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THE MICROENVIRONMENT AND KINETICS OF HUMAN ERYTHROCYTE ACETYL CHOLINESTERASE

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Human erythrocyte acetylcholinesterase is an externally oriented intrinsic membrane-bound enzyme whose kinetics were studied under a variety of different conditions. Activities of the enzyme in different preparations were measured spectrophotometrically at pH 7.4 and 30°. The enzymic velocity (v), thermal decay constant (λ) and the apparent Michaelis–Menten parameters (Kapp and Vapp) were used as probes to follow changes in the enzyme and/or its microenvironment.

INTRODUCTION

Cholinesterases, i.e. acetylcholinesterase (AChE; EC 3.1.1.7) and pseudocholinesterase (PChE; EC 3.1.1.8), are enzymes that catalyse the hydrolysis of acetylcholine into choline and acetic acid. AChE is found in the nervous tissue of all animals and in the erythrocytes of most of them whereas PChE is found in lesser amounts in the nervous tissue of all animals and in the serum of most of them [1] Erythrocyte AChE was first reported in the stroma of the human erythrocyte [2] and then on its outer surface and in the external half of the lipid bilayer of the membrane [3,4]. Later, the enzyme was purified and shown to be a dimer (mol. wt. 160,000), consisting of two unlike, but equally sized subunits, "a" and "b" [5] (Fig. 4, c). By analogy with other enzymes [6], we speculate that the monomers have different functions e.g. enzymic and allosteric. Subunit "a" may be regarded as an anchoring subunit with an anionic site and an esteratic site. Subunit "b" may be termed as the allosteric or regulatory subunit of the enzyme with an allosteric site.

Recently, it has been claimed that the enzyme is a glycolipoprotein [7]. Amino acid analysis demonstrated that the enzyme contains all the common amino acids including half-cystine [7]. The carbohydrate portion of the enzyme was shown to consist of mannose, galactose, glucose, glucosamine, galactosamine and sialic acid [7]. The detection of small amounts of phospholipids and cholesterol in purified AChE supported previous claims [e.g. 8].

The detailed kinetics of the enzyme were studied both in *in vitro* and *in vivo* in apparently normal and abnormal subjects of different ethnic origin. The thermal stability, initial velocity and apparent Michaelis-Menten parameters of the enzyme [9] were used as probes to detect changes in the behaviour of the enzyme.

In general, the kinetics of the enzyme were found very complex, because: (1) they are not immutable [10] and (2) they depend on the intactness of the cell or the microenvironment of the enzyme in the membrane [11,12]. We, therefore, developed a model (see under Results and Discussion) which interprets the above changes and also some of the enigmatic or ambiguous problems associated with this or other externally–oriented membrane–bound enzymes.

MATERIALS AND METHODS

Preparation of Washed Erythrocytes. Whole blood was collected by cubital venepuncture into heparinized tubes from healthy and abnormal individuals (with their informed consent) of different ethnic groups. Erythrocytes were washed four times with 4 vol. of ice-cold 0.9 % (w/v) NaCl [9].

Age-dependent separation of erythrocytes was performed by a modification of the method of Murphy [13]. Fresh heparinized blood (5–10 ml) was centrifuged (room temperature) twice at 2200 x g for 5 min to remove the plasma and buffy coat. Then the packed cells were mixed with the plasma and the suspension was centrifuged at 30,000 x g for 1 hr at 30°, using a Sorvall superspeed R C-B automatic refrigerated centrifuge fitted with an SM rotor. The plasma was removed and the resulting red cell column was separated into three fractions: young cells (top 10 %

cells), mature cells (middle 80 % cells) and old cells (bottom 10 % cells). The cells were washed four times with 4 vol. of ice-cold 0.9 % NaCl and then kept at 4^{o} until required.

Preparation of Enzyme. Stock haemolystate was prepared by adding 0.06 ml of packed washed erythrocytes to 100 ml ice-cold 5mM potassium phosphate buffer, pH 7.4. After 30 min the stock haemolysate was diluted with an equal vol of ice-cold phosphate buffer (0.195 M, pH. 7.4).

Erythrocyte ghosts were prepared according to Dodge *et al.* [14] Triton X-100 (1.8 ml/100 ml) was used to solubilize the membrane–bound enzyme protein in the ice-cold buffered preparations (haemolysate or ghosts, pH 7.4). The whole cell suspension was prepared by suspending 0.03 % (v/v) of packed washed erythrocytes in potassium phosphate buffer (0.1 M, pH 7.4, 4^{o}). The suspension, before assaying, was usually stored at 4^{o} for 4 hr , this was done because stored cells did not settle in the cuvette for at least 4 min.

AChE was extracted from intact erythrocytes (0.05–2% of packed cells) in hypertonic media e.g. Sucrose and NaCl (0.5 M, pH 5–6, 37^{o}). The supernatant obtained by centrifugation at 4^{o} (30,000 x g, 1hr) was used for enzymic activity.

In heating experiments, the enzyme was incubated in 5mM or 0.1M potassium phosphate buffer of pH 7.4 at 60^{o} for different periods of time, then kept on ice for 15–120 min and assayed. In some experiments, physostigmine sulphate ($1x10^{-2}$ to $1x10^{-6}$M) was used. In each separate experiment assays at zero time were run as controls.

Estimation of Enzymic Activity. The enzyme was assayed in the above preparations at pH 7.4 and 30^{o} with acetylthiocholine as substrate by using a spectrophotometer linked to a chart recorder [9].

Statistical Treatment of Data. To minimise experimental errors, all assays were performed in replicates either in a direct systematic order or in Latin Square design [15]. The data were processed by computer programs written in Fortran and run on the Edinburgh Multi-Access System. Apparent Michaelis-Menten parameters (Kapp and Vapp) were computed by non-linear unweighted least squares [16]. The thermal decay constant (λ) was calculated by fitting a single exponential to the data [17]. In addition, computer simulations were used to find out the degree of variability in the kinetics of the enzyme that could be caused by experimental error.

RESULTS AND DISCUSSION

In general, the kinetics of the human erythrocyte AChE were found to change, for example, on washing and lysing the cells, on storage and heating, and with ionic strength and the age of the cells. The present model was, therefore, proposed which describes mainly the precise orientation and interaction of the enzyme in the membrane and interprets the changes in the kinetics of the enzyme which could be used as sensitive tool to signal changes in the organization of the membrane and the enzyme embedded in it.

State of the Enzyme in the Membrane. The enzyme is described as an elongated structure [18]; however, in the membrane the molecules of the enzyme appear to be spherical in shape and dimeric [19]. They are presumed to be studded in the outer lipid bilayer of the membrane [4] with their active centres exposed outward [20] either as individuals or in chains, or as large aggregates [21]; the latter two cases or several other assemblies of the enzyme molecules may be presumed to exist under abnormal circumstances such as deepfreezing or heating. Nonetheless, the enzyme may be called a periphrointegral glycolipoprotein. A conceptual ultrastructure of the human erythrocyte is given in Fig. 4 (a).

Molecular Interactions of the Enzyme in its Microenvironment. The enzyme being a glycolipoprotein [22] may undergo five sets of interactions in the aggregated form in the membrane between (1) enzyme molecules (2) enzyme-non-enzyme protein (3) enzyme-lipid (4) enzyme-carbohydrate and (5) enzyme-external environment of the enzyme (Fig. 4b).

The distal end of the enzyme molecule burried in the outer lipid bilayer of the membrane is presumed to be held by hydrophobic interactions (6). Such interactions are thought to cause a change in the conformation of the enzyme when exposed to cold [6]. For example, when the erythrocyte membranes were solubilized or stored at -20^{o} for 24 hr (Table 1) the activity of the enzyme fell, its Kapp rose, but its Vapp remained unchanged.

Similarly in cholesterol–depleted membranes (Table 2) the Vapp of the enzyme was unaffected, but its Kapp declined indicating that *in vitro* a decrease in the membrane fluidity [23] increases the interaction between the enzyme and its substrate. In contrast when the enzyme, whether membrane bound or solubilized was suspended in low ionic phosphate buffer (5mM pH 7.4) heated at 60^{o}, cooled on ice and then assayed its activity oscillated (Fig. 1). The origin of the oscillation is not clear. However, it is possible that interaction of the enzyme molecules at their active sites which are thought to be exposed outward [20] could result in such oscillations.

The side groups of the hydrophobic amino acids (e.g. Val, Leu and Phe) of the enzyme are likely to be directed

Table 1. Effect of storage and solubilization on A ChE.

The enzyme (haemolysate) was either stored (-20^{o}, 24hr) or solubilized in Triton X-100 (1.8 %) and then assayed in replicates.

Haemolysate	Mean ± S.E. (units)		Kapp ± S.E. (μM)	Vapp ± S.E. (units)
Untreated	10.6 ± 0.3	35.1 ± 0.9	64 ± 7	44 ± 1.0
Deep-Frozen	6.7 ± 0.7	30.4 ± 1.2	112 ± 9	44 ± 1.8
Solubilized	4.8 ± 0.6	25.3 ± 1.1	146 ± 9	40 ± 1.5

Values in column 1 and 2 were obtained at substrate concentration 20 and 250 μM respectively.

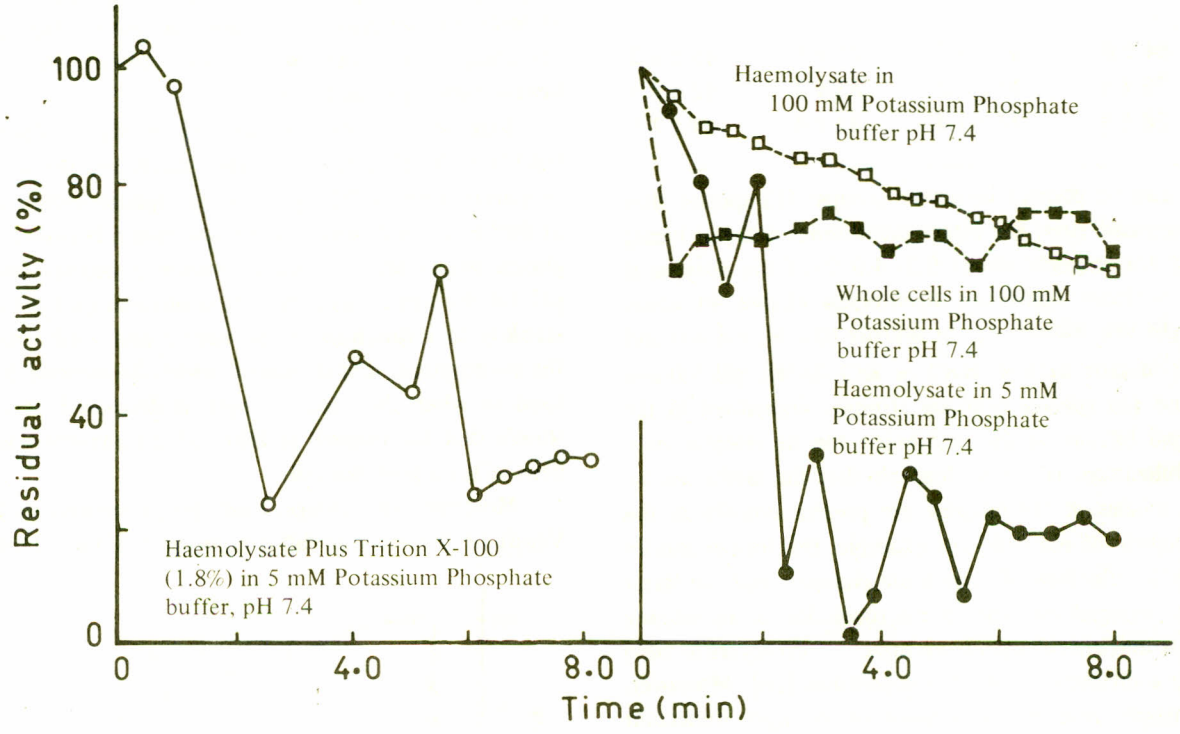


Fig. 1. Relationship between ionic strength and thermal stability of AChE enzyme preparations were prepared from freshly-drawn washed erythrocytes, heated at 60^{o} for up to 8 min, cooled on ice for 2hr and then assayed.

towards the interior of the molecule and its middle may be bounded with other membrane proteins. The proximal end and the N-terminal ends of the two subunits of the dimer [5] may be linked covalently, as in MN glycoproteins, to sialic acid residues and exposed to the external environment of the cell (Fig. 4, c). It is emphasized that the superior peripheral hydrophilic binding with sialic acid residues and the superior side chains of amino acids as well as the catalytic and non-catalytic combining sites of the enzyme moelcules are exposed to the external aqueous environment of the cell.

The Molecular Nature of the Enzyme. Genetic variants of the enzyme were sought. Our results (Table 3) and those of others [24,25] have failed to demonstrate that there are variations between individuals. However, the presence of different molecular forms of the enzyme in the membrane is a matter of conjecture. In the intact erythrocyte the enzyme seems to have labile and stable forms, as depicted from Fig. 2. The labile species of the enzyme comprise about 30 % of the total and are inactivated when the cells are exposed to any stimulus that perturbes the membrane such as lysis or brief heating. The stable species of the

Table 2. Effect of erythrocyte-membrane cholesterol depletion on AChE.

Freshly-drawn washed erythrocytes after they had been incubated at 30° in unheated plasma or plasma heated at 60° for 10 min were re-washed, lysed and assayed.

Values are relative (%) Means ± S.E.M. (2)

Incubation time (hr)	Unheated plasma		Heated plasma (60° /10 min)	
	Kapp (μM)	Vapp (units)	Kapp (μM)	Vapp (units)
0	84 ± 6	42 ± 1.7	85 ± 5	43 ± 1.3
1	78 ± 5	41 ± 1.2	83 ± 3	42 ± 0.9
3	58 ± 3	42 ± 1.4	83 ± 4	44 ± 2.1

enzyme can be divided into two groups: (1) species that can be released from the intact membranes without lysing the cells; for example about 0.2–4.0 % of the enzyme is released without significant haemolysis (Table 4) when whole cells are washed e.g. in sucrose (0.5 M, pH 6.0 and 37°), or in salts such as NaCl or KCl (0.5M, pH 5.0 and 37°); and (2) species that are strongly embedded in the outer lipid bilayer of the membrane can be released only on solubilization. It is not clear whether the labile and releasable species of the enzyme are present equally in the young, nature of old cells; for example, the former species could be a reflection of membrane changes, and the latter the same integral enzyme in microvesicles or membrane fragments that are released from intact erythrocytes *in vitro* and have a different chemical composition [26]. Moreover, on electrophoresis the occurrence of multiple molecular forms of the enzyme has been interpreted as a result of either polymerization of the monomers of the presence of polymorphic forms of the enzyme [27,28]. The question as to whether or not there is a single form of the enzyme in the membrane is clearly complicated by the possibility that the membrane affects the expression of enzymic activity.

Membrane Changes in Health. There seems to be a correlation between membrane changes and the structure and behaviour of the enzyme. Preliminary experiements indicated that when cells are heated or lysed, some of the kinetic properties of the enzyme change. For example, when fresh cells are heated at 60° for up to 5 min, they lose about 30 % of activity; the Vapp declines (12 %) and the Kapp rises (32 %) (See Fig. 2). It seems that heating either alters the organization of the enzyme protein or the membrane. The behaviour of the enzyme to heating was different in intact cells and in the haemolysate; for example, in the former case the enzyme was more resistant to heating than in the latter. These results (Fig. 2) could be due to heat–dependent alterations in the membrane because heat-induced changes in the shape of the cell or in the membrane were also demonstrated microscopically. Moreover, the enzyme was found more sensitive to inhibition by phosostigmine (eserine) in isolated membranes that in intact erythrocytes (Fig. 3). The reason could be that in intact erythrocytes the enzyme molecules are burried in the outer lipid bilayer and thus protected. Similary, when cells are lysed, they lose about the same amount of activity (Fig. 2); the Vapp falls (11%) and Kapp rises (45%). It appears that on lysis the membranes lose (or have altered) the components essential to protect the enzyme from the heat or inhibitor inactivation.

Estimates of the half life ($t_{½} = \ln 2/\lambda$; where ln2 = 0.693) of AChE in haemolysates, ghosts and the solubilized preparation (in 0.1M-potassium phosphate buffer, pH 7.4) at 60° were 38,29 and 3 min respectively. However, for the ghosts suspended in 5mM-potassium phosphate buffer of pH 7.4 the value was 2 min. It is obvious that the thermal stability is a function of the nature and ionic strength of the preparation; it is decreased when the enzyme is solubilized or when the ionic strength is decreased. It was also shown that the thermal stability of the enzyme is independent of the age of the cell.

Moreover, on storage, both the parameters (Kapp and Vapp) of the enzyme fall progressively [10b]. This fall in

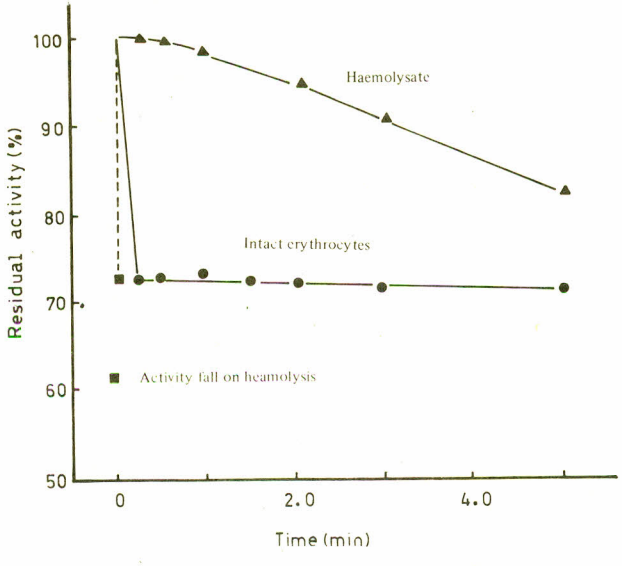


Fig. 2. Effect of haemolysis and heating (60°) on activity of AChE in freshly-drawn washed erythrocytes.

Table 3. The Kinetic constants and thermal stability of AChE from different healthy individuals.

Values are means ± S.D. in the upper row, and the coefficient of variation CV (%) and number of haemolysates assayed are in parentheses in the lower row.

The computer simulations were carried out by setting Kapp = 100 μM. Vapp = 40 units (1 unit = 1 μM/min per g.Hb) and using the known CV of the assay at 20 and 250 μM-substrate 4.4% and 3.0% respectively.

	Kapp (MM)	Vapp (units)	λ_{-1} (min)
Individuals	83 ± 6.2 (7.5 %, 23)	44 ± 4.2 (9.5 %, 23)	0.017 ± 0.002 (8.7 %, 23)
Single subject	92 + 4.5 (4.9 %, 10)	42 ± 2.5 (5.9 %, 10)	0.016 ± 0.001 (4.9 %, 10)
Computer	100 ± 4.0 (4.3 %, 500)	40 ± 1.16 (2.9 %, 500)	Not done

Table 4. Effect of media on the extraction of proteins from intact erythrocytes.

Extraction of A ChE and other proteins from freshly-drawn washed erythrocytes was carried out at 37° and pH 5.0 (salts) or 6.0 (sucrose).

Extraction media	Release of component		
	Haemoglobin (%)	A ChE (%)	Total proteins (μg/0.05 ml packed cell)
NaCl (M)			
0.15	0.75	0.16	8.0
0.25	0.91	0.17	12.0
0.35	0.94	0.19	14.0
KCl (M)			
0.15	0.11	0.27	1.2
0.25	0.15	0.29	1.4
0.35	0.19	0.35	2.1
Sucrose (M)			
0.31	0.18	2.80	3.5
0.62	0.18	2.82	4.0
0.78	0.19	3.19	8.9

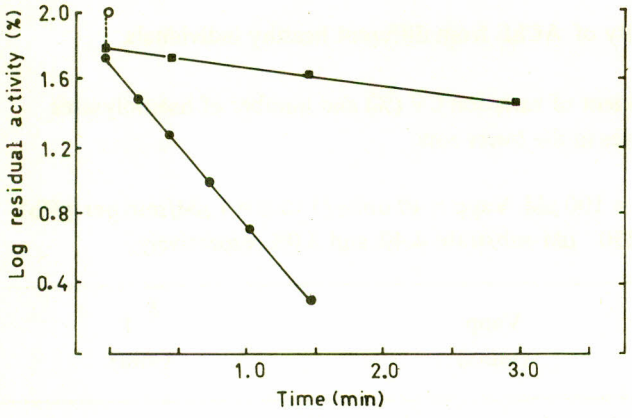


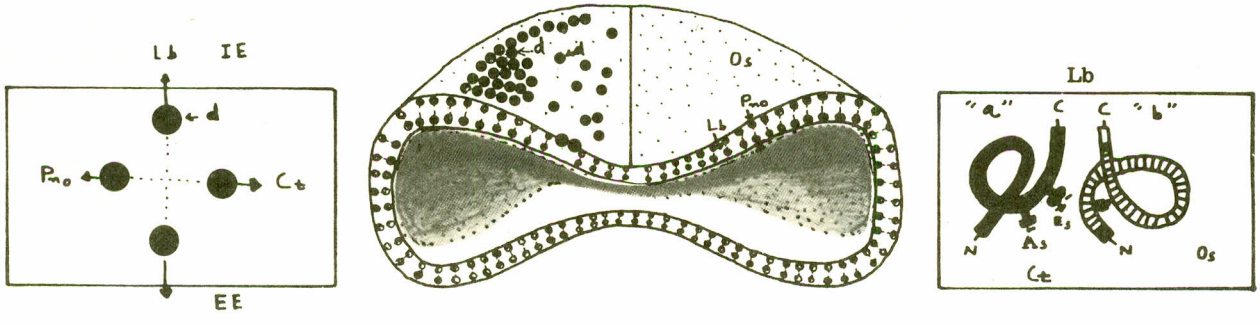
Fig. 3. The Time courses of inhibition of AChE by Physostigmine. Whole cell suspension (▪) and haemolysate (•) were assayed in triplicate before (controls = 0) and after they had been incubated with Physostigmine (1μM).

the parameters is interpreted as manifestation of the storage-dependent loss in the non-Michaelian (allosteric) behaviour of the enzyme. The behaviour of the enzyme is dependent on the age and nature of the preparation, e.g. it decreases with time in the source in the order: erythrocytes > haemolysate > ghosts > Tritontreated haemolysate.

Membrane Changes in Disease State. It is, in fact surprising that for the erythrocyte membrane which contains more than a dozen enzymes abnormality has been reported only in AChE [29]. This suggests that the biochemical status of the AChE is more closely linked to that of the erythrocyte membrane than the others. A survey of the literature indicated that in more than a dozen abnormalities there is low activity of the enzyme [10,a]. The cause for the subnormal activity of the enzyme is a matter of contradiction. Possibilities include: (1) coating of the enzyme molecules by immunoglobulin antibodies [30]; (2) loss of membrane constituents essential for activity [30]; (3) loss of the enzyme molecules in circulation from the membranes [31]; (4) a fall in circulating reticulocytes (Normal or defective) [32]; and (5) an altered composition of the memrane with regard to lipid (it is likely that only lipids firmly bound to the enzyme influence the enzyme activity [33], or a change in the fluidity of the membrane which influences the characteristics of the enzyme [34].

Inter-day Variation. Day to day variation in the kinetic parameters and thermal stability of the enzyme from a single subject was observed over a period of three years. The variation was usually statistically not significant; however, in some instances it was considerable. The possiblities must be considered that the variation is a real one, and not just due to some sort of systematic experimental error [35]. The magnitude of the error was reduced when solubilized AChE, or for comparison, the water-soluble enzyme horse liver alcohol dehydrogenase (10b) were assayed suggesting that part of the variation could be due to changes in the composition of the membrane.

Diurnal Variation. Some times huge biological oscillations, notably of circadian or semi-circadian nature, in the activity of the enzyme from a single individual have been reported [36]. The oscillations have been observed in both normal and pathological cells, either *in vivo* or *in vitro*. We



(b) Molecular interactions in aggregated AChE molecules

(a) Conceptual ultrastructure of erythrocyte membrane

(c) Subunit structure of a dimer of AChE

Fig. 4. (a). Shows a conceptual ultrastructure of erythrocyte where on a portion of its outer surface (Os) is seen a distribution of individual or aggregated spherical dimers of AChE(d) and on its inner membrane surface (Is) internally located particles. (b). Shows interactions: (1) between dimers of AChE in an aggregate and (ii) between a dimer and its chemical microenvironment constituting e.g. non-enzymic protein coat on the outer surface of the lipid bilayer (Lb) and the externally oriented carbohydrate (Ct) as well as lipid of Lb. The Pni indicates the non-enzymic protein coat on the Is of Lb. EE and IE indicate the extracellular and intracellular environments respectively. (c). Shows a dimer of AChE. The sub unit'a' contains an anionic site (As) and an esteratic site (Es); the latter further comprises an electrophilic esteratic site (+) and a nulcleophilic esteratic site (−). The sub unit'b' contains an allosteric site (Als). C and N indicate the corboxyl and amino terminals of the sub units respectively.

repeated these experiments and concluded that the variations of the kind could be partly due to the experimental error and partly due to membrane dependent changes in the kinetics of the enzyme [37].

Role of the Enzyme. The exact role of the erythrocyte membrane is still obscure [38]. However, there have been some reports indicating that the enzyme is involved in the active transport of K ions in erythrocytes [39,40]. To test this, experiments were done with normal erythrocytes and with erythrocytes which were known to leak K ions [41]. Our results indicated that there is no direct relationship of the enzyme with transport of K ions in erythrocytes; however, there could be another transport mechanism in the membrane which seems to depend on changes in the conformation of the enzyme. We presume that such changes in the conformation of the enzyme may be involved in the passive trans-membrane transfer of ions (cations and/or anions) in erythrocytes. It is based on the fact that the enzyme is allosteric in nature, very sensitive to ions and the orientation is such that part of the molecule containing the active centre is exposed and the rest is presumed to be embedded in the outer lipid bilayer of the membrane. The events that lead to modulation of ion channels and the exchange of ions across the membrane could be put in the order: A change in conformation in the external half of the enzyme molecule at the interaction with the inhibitor or activator (substrate) – the transfer of the change to the internal half of the membrane-structural rearrangement of the membrane-modulation of ion channels-exchange of ions. A similar regulatory role of AChE in the transport of anions in human erythrocytes as described recently [42] supports the above mechanism.

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REFERENCES

1. K.B. Augustinsson, Acta Physiol. Scand., **15**, 1 (1948).
2. J. Metz. B.A. Bradlow, S.M. Lewis and J.V. Dacie, Brit. J. Maematol., **6**, 372 (1960).
3. B.D. Firkin, R. W. Beal and G. Mitchell, Australian Ann. Med., **12**, 6 (1963).
4. T.L. Steck, J. Cell. Biol., **62**, 1 (1974).
5. T. Shafai and J.A. Cortner, Biochim. Biophys. Acta, **236**, 612 (1971).
6. E.R. Stadtman, *In Advances in Enzymology,* edited by F.F. Ford (Interscience Publishers, John Wiley and Sons, New York, 1966), vol xxviii.
7. E. Niday, C.S. Wang and P. Alaupovic, Biochim, Biophys. Acta, **469**, 180 (1977).
8. K. Sihotang, Eur. J. Biochem., **63**, 519 (1976).
9. S.F. Mabood, P.F. Newman and I.A. Nimmo, Biochem. Soc. Trans., **5**, 1540 (1977).
10. (a) S.F. Mabood. (M. Phil Thesis, Edinburgh University, 1979).
    (b) S.F. Mabood. P.F. Newman and I.A. Nimmo, Biochem. Soc. Trans., **7**, 924 (1979).
11. B. Aloni and A. Livne, Biochem. Biophys. Acta, **339**, 359 (1974).
12. I.D. Volotovakii. LM. Sheiko and S.V. Konev. *In Enzymes*, Vol. I, edited by P.D. Boyer, H. Larely and K. Myraback (Academic Press, New York, 1959).
13. J.R. Murphy, J. Lab. Clin. Med., **60**, 86 (1962).
14. J.T. Dodge, B. Mitchell and D.J. Hanahan, Arch. Biochem. Biophys., **100**, 119 (1963).
15. R.A. Fisher and F. Yates, Statistical Tables for Biological, Agricultural and Medical Research, 6th Edn., Table IX. p. 69 (1963).
16. G.N. Wilkinson, Biochem. J., **80**, 324 (1961).
17. I.A. Nimmo and G.L. Atkins, Biochem, Soc. Trans., **6**, 548 (1978).
18. J. Massoulie, F. Rieget and S. Bon, Eur. J. Biochem., **21**, 542 (1971).
19. G. Ciliv and T.P. Ozand, Biochem, Biophys. Acta, **284**, 136 (1972).
20. M.G. Low and B. Finean, FEBS Lett. **82**, 143 (1977).
21. M. Brocheck, P. Ott and J. Wiedmer, Croatioa Chemica Acta, **47**, 201 (1975).
22. O.J. Bjerrum and T. C. Hansen, Biochem. Biophys. Acta, **455**, 66 (1976).
23. T. Shiga, N. Maeda, T. Suda, K. Kon and M. Sekiya, Biochim. Biophys. Acta, **553**, 84 (1979).
24. P. Ozand, S. Artivinli and S. Yatimagan, Turk. J. Pediat.,**12**, 1 (1970).
25. F. Herz and E. Kaplan, Brit. J. Haematol., **26**, 165 (1974).
26. R. Coleman and G. Holdsworth, Biochim. Piophys. Acta, **426**, 776 (1976).
27. D.L. Wright and D.T. Plummer. Biochem. J., **133**, 521 (1973).
28. P.Ott, B. Tenny and U. Brodbeck, Eur. J. biochem., **57**, 469 (1975).
29. F. Herz and E. Kaplan, Pediat. Res., 7, 204 (1973).
30. G.L.M.B. Scott and M.R. Rasbridge, Scand. J. Haematol., **8**, 53 (1971).

31. S.Shrier, Blood, **50**, 227 (1977).
32. J. Metz, B.A. Bradlow, S.M. Lewis and J.V. Dacie, Brit. J. Haematol., **6**, 372 (1960).
33. J. Siriwattayakorn and Y. Yuthavong, Brit. J. Haematol., **41** 383 (1979).
34. S.S. Kaplay, Ind. J. Biochem. Biophys., **12**, 284 (1975).
35. D.J. Hanahan and J.E. Ekholm, Arch. Biochem. Biophys., **187**, 170 (1978).
36. Y.E. Ashkenazi, H. Hartman, B. Strulovitz and O. Dar, J. Interdiscip. Cycle Res., **6**, 291 (1975).
37. S.F. Mabood, P. F. Newman and I.A. Nimmo, Biochem. Soc. Trans., **6**, 305 (1978).
38. F. Herz and E. Kaplan, Proc. Soc. Exp. Bid. Med., **140**, 720 (1972).
39. M.E. Greig and W.C. Holland, Arch. Biochem., **23**, 370 (1949).
40. I.M. Taylor, J.M. Welle and A.B. Hastings, Amer. J. Physiol., **168**, 658 (1952).
41. G.W. Stewart, R.J.M. Corrall, J. A. Fyffe, G. Stockdill and J.A. Strong, Lancet, **2**, 175 (1979).
42. V.S. Finin, I.D. Voltovsky and S.V. Konev, Biojizika, **24**, 96 (1979).