ± 2.5 (12.3)	13.6 ± 3.4 (20.0)	2.8 ± 0.6 (21.5)
5'-dTMP-p-nitrophenyl ester phosphodiesterase	0.60 ± 0.06 (100)	0.80 ± 0.07 (17.1)	0.60 ± 0.07 (2.7)	0.56 ± 0.04 (13.6)	0.70 ± 0.15 (60.5)
3'-dTMP-p-nitrophenyl ester phosphodiesterase	0.19 ± 0.07 (100)	0.39 ± 0.04 (13.3)	0.58 ± 0.02 (4.6)	0.82 ± 0.15 (30.4)	0.22 ± 0.01 (49.6)
$\beta$ -N-acetylglucosaminidase	0.72 ± 0.14 (100)	0.69 ± 0.26 (5.8)	1.1 ± 0.3 (6.2)	2.4 ± 0.1 (27.0)	1.1 ± 0.2 (55.7)
Glucose-6-phosphatase	0.61 ± 0.04 (100)	1.9 ± 0.6 (19.2)	8.9 ± 1.0 (28.1%)	5.0 ± 0.3 (41.3)	0.35 ± 0.10 (7.3)
Acid phosphatase	15.9 ± 2.2 (100)	12.7 ± 0.9 (20.2)	45.5 ± 2.6 (15.5)	53.4 ± 3.3 (43.9)	5.8 ± 1.6 (13.4)
Succinate dehydrogenase	0.37 ± 0.06 (100)	0.01 ± 0.01 (0.32)	0.31 ± 0.22 (1.1)	3.25 ± 0.29 (90.4)	0.04 ± 0.03 (3.1)
Lactate dehydrogenase	40.2 ± 2.5 (100)	0	0	0	75.8 (99.4)

Values are the means of specific activity of marker enzymes expressed as micromole product formed (p-nitrophenol, phosphate, formazan, or lactate)/min. · milligram of protein ± SD from three preparations assayed. The data of the percentage distribution of enzyme activities are shown in the parentheses.

**Table 2. Subcellular Location of Acetylcholinesterase From Sheep Platelets**

	Fraction				
	Homogenate	As	Bp	Cp	Dp
Specific activity	4.50 ± 1.40	1.82 ± 0.40	21.04 ± 1.60	12.40 ± 1.60	11.11 ± 1.48
Purification factor	1	0.4	4.7	2.7	2.4
Enzyme activity percentage	100	26	41	12	21

Values of specific activities expressed as mU/mg of protein are the means ± SD of five assays.

most of the granules and mitochondria are included in the Dp fraction. The As corresponds to the soluble fraction. The results are in accordance with those obtained previously.<sup>24</sup>

**Distribution of AChE activity in subcellular fractions.** The results shown in Table 2 indicate that platelet AChE activity was located in all subcellular fractions, being recovered to the largest extent in the plasma membrane fraction (41%). Specific activity in plasma membranes demonstrated an enrichment of about 4.7-fold over that of the homogenate. The remaining particulate fractions showed similar enrichments and yields in this enzyme.

**Physicochemical and kinetic characterization of AChE.** Optimal AChE activity from the particulate subcellular fractions was achieved at around pH 7.5 (data not shown).

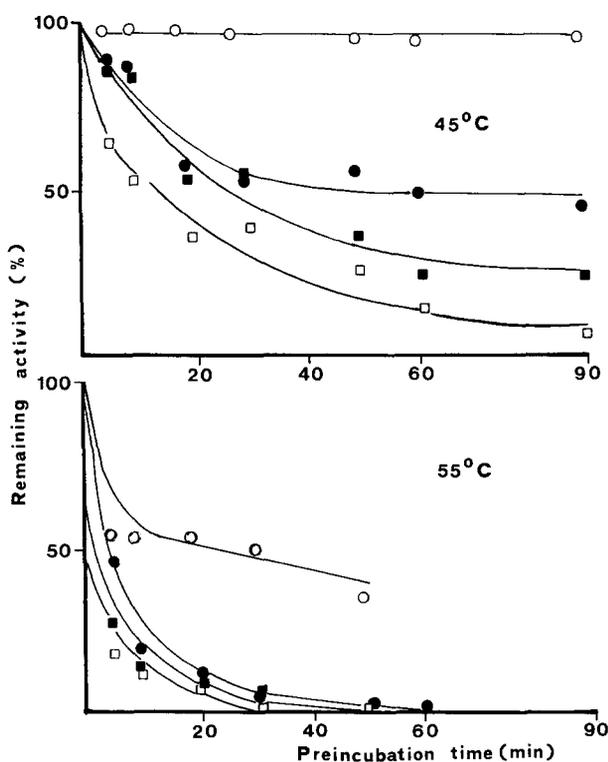
The AChE activity from the particulate subcellular fractions shows different profiles of thermal stability (Fig 1). At 35°C, all enzyme activities were very stable throughout the preincubation time (data not shown). The AChE from fraction As showed the most thermostable behavior, losing less than 10% of its activity after preincubation at 45°C for

90 minutes. Nevertheless, AChE from the particulate fractions lost its original activities in a range from 50% (fraction Bp) to 90% (fraction Dp) after 90 minutes of preincubation. When temperature was increased to 55°C, the particulate enzymes were almost inactivated after 30 minutes of heating.

All AChE activities from sheep platelets follow typical Michaelis-Menten kinetics for the hydrolysis of the acetylthiocholine substrate. The apparent Michaelis constant ( $K_m$ ) and maximal velocities are given in Table 3. AChE from Cp showed the highest  $K_m$  (0.183 mmol/L). The  $V_{max}$  are almost constant for the different particulate fractions.

To be sure that under the experimental conditions used in the temperature-dependence studies AChE does not suffer from either unsaturation with or inhibition by its substrate, the  $K_m$  values for acetylcholine at different temperatures (12°C, 25°C and 37°C) were initially determined in the three particulate fractions (Bp, Cp, and Dp). The values obtained in the presence or absence of Triton X-100 were essentially identical in all cases (data not shown). The Lineweaver-Burk plots of the data from AChE activities assayed in the presence of  $F^-$  showed a noncompetitive inhibition of the enzyme by this ion. The  $K_i$  values obtained from Dixon plots are also given in Table 3. AChE from Cp fraction is inhibited to a greater extent than any other AChE activity.

**Temperature-dependence of AChE activity.** The temperature dependence of AChE activity in particulate subcellular fractions was visualized as a plot of log activity versus  $1/T$ , which for homogeneously reacting systems has been called the Arrhenius-plot. In the three particulate fractions a clear break in the plot was observed around 17°C. When these activities were assayed in the presence of 0.02% Triton X-100, linear relationships in both of these plots were obtained (Fig 2). The apparent activation energies ( $E_a$ ) for the AChE activities from the Bp and Cp fractions were 29.0 and 22.8 kJ · mol<sup>-1</sup> above the break point and 61.1 kJ · mol<sup>-1</sup> below the discontinuity point. AChE from the Dp fraction and all particulate forms after treatment with 0.02% Triton X-100 exhibited an activation energy of  $25.4 ± 3.8$  kJ



**Fig 1. Thermal stability of acetylcholinesterase from soluble (O), Bp (●), Cp (■), and Dp (□) fractions of sheep platelets after heating at 45°C and 55°C for different times.**

**Table 3. Kinetic Parameters for Acetylcholinesterase Located in Subcellular Fractions From Sheep Platelets**

Fraction	$K_m$ (mmol/L)	$V_{max}$ (mU/mg)	$K_i$ (mmol/L)
Soluble	0.045 ± 0.005	4.7 ± 0.5	3.47 ± 0.16
Bp	0.121 ± 0.020	20.4 ± 2.0	1.83 ± 0.10
Cp	0.183 ± 0.10	17.2 ± 2.3	0.55 ± 0.03
Dp	0.075 ± 0.015	11.1 ± 1.9	0.89 ± 0.11

Data are the means ± SD of three duplicate determinations. The  $K_m$  and  $V_{max}$  values were determined with acetylthiocholine chloride as substrate, and the  $K_i$  were calculated using NaF as inhibitor.

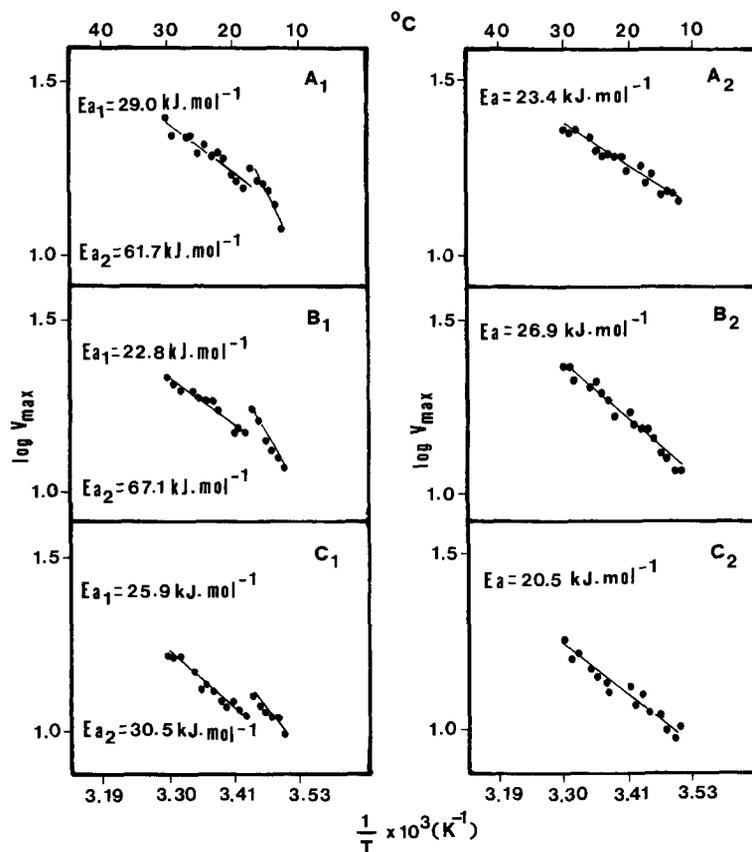


Fig 2. Temperature-dependence of acetylcholinesterase activity in native plasma membrane (A), Cp (B), and Dp (C) fractions from sheep platelets in the absence (A<sub>1</sub>, B<sub>1</sub>, and C<sub>1</sub>) or presence (A<sub>2</sub>, B<sub>2</sub>, and C<sub>2</sub>) of detergent (0.02% Triton X-100, final concentration). For experimental details see Materials and Methods. Each point represents the average value of duplicate from a typical experiment repeated three times. The straight lines were fitted by the least-squares method.

· mol<sup>-1</sup>, which is similar to that found above the break point in the particulate AChE forms.

As in the works of Bloj et al,<sup>14</sup> Farias et al,<sup>15</sup> and Morero et al,<sup>16</sup> the possible influence of the lipid environment and its fluidity on the inhibition of the AChE by various concentrations of fluoride ions was studied in the three membrane fractions at 12°C and 37°C (temperature below and above the breakpoint observed in the Arrhenius plot). In each of the

cases studied, a Hill coefficient (*n*) of about 1 was found (Table 4).

#### DISCUSSION

The goal of the studies described here was to establish the subcellular location and physicochemical and kinetic characterization of platelet AChE because hitherto no clear reports have been offered about the subcellular distribution of AChE

Table 4. Values of the Hill Coefficient (*n*) for Inhibition of Acetylcholinesterase Plasma Membrane From Sheep Platelets by Fluoride

	- log [F <sup>-</sup> ]									
	3.60	3.30	3.12	3.00	2.80	2.70	2.60	2.50	2.45	2.40
12°C										
log $\frac{V}{V_0 - V}$ *	0.07	-0.2	-0.43	-0.48	-0.68	-0.68	-1.14	-0.93	-1.14	-1.11
<i>n</i> *					1.02					
log $\frac{V}{V_0 - V}$ †	0.40	0.12	0.025	-0.39	-0.60	-0.46	-0.68	-0.60	-0.78	-0.89
<i>n</i> †					1.03					
37°C										
log $\frac{V}{V_0 - V}$ *	1.30	1.13	0.94	1.13	0.86	0.50	0.31	0.21	0.34	0.21
<i>n</i> *					0.99					
log $\frac{V}{V_0 - V}$ †	1.34	1.10	1.16	0.83	0.69	0.50	0.42	0.22	0.29	0.17
<i>n</i> †					1.03					

The *n* values are the means of two duplicate assays and were calculated from the slope of each straight line obtained in the graphic plot of log  $V/V_0 - V$  versus - log [F<sup>-</sup>], where *V* is the reaction rate in the presence of F<sup>-</sup>; *V*<sub>0</sub> is the rate of the reaction in the absence of F<sup>-</sup>. The assays were made in the presence (\*) or absence of Triton X-100 (†) under standard conditions (detailed in Materials and Methods) at 12°C and 37°C.

in platelets.<sup>8,34</sup> In particular, the physicochemical and kinetic characteristics of the enzyme in relation to its subcellular distribution are unknown. To study the properties of a protein in its natural environment it is essential to have knowledge of its location within the cell, especially in the case of an enzyme, where it is possible that several different molecular forms might exist.

In studies on the subcellular location of enzymes, the use of intact cells prevents breakage of the subcellular organelles and avoids probable loss of the enzyme assayed. Thus, in our studies we previously corroborated the integrity of the sheep platelets isolated, studying the standard distribution of PE and PS in the plasma membrane from whole platelets by a nonpermeable probe, as described by Sánchez-Yagüe and Llanillo.<sup>26</sup> The subcellular location of AChE in sheep platelets was studied following isolation of four different subcellular fractions in a discontinuous sucrose gradient.<sup>25</sup> The lipid composition and the values of marker enzymes in these fractions are in agreement with our previous work.<sup>24,26</sup> The enzymatic criteria shown here and the characterization by electron microscopy, marker enzymes, and chemical studies previously carried out by Llanillo and Cabezas<sup>24</sup> demonstrate that the sheep platelet fractions were enriched in plasma membrane (fraction Bp), endoplasmic reticulum (fraction Cp), and intracellular granules and mitochondria (fraction Dp). The fraction As, called "soluble fraction," has 99% of total lactate dehydrogenase activity. Identical findings have been reported in pig platelets using a similar procedure.<sup>35</sup>

Sheep platelet plasma membranes show a similar density ( $d = 1.09$  to  $1.12$ ) to those found in humans ( $d = 1.066$ ,<sup>25</sup>  $d = 1.11$ <sup>36</sup>) and a similar purity when compared to those prepared from human platelets by other techniques, such as polylysine beads,<sup>37</sup> affinity chromatography,<sup>38</sup> cushion sucrose at 27%, and continuous sucrose gradient<sup>26</sup> or sodium diatrizoate linear gradient,<sup>36</sup> as indicated by the marker enzyme analyses. These facts, together with other biochemical parameters, suggest that the platelet plasma membrane from different sources are quite similar in many characteristics.

Assessment of the relative purity of prepared subcellular fractions from sheep platelets enabled us to determine the distribution of AChE activity. The hydrolysis of acetylthiocholine chloride is exclusively performed by AChE activity in all fractions because this reaction is fully inhibited by  $10^{-5}$  mol/L eserine, a compound that specifically inhibits AChE and butyrylcholinesterase activity (BChE, EC 3.1.1.8). The latter is excluded because the overall rate of substrate hydrolysis is the same in the presence or absence of a specific inhibitor for BChE, ethopropazine. The AChE from sheep platelets is found in the four subcellular fractions (Table 2), the principal one being recovered in the particulate fractions (about 74% of the total activity from the homogenate). Similar values in distribution were obtained in canine platelets,<sup>8,34</sup> where AChE activities from intact cells and from plasma membrane fractions were exoenzymes and were comparable, since their kinetic data were strikingly similar.<sup>34</sup> This enzyme has not been found to be directly involved in the acetylcholine-induced release and aggregation of canine

platelets, although it seems probable that this phenomenon is mediated by an acetylcholine receptor, even though such a receptor has not yet been demonstrated clearly. However, the simultaneous release of AChE activity with adenine nucleotides and serotonin suggest that this releasable AChE may be closely related to the function of acetylcholine in canine platelets.<sup>8</sup> The AChE and serotonin-sensitive aryl acylamidase (AAA) activities from different sources have been identified with the same protein.<sup>4,9</sup> The studies of Majumdar and Balasubramanian<sup>39</sup> suggest a nonidentical overlapping of active centers for its esterase and amidase activity. It appears, therefore, that serotonin sensitivity may be an exclusive property of aryl acylamidase associated with AChE. Possibly this association facilitates the action of AChE in the metabolism of serotonin. Thus it is postulated that platelets represent a useful model for neural cells (with certain limited pathways) in terms of the metabolism of biogenic amines and acetylcholine.<sup>40</sup> These and other functions could account for the heterogeneous subcellular distribution of AChE reported here and in earlier studies.<sup>34,41</sup>

The different subcellular distribution found for AChE from sheep platelets prompted us to study whether the various AChE activities found are or are not the same molecular form. We have found significant physicochemical and kinetic differences among the AChE activities associated with the different subcellular fractions. Membrane-bound AChE has a neutral optimal pH, similar to that described in other materials.<sup>34,42</sup> The soluble enzyme shows the most stable thermal behavior and the lowest  $K_m$  against acetylthiocholine chloride. The plasma membrane-associated AChE (AChEm) shows a  $K_m$  value similar to that found in human and intact canine platelets and erythrocytes.<sup>8,43</sup> The observed differences could be attributed to changes in the environment of the enzyme, although the influence of this surrounding must be less marked than for proteins which penetrate more deeply into the lipid bilayer.

Sheep platelet-soluble AChE (AChEs) has very different physicochemical and kinetics properties with respect to those found in membrane-bound activities. By contrast, data on canine platelet AChE from whole platelets, plasma-membrane preparations, and platelet-released supernatants are strikingly similar.<sup>34</sup> Considering that the substrate used (acetylcholine) by the authors does not penetrate into the cell, these results are not surprising because they are probably assaying the same activity, especially if this is an exoenzyme.<sup>8</sup> The presence of AChE in the supernatant of the canine platelet-release reaction medium, mediated by acetylcholine, could be explained not only by a soluble enzyme from the internal granules, but also by a shedding of the plasma membrane enzyme, in particular because the  $\text{CaCl}_2$  added to the preparation could be an activation factor for specific phospholipases, which might facilitate enzyme solubilization. These facts may explain the great similarity found in optimal pH and kinetic data of canine and sheep AChEs.<sup>34</sup>

In the presence of  $\text{F}^-$ , all AChE activities from sheep platelets are noncompetitively inhibited, which is in agreement with data from rat AChE.<sup>16</sup> The relative rate of substrate hydrolysis in the presence of  $\text{F}^-$  by AChEm is

reduced more rapidly than with AChEs; similar results were obtained using electric eel AChE.<sup>44</sup>

Discontinuities in the Arrhenius plots of membrane-bound enzymes have generally been considered to be a reflection of a phase transition in the lipid environment of the activities and therefore to indicate the lipid dependence of the enzyme. However, it sometimes happens that a break in the Arrhenius plot is due to some intrinsic property of the enzyme itself<sup>45</sup> or to the temperature-dependent changes in the  $K_m$ .<sup>46</sup> With sheep platelet AChE from all subcellular fractions studied, the  $K_m$  values remained constant throughout the temperature range investigated (12°C to 37°C) in the presence or absence of Triton X-100. Nevertheless, the membrane-bound AChE assayed in the absence of Triton X-100 showed a deviation from linearity in the Arrhenius plots, with a break point at 17°C similar to that found in bovine erythrocyte AChE.<sup>47</sup> Barton et al<sup>48</sup> claimed that both the membrane-bound and enzymically solubilized rat erythrocyte AChE shows a discontinuity in the Arrhenius plot, which seems to support the view of a conformational change in the enzyme protein. Nevertheless, the same authors observed that in the *Torpedo* electric organ, the enzymatic solubilization of AChE abolished the breakpoint, despite the fact that both are PI-anchored AChE. These results could indicate a species-specific or tissue-specific influence of the lipidic environment on the catalytic activities of PI-anchored enzymes. Our data reveal abrupt changes in the Arrhenius plot slopes that can be ascribed to the effect of the physical environment on the catalytic properties of the enzyme because the biphasic nature of the Arrhenius plots is abolished when the environments of the platelet AChEm are modified by Triton X-100 or when the cholesterol/phospholipid molar ratio of the plasma membrane is increased.<sup>49</sup> The  $K_m$  and activation energies ( $E_a$ ) that we report for the AChE of the Dp fraction are of the same order as those reported in rat erythrocyte AChE (0.04 mmol/L, 15 to 25 kJ · mol<sup>-1</sup>).<sup>48</sup> However, sheep platelet AChE associated with the plasma membrane and Cp fractions shows a higher  $K_m$  (0.12 to 0.18 mmol/L) and a larger  $E_a$  below the breakpoint (67 kJ · mol<sup>-1</sup>).

Bløj et al,<sup>14</sup> Farias et al,<sup>15</sup> and Morero et al<sup>16</sup> reported that

the Hill coefficient for the inactivation of AChEm from rat erythrocytes by F<sup>-</sup> varied considerably when the fluidity of the membrane was changed. Thus an allosteric behavior with this AChE was evident. In our studies such findings could not be confirmed because the Hill coefficients are about one and appear to be independent of the nature of the subcellular fraction and the fluidity of the lipid matrix. Such results are in agreement with those of Frenkel et al<sup>17</sup> and point to a nonallosteric behavior of the enzyme.

The observations described in this paper show that the AChE activity of the membrane-bound systems may be modulated by the physical state of their lipid environment despite the fact that the enzyme might be lipid or nonlipid dependent. Recent experiments from our laboratory seem to indicate that plasma membrane AChE could be modulated by the progressive inclusion of free cholesterol into the sheep platelet plasma membrane.<sup>49</sup> This inclusion, which could be produced under conditions of atherosclerosis, causes a gradual movement of PS from the inner to the outer side of the lipid bilayer<sup>27</sup> and would be expected to increase the negative-charge density of the membrane, promoting a hypercoagulation process accompanied by changes in the catalytic properties of plasma-membrane AChE. The relationships between cholesterol-AChE modulation and the outlet of the procoagulant phospholipid PS upon platelet functionality are currently being investigated in our department. Hence the studies reported here offer base-line data with a view to discovering the AChE activities present in platelet subcellular fractions.

Our experimental results are suggestive of different forms of sheep platelet AChE, since several subcellular locations and physicochemical and kinetic differences were found for each activity. Although further experiments are needed to confirm this fact, at least two forms could be established: a soluble and a membrane-bound AChE.

#### ACKNOWLEDGMENT

The authors are grateful to N. Skinner for correcting the manuscript.

#### REFERENCES

1. Massoulié J, Bon S: The molecular forms of cholinesterase and acetylcholinesterase in vertebrates. *Ann Rev Neurosci* 5:57, 1982
2. Silman I, Futerman AH: Modes of attachment of acetylcholinesterase to the surface membrane. *Eur J Biochem* 170:11, 1987
3. Chatonnet A, Lockridge O: Comparison of butyrylcholinesterase and acetylcholinesterase. *Biochem J* 260:625, 1989
4. George ST, Balasubramanian AS: The identity of the serotonin-sensitive arylacylamidase with acetylcholinesterase from human erythrocytes, sheep basal ganglia and electric eel. *Eur J Biochem* 111:511, 1980
5. Brimijoin S: Molecular forms of acetylcholinesterase in brain, nerve and muscle: Nature, localization and dynamics. *Prog Neurobiol* 21:291, 1983
6. Ott P: Membrane acetylcholinesterase: Purification, molecular properties and interactions with amphiphilic environments. *Biochim Biophys Acta* 822:375, 1985
7. Majumdar S, George ST, Balasubramanian AS: Serotonin-sensitive aryl acylamidase activity of platelet acetylcholinesterase. *Biochem Pharmacol* 31:2319, 1982
8. Chuang HYK, Mohammad SF, Mason RG: Acetylcholinesterase, choline acetyltransferase, and the postulated acetylcholine receptor of canine platelets. *Biochem Pharmacol* 25:1971, 1976
9. Majumdar R, Balasubramanian AS: The solubilization of platelet membrane-bound acetylcholinesterase and aryl acylamidase by exogenous or endogenous phosphatidylinositol specific phospholipase C. *Biochem Pharmacol* 34:4109, 1985
10. Low MG, Saltiel AR: Structural and functional roles of glycosyl-phosphoinositol in membranes. *Science* 239:268, 1988
11. Roberts WL, Kim BH, Rosenberry TL: Differences in the glycolipid membrane anchors of bovine and human erythrocyte acetylcholinesterase. *Proc Natl Acad Sci USA* 84:7817, 1987
12. Beauregard G, Roufogalis BD: Characterization of lipid-protein interactions in acetylcholinesterase lipoprotein extracted from bovine erythrocytes. *Biochem J* 179:109, 1979

13. Sihotang K: Acetylcholinesterase and its association with lipids. *Eur J Biochem* 63:519, 1976
14. Bloj B, Morero RD, Farias RN, Trucco RE: Membrane lipid fatty acids and regulation of membrane-bound enzymes. Allosteric behaviour of erythrocyte  $Mg^{2+}$ -ATPase,  $(Na^+-K^+)$ -ATPase and acetylcholinesterase from rats fed different fat-supplemented diets. *Biochim Biophys Acta* 311:67, 1973
15. Farias RN, Bloj B, Morero RD, Siñeriz F, Trucco RE: Regulation of allosteric membrane-bound enzymes through changes in membrane lipid composition. *Biochim Biophys Acta* 415:231, 1975
16. Morero RD, Bloj B, Farias RN, Trucco RE: The allosteric transitions from membrane-bound enzymes: Behaviour of erythrocyte acetylcholinesterase from fat-deficient rats. *Biochim Biophys Acta* 282:157, 1972
17. Frenkel EJ, Roelofsen B, Brodbeck U, Van Deenen LLM, Ott P: Lipid-protein interactions in human erythrocyte-membrane acetylcholinesterase. *Eur J Biochem* 109:377, 1980
18. Spinedi A, Rufini S, Luly P, Farias RN: The temperature dependence of human erythrocyte acetylcholinesterase activity is not affected by membrane cholesterol enrichment. *Biochem J* 255:547, 1988
19. Rosenberry TL, Scoggin DM: Structure of human erythrocyte acetylcholinesterase. *J Biol Chem* 259:5643, 1984
20. Fambrough DM, Engel AG, Rosenberry TL: Acetylcholinesterase of human erythrocytes and neuromuscular junctions: Homologies revealed by monoclonal antibodies. *Proc Natl Acad Sci USA* 79:1078, 1982
21. Saba SR, Mason RG: Acetylcholine, cholinesterase activity and the aggregation of human platelets. *Proc Soc Exp Biol Med* 135:104, 1970
22. Shermar RW, Chuang HYK: Acetylcholine-induced aggregation of canine platelets. *Fed Proc* 32:844, 1973
23. Schrier SL: Human erythrocyte membrane enzymes: Current status and clinical correlates. *Blood* 50:227, 1977
24. Llanillo M, Cabezas JA: Isolation, characterization and chemical composition of the membrane from sheep platelets. *Biochim Biophys Acta* 649:229, 1981
25. Barber AJ, Jamieson GA: Isolation and characterization of plasma membranes from human blood platelets. *J Biol Chem* 245:6357, 1970
26. Sánchez-Yagüe J, Llanillo M: Lipid composition of subcellular particles from sheep platelets. Location of phosphatidylethanolamine and phosphatidylserine in plasma membranes and platelet liposomes. *Biochim Biophys Acta* 856:193, 1986
27. Sánchez-Yagüe J, Cabezas JA, Llanillo M: Rearrangement of aminophospholipids in bilayers from sheep platelet plasma membranes and platelet liposomes by increasing their cholesterol levels. *Biochem Biophys Res Commun* 145:1362, 1987
28. Pennington RJ: Biochemistry of dystrophic muscle: mitochondrial succinate-tetrazolium reductase and adenosine triphosphatase. *Biochem J* 80:649, 1961
29. Wroblewski F, La Due JS: Lactate dehydrogenase activities in blood. *Proc Soc Exp Biol Med* 90:210, 1955
30. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265, 1951
31. Ellman GL, Courtney KD, Andres V, Featherstone RM: A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 7:88, 1961
32. Lineweaver H, Burk D: The determination of enzyme dissociation constants. *J Am Chem Soc* 56:658, 1934
33. Dixon M, Webb EC: *Enzyme Kinetics*. London, England, Longman, 1964, p 54
34. Lovette KM, Chuang HYK, Mohammad SF, Mason RG: The subcellular distribution and partial characterization of cholinesterase activities of canine platelets. *Biochim Biophys Acta* 428:355, 1976
35. Harris GLA, Crawford N: Isolation of pig platelet membranes and granules distribution and validity of marker enzymes. *Biochim Biophys Acta* 291:701, 1973
36. Rittenhouse-Simmons S, Deykin D: Isolation of membranes from normal and thrombin-treated gel-filtered platelets using a lectin marker. *Biochim Biophys Acta* 426:688, 1976
37. Kinoshita T, Nachman RL, Minick R: Isolation of human platelet plasma membranes with polylysine beads. *J Cell Biol* 82:688, 1979
38. Kambayashi J, Sakon M, Ohno H, Kósaki G: Isolation of plasma membrane vesicles of human platelets by affinity chromatography. *Thromb Res* 21:129, 1981
39. Majumdar R, Balasubramanian AS: Chemical modification of acetylcholinesterase from eel and basal ganglia: Effect on the acetylcholinesterase and aryl acylamidase activities. *Biochemistry* 23:4088, 1984
40. Pletscher A: *Assays in neurochemistry and neuropharmacology*, in Youdin MBH, Lovenberg W, Sharmar DF, Lagnado JR (eds): vol 3. New York, NY, Wiley, 1978, p 49
41. Ravazzolo R, Garré C, Bianchi-Scarrá G, Barresi R, Damiani G, Capra V, Ajmar F: Characterization, localization and biosynthesis of acetylcholinesterase in K562 cells. *Arch Biochem Biophys* 267:245, 1988
42. Taguchi R, Suzuki K, Nakabayashi T, Ikezawa H: Acetylcholinesterase release from mammalian erythrocytes by phosphatidylinositol specific phospholipase C of *Bacillus thuringiensis* and characterization of the released enzyme. *J Biochem* 96:437, 1984
43. Chuang HYK: Cholinesterase activities of intact platelets as measured by an improved radiometric method. *Biochim Biophys Acta* 321:546, 1973
44. Lenz DE, Maxwell DM, Walden MB: Kinetic properties of soluble and membrane-bound acetylcholinesterase from electric eel. *Life Sci* 34:219, 1984
45. Madden TB, Guin PJ: Arrhenius discontinuities of  $Ca^{2+}$ -ATPase activity are unrelated to changes in membrane lipid fluidity of sarcoplasmic reticulum. *FEBS Lett* 107:110, 1979
46. Silvius JR, Read BD, McElhaney RN: Membrane enzymes: Artifacts in Arrhenius plots due to temperature dependence of substrate-binding affinity. *Science* 199:902, 1978
47. Beauregard G, Roufogalis BD: Involvement of calcium ions in the properties of cardiolipin-associated erythrocyte acetylcholinesterase. *Biochim Biophys Acta* 557:102, 1979
48. Barton PL, Futerman AH, Silman I: Arrhenius plot of acetylcholinesterase activity in mammalian erythrocytes and in Torpedo electric organ. *Biochem J* 231:237, 1985
49. Sánchez-Yagüe J, Cabezas JA, Llanillo M: Regulation of acetylcholinesterase associated with platelet plasma membranes by changes in the environment. Joint Meeting of Membrane Groups from Biochemical Society and Sociedad Española de Bioquímica. Bilbao, Spain; July 1989 (abstr 38)