LIPID PROTEIN INTERACTIONS IN BOVINE
ERYTHROCYTE ACETYLCHOLINESTRASE

by

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ABSTRACT

Involvement of lipid in the activity of mammalian erythrocyte acetylcholinesterase (AChE) has been proposed by various workers. In bovine erythrocyte AChE a tightly bound fraction of cardiolipin (CL) was proposed to be involved in the modulation of AChE catalytic activity. Methods previously used for the isolation of CL resulted in enzyme denaturation. In the present study various methods for the separation of CL under non-denaturing conditions have been investigated.

It was reported earlier that all the lipoprotein forms of the enzyme containing CL showed a biphasic Arrhenius plot with a break around 20°C. It was suggested that treatment of the enzyme with 1.8M sodium chloride, 2mM sodium phosphate, pH 7.4 or 1M sodium bicarbonate pH 8-10, caused dissociation of cardiolipin and it was accompanied by abolition of the Arrhenius plot break. Methods used for the separation of CL from AChE were based on the difference in size and density between the two components. Enzyme was treated with "high salt" conditions which were postulated to cause dissociation of CL. The resulting mixture was passed through a Sephadex gel column so that CL can be separated from the enzyme because of its size difference. The enzyme obtained from the Sephadex gel column gave a partial specific volume of 0.81 ml/g, which is higher than that expected from the amino acid composition.
of the protein, indicating that CL is still bound to the enzyme.

In another experiment ("flotation experiment") an attempt was made to separate the dissociated CL from the enzyme on a sucrose gradient, based on the density difference between the phospholipid and protein. Arrhenius plots were obtained at different time intervals on the enzyme recovered from the sucrose gradient. A linear Arrhenius plot was observed after 24 h. Storage of the enzyme for 5 to 8 days gave rise to a distinct break in the Arrhenius plot. The reappearance of the break was observed even when the centrifugation was done in the presence of 0.09% Triton X-100. This was interpreted to indicate that endogeneous CL was bound to the enzyme through ionic and hydrophobic interaction. "High salt" treatment may abolish the ionic interaction, causing "functional dissociation" of CL (as shown by disappearance of the Arrhenius plot break) but simultaneous strengthening of the hydrophobic interactions may account for the reappearance of the break.

The next method attempted for the separation of CL was based on the principle that, if enzyme could be bound to a solid support, then washing of the enzyme with chaotropic agents, detergents and "high salt" may result in the release of CL. The suitability of the N-methylacridinium (MAC) affinity column as a solid support for this enzyme was investigated. The choice of MAC as an affinity ligand was based on the recent reports regarding its suitability as an affinity ligand for purification of eel and pig brain AChE. The elution profile of the enzyme in 0.1M NaCl, 20mM sodium phosphate, pH 7.4
at different ligand (MAC) concentrations indicated that a minimum of 2.8 umole/ml gel required for sufficient retention of the enzyme. As the affinity of the ligand for the enzyme will further decrease with increasing ionic strength, the MAC affinity column is unsuitable as a solid support for bovine erythrocyte AChE. It was found that the lower retention of the bovine erythrocyte AChE compared to the eel enzyme on the MAC affinity columns was due to the lower affinity of the former for the ligand, rather than to any structural difference or a different mode of binding. Preliminary findings suggest that CL dissociation did not alter the affinity of the enzyme for the ligand.

Finally, as a primary requirement for the preparation of large quantities of pure AChE, so that CL can be exchanged by a detergent exchange method, various steps for the purification of the native forms of AChE by a detergent free method have been characterized. The following findings were made. Butanol treatment enhanced the enzyme release from the membrane, from 40 to 80 percent, by extracting the "mobile" phospholipids. The extraction of AChE can be increased by increasing the ionic strength of the medium and by calcium chelation. Purification of the above enzyme can be achieved by affinity purification but optimum conditions required for the above purification are still under investigation. Characterization of the molecular forms of the enzyme on sucrose density gradient indicates extensive aggregation at low ionic strength, while a lower degree of aggregation with a prominent 11S peak was observed in the presence of 0.1M sodium chloride, 20mM sodium phosphate.
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INTRODUCTION

Acetylcholinesterase (AChE) plays a critical role in terminating the action of the neurotransmitter, acetylcholine, liberated at cholinergic synapses (1,2). Recently, this enzyme has also been considered to be a part of the basic excitation unit or "gateway" regulating ion flow (3). AChE has been isolated from a wide variety of sources, including muscle, nerve, electric organ, brain, erythrocytes and others (4,103). Because of the large scale availability of this enzyme in the electric organ of Electrophorus electricus (Eel) and Torpedo marmorata, most work has been done on the enzyme isolated from these sources (5-8).

The lack of complex intracellular organisation in erythrocytes makes them an ideal system for plasma membrane studies. Evidence available so far suggests that AChE is a membrane bound enzyme in erythrocytes (9-12); this makes the enzyme suitable for studying lipid-protein interaction. Sihotang (13) first suggested the requirement of phospholipid for the activity of human erythrocyte AChE. Based on their studies with bovine erythrocyte AChE, Beauregard and Roufogalis (14,15) postulated that tightly bound cardiolipin was involved in modulating the activity of bovine erythrocyte AChE. The separation of cardiolipin from AChE (14,15) was achieved by treatment of the enzyme with "high salt" (1.8M sodium chloride.
and 2mM phosphate) or 1M ammonium hydroxide, followed by chloroform-methanol extraction. The abovementioned conditions of extraction led to enzyme denaturation. In order to obtain a better understanding of the role of cardiolipin, it is essential to separate cardiolipin from AChE under non denaturing conditions. In the present study the following methods for removal of cardiolipin without denaturing the enzyme were examined.

1. "Flotation" method: This method is based on the principle that during centrifugation in a gradient with distinct density regions, the lighter component cardiolipin, which has been postulated to be dissociated from the enzyme in 1M sodium bicarbonate or 1.8M sodium chloride and 2mM sodium phosphate (high salt) will float to the top, while the heavier component, AChE, will stay at the bottom. A somewhat similar technique has been used by Watkins et al. (16) for lipid binding studies with AChE.

2. Gel filtration: Sigma AChE (0.5 mg/ml) was treated with "high salt", conditions which were postulated to dissociate the cardiolipin from the enzyme, and passed through a Sephadex G-200 column equilibrated with "high salt". During this process, because of the large molecular size of the protein, it will appear in the void volume, while cardiolipin being much smaller will be retarded by the column.

The availability of the delipidated preparation of the enzyme would allow examination of the following aspects of the lipid-protein interaction in AChE:
(a) Specificity of the cardiolipin for the enzymatic activity.

(b) Minimum number of phospholipid molecules essential for the enzymatic activity.

(c) Mechanism responsible for the break in the Arrhenius plot; whether it is due to the alteration in the fluidity of the fatty acyl chain or due to alteration in the configuration of cardiolipin from hexagonal (H$_n$) to bilayer phase (17).
Isolation and Purification Of AChE

There are contradictory reports regarding the binding of AChE to membrane. Many workers consider AChE to be an integral membrane protein of the erythrocyte (9-12). The enzyme can be released from human erythrocyte ghosts by detergents (11,12) or high salt treatment (9,12), but high salt treatment doesn't release the enzyme from intact human erythrocyte (10). The above difference can be attributed to an alteration in membrane structure resulting from hemolysis. Bovine erythrocyte AChE appears to be less tightly bound to the membrane as it can also be released from the membrane in a lipoprotein form in hypo-osmotic media (18).

Other methods for the extraction of erythrocyte AChE have also been used recently. Incubation of phosphatidylcholine vesicles with intact human erythrocytes results in the extraction of more than 80% of the AChE with few other outer membrane proteins (20). Treatment of the intact ox erythrocyte with 10 units/ml of phosphatidylinositol-specific phospholipase C causes almost the complete release of AChE (21), indicating that phosphatidylinositol may be involved in the binding of AChE to the membrane.

The introduction of affinity chromatography for the purification of AChE (7,19,25) has completely eclipsed more
conventional methods, such as ammonium sulfate precipitation (24), gel chromatography (23) and ion exchange chromatography (22) as a method of choice for the purification of this enzyme. Berman and Young (25) purified the eel and bovine erythrocyte AChE to a specific activity of 10,000 and 3,500 units, respectively, using N-trimethyl(aminophenyl)ammonium (PTA) derivative as an affinity ligand. For optimum retention the two enzymes require different isomers; while the eel enzyme is optimally retained with the para-amino isomer, bovine AChE requires the meta-isomer for retention. Niday et al. (19) purified the human erythrocyte AChE to a specific activity of 418 units/mg by double chromatography on a PTA column. A ten-fold higher specific activity of the AChE from human erythrocyte was reported by Ott and Brodbeck (26) (3500-4000 units/mg) using detergent solubilisation and ammonium sulfate precipitation. This high specific activity was attributed by Niday et al. (19) to a ten-fold underestimation of protein due to the presence of Triton X-100.

The main drawbacks of the PTA ligand are its low affinity for AChE at high ionic strength (28) and a high ionic strength dependence of the affinity of the ligand for AChE (27). The use of N-methylacridinium (MAC) as an affinity ligand (28) overcame the above problems with eel AChE. Recently, Reavill and Plummer (29) have also reported the suitability of this ligand for the purification of pig brain AChE. In the present
study the use of MAC for the purification of bovine erythrocyte AChE has been investigated.

**AChE Structure**

Most of the present knowledge of AChE structure is based on the enzyme obtained from eel electric organ. A fresh solubilised preparation of eel AChE consists of a mixture of three forms of the enzyme (6), termed A, C and D, with sedimentation coefficients of 9.2S, 14.2S and 18.4S. The asymmetric nature of the three forms were shown by electron microscopy (6) and column chromatography (30). In Rosenberry's model (fig. 1, in which is shown the D form) the A, C and D forms consist of one, two or three tetramers, respectively, attached together by a collagen like tail (8). The tail has been implicated in binding to the synaptic cleft (31) and in aggregation (32).

The appearance of the multiple molecular forms of AChE has been shown in enzymes obtained from other sources such as rat diaphragm (133), mammalian brain (33,120) and erythrocytes (34). In rat brain (33) the origin of multiple molecular form of AChE was found to depend on the ionic strength of the microenvironment. Ott and Brodbeck (34) showed that the sedimentation profile of the human erythrocyte AChE depended on the condition of study. In the presence of Triton X-100 a single molecular form with a sedimentation coefficient of 6.3S is obtained, while in the absence of detergent five forms of the enzyme varying from 6.3S to 16S were observed. Triton X-100 is very often used in the extraction of
Fig. 1. Model of the 18S asymmetric form of eel electric organ AChE as proposed by Rosenberry and Richardson (8).
erythrocyte AChE and the fact that Triton X-100 can cause
dissociation of multiple molecular forms into dimers in vitro,
leaves the question of the nature of the native form of AChE
in erythrocyte membrane unanswered.

The physiological significance of the appearance of
various multimeric forms of AChE is unknown. Appearance of a
new molecular form of the enzyme in rectal tissue was seen
under the condition of Hirschsprung's disease (35). In birds and
mammals, the asymmetric 16S form localised in muscle was
found to disappear on denervation (36). Different molecular
forms of Electrophorus, Torpedo, rat and chicken were found
to be catalytically equivalent (37). Similar turnover rates
for globular and asymmetric forms of the eel enzyme (37)
suggests that quaternary interactions among the catalytic
subunits or with the tail do not influence the catalytic
efficiency. The catalytic domain was found to be relatively
insensitive to the tertiary structure of the enzyme.

Subunit structure of erythrocyte AChE: Berman and
Young (25) reported the dissociation of a partially purified
bovine AChE of apparent molecular weight 200,000 to two
unequal subunits of molecular weight 126,000 and 75,000 on
SDS gel electrophoresis. Both of these subunits contained
one catalytic active site. Shafai and Cortner (39) have also
reported the presence of two dissimilar \( \alpha \) and \( \beta \) subunits from
eel AChE. Except for the above two reports, most of the
other findings (19,40,132) support the presence of equivalent
subunits in various molecular forms of the enzyme. Bellhorn et al. (40) and Niday et al. (19) found the molecular weight of human erythrocyte AChE on SDS gel electrophoresis to be 180,000 and 160,000, respectively, which dissociated into two equal subunits of 90,000 and 80,000 in SDS and mercaptoethanol. Niday et al. (19) and Ott et al. (34) reported the human erythrocyte enzyme to be glycoprotein in nature. The lipoprotein form of the bovine erythrocyte enzyme obtained by Lubrol WX solubilisation (15) was found to have a molecular weight of 156,000 with a sedimentation coefficient of 7.3S and Stokes radius of 6nm.

In summary, erythrocyte AChE appears to be a tailless dimer of molecular weight between 142,000 and 200,000 with a sedimentation coefficient of 6.3-7.3S, which can further aggregate at low ionic strength to less symmetric forms of higher molecular weight.

Lipid-Protein Interactions In AChE

AChE is embedded in the membrane and it catalyses acetylcholine hydrolysis on the external surface of the plasma membrane (9,10). The extent of its membrane association is not clear. Release of bovine erythrocyte AChE by hypotonic shock (15) or by extraction with high salt from human erythrocyte ghosts (9,12) suggests that it is an extrinsic membrane protein. However, other findings, such as the requirement of detergent for extraction of AChE from intact human erythrocytes (10),
the release of AChE in lipoprotein form (12,13,15,19),
extraction of AChE by phosphatidylcholine vesicles (20) and
the release of the enzyme by treatment with phosphatidylinositol
specific phospholipase C (21) suggest that AChE is an integral
membrane protein.

The presence of "tightly associated" lipid with
mammalian erythrocyte AChE has been proposed by various workers
(13-15). Sihotang (13) found that the maximum specific activity
of the released AChE from human erythrocyte was obtained at
a deoxycholate concentration of 60mM; further increase in
deoxycholate concentration resulted in decreased enzymatic
activity accompanied by a simultaneous decrease in the phospho­
lipid concentration. At maximum specific activity the phospholipid
content of the enzyme was found to be 0.3±0.1 ug/ug of protein.
The delipidated enzyme preparation obtained at a higher
deoxycholate concentration can be reactivated by adding back
membrane lipids or phosphatidylserine (13).

In bovine erythrocyte AChE cardiolipin was found to be
tightly bound to the enzyme (14) and based on the following
observations it was suggested to be involved in the modulation
of AChE activity. If the Arrhenius plot was done on "Sigma"
or Lubrol solubilised bovine erythrocyte AChE in distilled water,
a break in the Arrhenius plot was observed; under these
conditions cardiolipin cannot be extracted by chloroform-methanol
treatment (14). Treatment of the enzyme with 1.8M sodium
chloride, 2mM phosphate pH 7.4, 1M sodium bicarbonate pH 8.0
or 0.7M sodium phosphate pH 7.4 abolished the Arrhenius plot
break; these conditions also made possible the extraction of cardiolipin by chloroform-methanol. From a study of the effect of endogenous cardiolipin (43) on various steps involved in acetylcholine hydrolysis, it was postulated that cardiolipin modulated the conformational rearrangement of the enzyme-substrate complex, which is the rate determining step at subsaturating acetylcholine concentrations (43). The tightly bound cardiolipin was proposed to be buried inside the three dimensional structure of the enzyme, rather than forming an annular ring as in cytochrome oxidase (67,90). This was concluded from the inaccessibility of the cardiolipin to phospholipase A2 as well as the failure of high salt treatment to alter the Stokes radius (13,44).

Wiedmer et al. (45) have recently studied the incorporation of bovine and eel AChE in lipid monolayers. An increase in surface pressure due to incorporation of the enzyme was observed both with bovine and eel enzyme. This is contrary to expectations, as eel enzyme is considered to be a peripheral membrane protein. On the other hand, incorporation of bovine AChE was much less than expected from its proposed integral nature. The lower incorporation of bovine AChE was attributed to the aggregation of the detergent-depleted form of the bovine enzyme, thus masking the hydrophobic site in the lipid interaction. Another unaccountable observation was the penetration to equal extent by both globular and asymmetric forms of the eel enzyme, thus making the tail nonessential for lipid binding. This is contrary to the findings of Watkins et al. (16) who showed the specific binding of
sphingomyelin to the tail region of the asymmetric form.

Membrane Structure

The "fluid mosaic" model proposed by Singer and Nicolson (46) regards the biological membrane as a homogeneous two dimensional fluid, with globular proteins floating on or in a "sea of lipid". The basic assumption inherent in the fluid mosaic model is that there is little or no specific intermolecular interactions among membrane components, which is contrary to the present day concept of protein-lipid or protein-protein interactions in the membrane. The above limitation was overcome in the "plate model" proposed by Jain and White (47). According to this model the membrane is a spatial and temporal mosaic of small scale aggregated systems, where the individual plates or island patches (Fig. 2) are in relative motion with respect to each other. This model accounts for the presence of "annular lipid" as well as the coexistence of several phases in the lipid bilayer.

Membrane proteins are classified into two operational categories i) peripheral proteins and ii) integral proteins (48). Peripheral proteins are those that appear to be only superficially bound to the membrane, and their attachment to membrane is brought about by interaction with integral membrane proteins rather than to membrane lipids (48). The result is a weak interaction which can be disrupted by relatively gentle techniques, such as sonication or alteration in ionic strength.
Fig. 2. Plate model of biological membrane as proposed by Jain and White (47)

a) Organized lipid molecules may form discreet plates (white circles) that are separated from each other by regions of relatively disorganized lipids (hatched circles)

b) The organized and disorganized regions are viewed as plates, each having characteristic system properties specified by its components.

c) Various molecules (within the bilayer of biomembrane) interact hydrophobically and are distributed asymmetrically.
Integral membrane proteins on the other hand are more strongly bound to the membrane by direct hydrophobic and ionic interactions with membrane lipids. These proteins require more drastic conditions, such as detergents or organic solvents, for their solubilisation. Table 1 shows some of the basic differences in properties between peripheral and integral membrane proteins.

### Table 1 Criteria for distinguishing peripheral and integral membrane proteins

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<th>Property</th>
<th>Peripheral protein</th>
<th>Integral protein</th>
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<td>Requirements for dissociation from membrane</td>
<td>Mild treatments sufficient: high ionic strength, metal ion chelating agents</td>
<td>Hydrophobic bond-breaking agents required: detergents, organic solvents, chaotropic agents.</td>
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<td>Association with lipids when solubilized</td>
<td>Usually soluble, free of lipids</td>
<td>Usually associated with lipids when solubilized</td>
</tr>
<tr>
<td>Solubility after dissociation from membrane</td>
<td>Soluble and molecularly dispersed in neutral aqueous buffers</td>
<td>Usually insoluble or aggregated in neutral aqueous buffers</td>
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Thermal Phase Transition And Phase Separation In Membrane Lipids

Lipids are capable of existing in two phases a) solid gel phase and b) liquid crystalline phase. In the gel phase, fatty acyl chains are arranged in all-trans conformations (49,114), while in the liquid crystalline phase they show a greater degree of mobility (49,52,114). Conversion from gel to liquid crystalline state is accompanied by lateral expansion and alteration in permeability, compressibility, packing density and thickness of the bilayer.(105).

Phase transitions have been studied in many pure phospholipid systems (52). It has been found that unlike "conventional melting points" which are discontinuous, the gel to liquid crystalline phase transition is continuous. In "melting" the solid and liquid phase coexist in equilibrium only at a single temperature called the melting point, but in a continuous transition, the equilibrium occurs over a wide range of temperature e.g. 1°C in pure bilayer of dimyristoylphosphatidylcholine (109). In a system consisting of a mixture of lipid, such as naturally occurring membrane, the transition is much broader (114). The transition temperature of a pure phospholipid is determined both by the size and nature of the head group and the fatty acyl chain (52,114). Similar to melting point of an organic compound, transition temperature increases with chain length and decreases with the incorporation of a double bond (110,114).

Similar to other melting processes, pre and post transition
phenomenon are associated with the phase transition (53). At all temperatures above zero a solid has defects; the number of defects increases with increasing entropy, which is a function of temperature. The defects can be simple vacant sites or complex arrangements, such as grain boundaries. A grain boundary is the boundary between two crystals with slightly different orientation. The "defect structure" formed during pretransition provides a simple explanation for gel to liquid crystalline phase transition (53). As the temperature approaches that of the transition, small random close packed regions of lipid will form throughout the relatively perfect gel lattice. The liquid crystalline phase will be contained in small islands or domains so that long range order will be retained in the bilayer. As the temperature increases, the area occupied by these regions will grow, until at some point they will coalesce. At this point, the continuity of the gel region is replaced by continuity of the liquid crystalline region, with the consequent loss of rigidity associated with the gel phase. The temperature at which the continuity of gel phase is replaced by liquid crystalline phase is called the transition temperature ($T_c$) (52). The gel to liquid crystalline phase transition exhibits the phenomenon of hysteresis, since the transition from gel to liquid crystalline state is not identical to that from the crystalline to gel state (54).

**Phase Separation**: Binary lecithin mixtures having components which vary by only two carbon atoms, such as
dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine, usually cocrystallise below the transition temperature (Tc). However, when the phospholipids are structurally so different that packing problems arise, lateral phase separation and crystallisation of the individual phospholipids will occur (solid phase immiscibility). Such a phenomenon has been demonstrated in model membranes containing a spin labelled steroid derivative (50). A "vertical" phase separation may also occur below Tc, by preferentially partitioning back into the water phase (51). The separation occurring above Tc (fluid/fluid phase separation) is of greater biological significance.

Limited miscibility above Tc is typically observed for mixtures of phospholipids and nonhydrating lipids, such as triglycerides or cholesterol esters. A lateral phase separation above Tc has also been described for the binary mixtures of dipalmitoylphosphatidylethanolamine and dielaidoylphosphatidylcholine (55).

Factors effecting phase transition and phase separation:

If the phase transition and phase separation were produced only through changes in temperature, it may not be a biologically important phenomenon. However, both of the above phenomenon can be produced by altering any of the following parameters:

a) **Cholesterol**: Exclusion of cholesterol from the "boundary lipid" layer of (Mg$^{2+}$+Ca$^{2+}$)-ATPase has been shown by Warren et al. (68). Recent studies with dimyristoyllecithin and dipamitoyllecithin dispersions indicate that the transition enthalpy decreases linearly with the increase in
the percentage of cholesterol and vanishes at 33 mole percent cholesterol (56). The addition of cholesterol acts as a plasticizer, allowing bilayers to exist in a semifluid state over a wide range of temperature. Cholesterol shows the following order of preference for phospholipid head group: sphingomyelin > phosphatidylcholine > phosphatidylethanolamine (119). In the case of phospholipids with similar head group, cholesterol preferentially partitions into the phospholipid having more unsaturated fatty acyl chains (117).

b) Metallic ions: Alteration in the metal ion concentration seems to be one of the most promising ways for modulating phase transition in biological membrane. Addition of divalent cations like Ca$^{+2}$ to unilamellar vesicles of phosphatidylserine or phosphatidylglycerol gives rise to large planar arrays that roll up to form cylinders (57), and in cardiolipin vesicles Ca$^{+2}$ causes conversion from the lamellar to the hexagonal ($H_3$) phase (17). In charged phospholipids the phase transition is sensitive to the ionisation of the polar groups. Therefore the transition temperatures of phospholipids such as phosphatidylcholine and phosphatidylethanolamine were found to be pH sensitive (52).

c) Protein/lipid ratio: Papahadjopoulos et al. (65) have classified proteins into three categories on the basis of how they effect the thermotropic transition in lipid bilayers.

i) Proteins which increase enthalpy, while the transition temperature is increased or remains unaffected e.g. ribonuclease and polymyxin.
ii) Proteins which drastically decrease both transition
temperature and enthalpy e.g. cytochrome C and A_1 proteins.

iii) Proteins which did not effect the transition
temperature but induce a linear decrease in enthalpy e.g.
apoprotein and gramicidin.

**Boundary Lipid And Other Lipids**

Studies on various membranes including those of
mycoplasma (72), E.coli (73), mitochondria (72) and sarcoplasmic
reticulum (51), using a variety of techniques such as X-ray
scattering (75), electron spin resonance (ESR) (51), and
fluorescent probes (73), have clearly indicated that 20-30%
of lipids in the membrane are immobilised by interaction
with protein, while the rest (70-80%) of the lipids are mobile
or fluid. Analysis of ESR spectra from a series of mixtures
containing various lipid:cytochrome oxidase ratios have been
interpreted in terms of two distinct lipid environments for
the spin probe (67). Hesketh et al. (66) have gone a step
further and classified the bilayer lipids into four zones,
based on the extent of perturbation by (Ca^{2+}-Mg^{2+})ATPase.

Other experiments with cytochrome oxidase lend support
to the concept of boundary lipid. Removing the lipid from
cytochrome oxidase-lipid complexes by acetone extraction is
relatively easy until the complex is reduced to about 0.2 mg
of lipid/mg protein. This is the amount of lipid estimated
to be in the boundary layer (67). Removing this final amount
of lipid, which is enriched in cardiolipin (69), results in
loss of enzymatic activity and in morphological changes (76).
The essential requirement of cardiolipin for the activity of cytochrome oxidase suggested previously (67,76) has been recently refuted by Watts et al. (89), who were able to retain the activity even after substitution of most of the cardiolipin with dimyristoylphosphatidylcholine.

The "boundary layer" of lipid surrounding the protein behaves in many ways analogous to that of the water of hydration (77,111). The boundary lipid doesn't undergo phase transition (73) and its viscosity is intermediate to that of gel and liquid crystalline phase (67,68,113). The equilibrium between the mobile and immobile phase was found to be temperature dependent. The concept of boundary lipid appears to be quite attractive, but except for studies with ESR probes, most of the evidence is indirect and circumstantial. A critical assumption in ESR studies, whose validity can be questioned, is that the behaviour of the spin probe is unaltered by the presence of protein and remains the same as in pure phospholipid bilayers.

Lipid Modulation Of Enzyme Activity

Many membrane bound enzymes, like cytochrome oxidase (67,70), (Na\(^+\)-K\(^+\)) ATPase (60,61,62), (Ca\(^{2+}\)-Mg\(^{2+}\)) ATPase (59), AChE (14,15) and others are isolated in lipoprotein form and have been found to require lipids for their enzymatic activity (61). A classical way to study the lipid dependence of enzymatic activity is to observe the loss of activity
accompanying lipid depletion followed by recovery during reconstitution (13, 59, 70). The lipid modulation of enzymatic activity could arise from any of the following mechanisms:

a) Solubilisation of substrate: In reactions involving water insoluble substrates, like long chain ubiquinones or plasmaquinones, the presence of lipid may be required for solubilisation of the substrate (49). In many of those cases it is possible to substitute detergents for lipids.

b) Protein conformation: One of the main functions of the lipid phase is to act as an anchor and maintain the enzyme in an active conformation (105, 112). There are many instances where changes in the lipid-bilayer phase is reflected by the modification of the enzymatic activity. The requirement of a fluid bilayer for activity has been shown in the case of cytochrome oxidase (67) and (Ca$^{2+}$-Mg$^{2+}$)ATPase (77) among others. Some membrane transporting proteins show maximum activity at the transition temperature, rather than above or below transition (71). This is attributed to the greater "porosity" of the membrane due to the simultaneous presence of gel and fluid phases (107).

c) Protein distribution and aggregation: CTP phosphocholine cytidyl transferase from fetal rat lung is activated by phosphatidylglycerol in a concentration of 4% by aggregation of the enzyme to a higher molecular weight form (58). Usually most proteins are randomly distributed above the transition temperature (108). Lowering of temperature below the transition temperature results in "freezing out" of the protein from the
newly formed gel region, due to favourable partitioning of proteins into the fluid region (as stated earlier many proteins require a lipid environment for their activity)

The Arrhenius Plot: Significance And Limitations

Functional activities of biological or reconstituted enzyme often show bi or triphasic Arrhenius plots (113). Non linearity in the Arrhenius plot could arise due to:

a) A change in the rate determining step of the enzymatic reaction.

b) A temperature dependent shift of equilibrium between two conformers.

c) Changes in fatty acyl chain mobility due to phase transition.

Temperature change can produce two type of effects in the lipid bilayer:

a) Interfacial effect: Lipid modulation of C₅₅ isoprenoid phosphokinase activity has been suggested to be brought about by this mechanism (79). Sandermann (79) found that lipids which are capable of activating the above enzyme can bind 2.5 to 5.3 nmoles of ³H₂O per mole of lipid, while the inactive cofactor lipids can only bind 0.14 nmoles of ³H₂O. The number of water molecules bound to the phospholipid head group is critical in determining the property at the lipoprotein/water interface.

b) Viscotropic effect: The transition from gel to liquid
crystalline phase brought about by increase in temperature is accompanied by a simultaneous increase in the mobility of the fatty acyl chain (52), thus altering the viscosity of the lipid phase. Viscotropic effects have been studied using spin labelled probes (114).

Arrhenius plots are usually obtained by plotting log rate versus the reciprocal of absolute temperature.

$$d \log k = \frac{E}{2.3 R} d(1/T)$$

Most soluble enzymes will yield a straight line, and in those cases activation energy of the reaction is calculated from the slope.

Many membrane bound enzymes, including adenylate cyclase (112), (Ca$^{+2}$-Mg$^{+2}$)ATPase (63), AChE (14,15,126) and (Na$^{+}$-K$^{+}$) ATPase (61,62,66) yielded a biphasic Arrhenius plot. The Arrhenius plot break has been attributed to various causes, such as phase transition (62,80), phase separation (71,80,81) and cluster formation (17,55). In early studies (113) the biphasic Arrhenius plot was almost exclusively interpreted in terms of phase transitions, but this can't account for the differences in the break point in Arrhenius plot of various enzymes obtained from the same membrane (64). Another limitation is that the transition doesn't occur at a single temperature but over a range of temperature.

The activity of sarcoplasmic reticulum (Mg$^{+2}$-Ca$^{+2}$)ATPase shows a discontinuity in the Arrhenius plot around 18°C, even though differential scanning calorimetry measurements
do not detect any phase transition at this temperature (63). Silvius et al. (118) have recently reported a temperature dependent variation in both $V_{\text{max}}$ and $K_m$ of $(\text{Na}^+ - \text{K}^+)\text{ATPase}$. This in turn will give rise to an artifactual break in the Arrhenius plot (118). 5'-Nucleotidase (116) is another example where the previously considered lipid-dependent Arrhenius plot break was found to be an intrinsic characteristic of the protein. The sharp change in activation energy observed in most of the systems showing a biphasic Arrhenius plot is not accompanied by a significant change in the reaction rate ($v$) (71). The change thus is in $dV/dT$ and not in $V$. This behaviour (change in enthalpy without significant change in reaction rate) is difficult to explain since it requires that the change in activation enthalpy be exactly compensated by a change in activation entropy (71).

The molecular mechanism underlying bi or triphasic Arrhenius plots have so far in no case been elucidated and therefore any interpretation of Arrhenius plot breaks has to be considered with reservation.
MATERIALS AND METHOD

Materials: Fresh bovine blood was obtained from a local slaughter house (Inter Continental Packers); (I-\textsuperscript{14}C) Acetylcholine iodide (1-5 C\textsubscript{i}/mole) was from New England nuclear. Acetylcholine perchlorate was from BDH. Sepharose 2B was from Pharmacia. Butanol (spectral quality) was from Fisher Scientific. Acetylcholine iodide, 5,5'-dithiobis (2-nitrobenzoic acid), decamethonium bromide, acridine, guanidine and iodomethane were purchased from Sigma. The 6 aminohexanoyl derivative of N-methylacridine and N(6-amino caproyl-m-aminophenyl)trimethyl ammonium bromide hydrobromide were gifts from Geoffry Webb, Department of Chemistry, UBC. All inorganic salts were of analytical reagent grade.

"Flotation" technique for cardiolipin separation:

All the sucrose solutions were prepared in 1M sodium bicarbonate (pH 8.0) in the presence or absence of 0.09% Triton X-100. Gradients were prepared by layering the following solutions (w/v) of sucrose: 1 ml of 60% sucrose (acts as a plug); 1 ml of 0.5 mg/ml of Sigma bovine erythrocyte AChE in 30% sucrose; 9.5 ml of 25% sucrose and 1 ml of water at the top. The tubes (12.5 ml) were
centrifuged for 66 h. at 100,000g in a Beckmann L2-65B ultracentrifuge. After centrifugation, 0.287 ml fractions were collected upwards from the bottom of the tube by the use of a Desaga peristaltic pump and Gilson microfraction collector. The most active enzyme fractions were combined and divided into four equal portions. Just before the assay each one of the portions was diluted 200 times with 0.1M sodium phosphate pH 7.4 and Arrhenius plots were determined at times indicated in the figure.

Cardiolipin separation by Sephadex gel filtration:

A 5x60 cm Sephadex G-200 column was prepared and equilibrated with 1.8M sodium chloride, 2 mM sodium phosphate pH 7.4 ("high salt") at 4°C. A 2 ml sample containing a solution of 0.5 mg/ml of Sigma AChE and 2 mg/ml of dextran blue in "high salt" was applied to the column. The flow rate was maintained at about 7 ml/h with a Desaga peristaltic pump. Fractions (1.2 ml) were collected. AChE was determined by the radiometric method and blue dextran at A240.

Partial specific volume:

The partial specific volume of AChE was determined by equilibrium sedimentation on a partially preformed sucrose gradient (15). The partially preformed sucrose gradient was made by layering 1.3 ml of each of the following sucrose
concentrations (gm/ml) in "high salt": 0.906, 0.541,
0.329, 0.213, 0.140, 0.097, 0.069, 0.052, 0.037, 0.010.
Refractive indices were determined with a Fisher refractometer
at 29.2°C.

Affinity chromatography of AChE on MAC columns

Synthesis and purification of MAC: MAC was synthesised
and purified as described by Mooser et al. (95). The iodide
derivative of the N-methylacridine, obtained by reaction of
acridine with iodomethane, was converted to its corresponding
chloride derivative by passing it through a column containing
anion exchange resin Ag 1-X8. A slight impurity could be
detected on TLC using precoated 60 F254 silica gel plates
and 1-butanol: acetic acid: water (8:12:3 v/v) as the develop­
ing solvent. The impurity was removed by passing the
N-methylacridinium chloride once through a 1x20 cm Sephadex G-10
column and eluting with water. Following lyophilization, the
yellow crystals were recrystallized from acetonitrile,176-179°C
decomposition (lit 176-179°C) (95).

Coupling of 6 aminohexanoyl derivative of MAC to
Sepharose 2B: The procedures for the cyanogen bromide activation
of Sepharose, the ligand coupling to the activated gel and
washing of the coupled gel to remove unreacted ligand were
those described by March et al. (127). The coupling
reaction was carried out at room temperature rather than at
4°C suggested by the authors.
Ligand concentration of 0.5, 1.0, 2.0 and 2.8 umole/ml of Sepharose were obtained by varying the cyanogen bromide:gel ratio in the activation step and the ligand: activated gel ratio in the coupling step. Coupled gel was washed with 0.2M sodium bicarbonate buffer pH 9.5. Free ligand concentration was determined by measuring absorbance of the wash at 412 nm. The difference in the initial ligand concentration and unbound ligand concentration was used to calculate the amount of ligand bound to the gel.

**Affinity chromatography:** One thousand units of Sigma AChE were dissolved in 200 ml of 0.1M NaCl, 20mM sodium phosphate, pH 7.4 (buffer L) to give a solution of 5 units/ml. This enzyme concentration was loaded on the column. Affinity chromatography was performed in miniature columns constructed from 5 ml disposable syringes in which tight fitting discs of porous polypropylene were inserted. Columns were packed with the resin and equilibrated with buffer (L) before use. A multichannel peristaltic pump was used to load, elute and wash the four columns simultaneously with a rate of 13 ml/h. Elution was done with 20mM decamethonium in buffer (L), and regeneration of the column was achieved by washing with 5M guanidine hydrochloride in buffer (L) followed by normal buffer (L) washing. All fractions were collected in a Gilson microfraction collector. Fractions obtained on elution of the enzyme with decamethonium were dialysed for 16 h against buffer (L), with one change before the activity of the eluted AChE was determined.
Protein estimation

Protein estimation was done both by the method of Murphy and Kies (82) and Lowry et al. (83). Fatty acid free bovine serum albumin was used as the standard. Standard curves by the method of Murphy and Kies were linear from 10 to 75 ug of protein, while that of Lowry was linear from 25 to 250 ug protein. At the lower concentrations of protein the former method was found to be more suitable.

Radiometric assay of AChE

The method was essentially the same as described by Beauregard and Roufogalis (14). 20 ul of the enzyme solution was added to 190 ul of 0.1M sodium phosphate, pH 7.4 in thick walled incubation tubes. After 30 min. of preincubation at the required temperature, the reaction was started by the addition of 20 ul (0.001 mCi) acetylcholine to give a final concentration of 1mM in acetylcholine. The reaction was allowed to proceed for 5 to 20 min. depending upon the enzyme activity. At the end of the reaction period 200 ul of absolute alcohol was added to stop the reaction. Unreacted acetylcholine was removed by adding 1 ml of 0.87 gm/ml Amberlite ion exchange resin (CGC 241). This reaction mixture was allowed to stand for 20 min. during which the resin was sedimented. Supernatant (480 ul) was transferred onto vials, 10 ml of aquasol added and the radioactivity counted in an Isocap
Kinetic studies

a. Spectrophotometric assay of AChE: Inhibition of AChE by MAC was determined by using acetylthiocholine as substrate, by the method of Ellman et al. (84). The assay conditions were similar to those described by Wermuth and Brodbeck (85). The reaction was started by the addition of various concentration of acetylthiocholine to a 3 ml reaction mixture in a spectrophotometer cuvette containing (in a final concentration) dithiodinitrobenzoic acid (0.125mM), bovine serum albumin (0.01%) and AChE (0.02 units/3 ml) in 25mM sodium phosphate pH 7.4. The change in absorbance at 412 nm was immediately recorded on a Beckman model 25 UV spectrophotometer. The initial velocity was determined by measuring the slope of the recording for the first 1-2 min.

b. pH stat Titrimetric assay: The effect of calcium on MAC inhibition was determined by the pH stat method, using the Radiometer titrimeter TTA31. The reaction was carried out at 25°C in a water jacketed reaction vessel. MAC-purified bovine erythrocyte AChE (0.5 units) and MAC were incubated for 5 min., in a CO₂ free nitrogen atmosphere, in the presence or absence of 0.1mM CaCl₂. The pH was adjusted to 7.4 with 0.01N sodium hydroxide and the reaction started by the addition of 0.4mM acetylcholine. Experiments were done in the absence of added ions or in the presence of 40mM sodium chloride as indicated in the figure.
Isolation and purification of AChE from bovine blood:

The method followed was similar to that described by Cohen and Warringa (24) with some modifications (Fig. 12). All the following operations were carried out at 4°C. 1.8 L of bovine blood (to which 0.37% citrate was added to prevent clotting) from freshly slaughtered animals was obtained from the slaughter house. It was centrifuged at 5,000 g in 250 ml buckets in the IEC B 20A centrifuge. The supernatant and top buffy layer were removed by suction. Considerable care was taken to remove the buffy coat as completely as possible, despite a considerable loss of blood cells. Packed cell (200 ml) were washed three times with 4 volumes of 0.9% sodium chloride, followed by centrifugation and removal of the supernatant, and any remaining buffy layer. The packed cells were hemolysed overnight in 15 volumes of distilled water after addition of a few lumps of dry ice.

The stroma which settled was collected and washed three times with 9 times diluted Tyrode's solution. After each washing, supernatant obtained after centrifugation at 15,000 g for 15 min. was removed by suction. The ghosts obtained from the final washings were suspended in 350 ml of distilled water, distributed into equal volumes in seven separate 250 ml bucket flasks and freeze dried in a Virtis lyophilizer. The freeze dried product (3.1 gm) was suspended in one liter of cold dry butanol and mixed for two min. in a Waring blender. The butanol extracted membrane.
"Post butanol" membrane) was separated from butanol on a Whatman number 1 filter by vacuum filtration. The residue thus obtained (2.7 gm) was dried in an evacuated dessicator in the presence of silica for several hours. The dried butanol extracted membrane was suspended in 0.1M NaCl, 0.2mM EDTA, 10mM sodium phosphate, pH 8 buffer (1 gm/100 ml) and homogenized in a Sorvall omnimixer at speed 5, with six homogenizations for 10 second periods, with 20 second intervals in between. The homogenate was centrifuged at 15,000g for 15 min. and the residue discarded. The supernatant was divided into two portions; one portion was purified by PTA affinity chromatography and the other by ammonium sulphate precipitation.

**AChE purification on PTA affinity column:** The PTA column was constructed from a 10 ml disposable hypodermic syringe, in a manner similar to that described for the MAC column. The column was packed with the affinity resin, containing the ligand (N-6-amino caproyl-m-aminophenyl) trimethylammonium bromide hydrobromide in a concentration of 4.6 umole/ml gel. After equilibrium of the column, with 0.1M NaCl, 0.2mM EDTA in 10mM sodium phosphate pH 8.0; a portion of the supernatant (obtained by homogenisation of butanol treated membrane in the above buffer) was loaded onto the column at a rate of 13 ml/h. Following washing of the column with 3 column volumes of buffer, the enzyme retained on the column was eluted with 20mM decamethonium bromide in buffer(L); 1% Triton in buffer (L); 1% Triton, 20mM decamethonium in buffer (L) and 50% polyethylene glycol. Fractions eluted from
the column were dialysed for 16 h against buffer (L) before assay. AChE activity was assayed by a Radiometric method in the presence of 0.1M sodium phosphate pH 7.4, as described above.

Ammonium sulphate precipitation: Another portion of supernatant (obtained by homogenisation of butanol treated membrane) was treated with an equal volume of cold saturated ammonium sulphate at pH 7.0 and kept in the refrigerator overnight. After precipitation, the precipitate was taken up in 150 ml of cold distilled water. Cold saturated ammonium sulphate (41.9 ml) was added and the pH adjusted to 6.0. A small precipitate formed after stirring and standing for 30 min. was centrifuged. To the supernatant was added 168 ml of saturated ammonium sulphate at pH 6.0. The precipitate obtained after 2 h of standing and centrifugation was taken up in buffer (L).

Sedimentation coefficients

The sedimentation coefficient of AChE purified on the PTA affinity column following butanol extraction was determined in a 5-20% (w/v) continuous sucrose gradient (15) prepared in distilled water or an isokinetic gradient (28) in buffer (L). Marker enzymes and AChE were placed at the top of the gradient and centrifuged in a Beckman SW 41 Ti rotor in a Beckman L2-65B ultracentrifuge at 4°C for the time and speed indicated in the figure legend. Fractions (18 drops) were collected by carefully lowering a capillary into the tube at the end of
the run and pumping the liquid from the bottom with a Desaga peristaltic pump. AChE activity was determined by the Radiometric method (14). Bovine serum albumin and Catalase were determined by measuring absorbance at 280 and 402 nm, respectively. -Galactosidase was assayed according to a previously published procedure (87).

**Phospholipid extraction and estimation:**

Phospholipids were extracted by the method of Bligh and Dyer (124). The phospholipid extracts were spotted on Kieselgel 60 F<sub>254</sub> precoated silica gel plates and the plates were developed in chloroform:methanol:ammonium hydroxide in the ratio of 60:30:5. The TLC spots were scraped off the plate after moistening and transferred to glass centrifuge tubes. Phospholipids were extracted three times with 2 ml methanol. After evaporation of the methanol, 100 µl water and 0.5 ml of 10N sulphuric acid were added and the mixture heated at 150-160°C for 3 h. Once the tubes were cooled down to room temperature, 3 to 4 drops of hydrogen peroxide were added and the mixture was heated for 2 h at 150-160°C. After the tubes were cooled to 50°C, 4.6 ml of ammonium molybdate and 80 ul of Fisk and Subbarow reagent were added. The solution was mixed well and incubated in boiling water for 7 min. The blue colour developed was measured at 830 nm.
RESULTS

1. Separation Of Cardiolipin From AChE

A number of experiments were performed in order to physically separate cardiolipin from AChE. These included gel filtration of the "high salt" treated AChE and sucrose gradient centrifugation in the presence of "high salt".

A. Partial specific volume of "high salt" treated AChE after Sephadex G-200 gel filtration: Following treatment of Sigma AChE with 1.8M NaCl, 2mM sodium phosphate, pH 7.4, the enzyme was applied to a Sephadex G-200 column equilibrated and run in the presence of the same salt concentration. The presence or absence of cardiolipin in AChE obtained from the Sephadex G-200 gel filtration under "high salt" conditions was determined by equilibrium sedimentation in a preformed sucrose gradient, as described in the methods. The peak of the enzyme activity was obtained in the 4th fraction from the bottom (Fig. 3), which corresponded to a density of 1.214 g/ml. The reciprocal of this density gave a partial specific volume of 0.823 ml/g, which is higher than the partial specific volume of 0.725 ml/g expected from the amino acid composition of the enzyme in the absence of lipid.(7).

B. Characterisation of cardiolipin-AChE interaction by Arrhenius plot: Sigma AChE was placed on a sucrose gradient in
Fig. 3. Determination of the partial specific volume of AChE obtained from Sephadex G-200 gel filtration under "high salt" conditions.
the presence of 1.0M sodium bicarbonate, pH 8.0, designed to separate cardiolipin by "flotation" from the denser AChE protein, (see methods for detail) if the cardiolipin was dissociated from the enzyme under "high salt" conditions. Alternatively, if the cardiolipin was not dissociated from AChE under these conditions, the extent of cardiolipin-AChE interaction could be monitored by determining the Arrhenius plot of the enzyme at various time intervals following the sucrose gradient centrifugation in the "flotation experiment". The cardiolipin did not appear to have been removed from AChE by the "high salt" treatment and the sucrose gradient centrifugation, as the biphasic Arrhenius plot was regenerated in time. A straight line was obtained in the Arrhenius plot 24 h after recovery of the enzyme from the sucrose gradient (Fig. 4A). After 48 h, the points on the Arrhenius plot were scattered, thereby it was difficult to decide between a straight line or two lines with a break; but storing the enzyme for a further period of 24 h (72 h after recovery of the enzyme from the gradient) gave an Arrhenius plot with a distinct break (Fig 4C). After 192 h the Arrhenius plot showed a distinct break at 21°C. Lines of best fit were drawn from linear regression analysis.

The sucrose gradient "flotation" experiment was then performed in the presence of 0.09% Triton X-100 in an attempt to solubilise any cardiolipin which may have dissociated from the enzyme in the presence of 1.0M sodium bicarbonate (pH 8.5). The Arrhenius plots were determined at various
Fig. 4. Arrhenius plots of AChE at various time periods following sucrose gradient centrifugation of AChE in 1M sodium bicarbonate, pH 8.0: A) 24 h., B) 48 h., C) 72 h., D) 192 h.
X axis - 1/T
Y axis - log cpm.
time periods after recovery of the enzyme from the sucrose gradient in "high salt" and Triton X-100 (Fig. 5). The Arrhenius plot after 24 h was intermediate between that of a straight line and a distinct break. A distinct break at 21°C was observed in the Arrhenius plot after 58 h. The above result suggests that a time dependent reassociation of cardiolipin might be taking place.

2. Purification Of AChE By Affinity Chromatography

Attempts were made to purify sufficient quantities of AChE for further studies on the AChE-cardiolipin interaction.

A. Affinity chromatography of bovine erythrocyte AChE:

The relative amounts of bovine erythrocyte AChE retained and eluted on MAC affinity columns of increasing ligand concentration are shown in Fig. 6. As the ligand concentration increased, less of the enzyme activity passed through the columns during the loading and buffer-wash steps. Very little enzyme activity was retained at ligand concentrations of 0.5 and 1.0 umole/ml, as shown by the low elution of enzyme activity on application of 20mM decamethonium. Efficient retention of the enzyme required a ligand concentration of 2.0 or 2.8 umole/ml. At these ligand concentrations less enzyme activity passed through the column during the loading and washing steps, and enzyme activity was eluted by 20mM decamethonium. A single affinity purification of the Sigma bovine erythrocyte AChE at the highest ligand concentration of 2.8 umol/ml of gel yielded preparations with a specific activity of 60 umole/mg. min;
Fig. 5. Comparison of Arrhenius plots obtained from the presence and absence of 0.09% Triton X-100 in the centrifugation media. A and C in presence of Triton at 24 h and 58 h; B and D in absence of Triton at 24 h and 48 h.

X axis - 1/T
Y axis - log cpm.
Fig. 6. Affinity chromatography of bovine erythrocyte AChE on MAC-Sepharose 2B columns at various ligand concentrations. The columns were loaded as described in methods. At the first arrow marked "Buffer", Buffer (L) was applied to the columns to remove unbound AChE and nonspecifically absorbed proteins. At the arrow marked "Deca", 20mM decamethonium was applied. Fractions (1.4 ml) were assayed for AChE activity by the radiometric assay (see Methods). The ligand concentrations were 0.49 umole/ml (●), 0.99 umole/ml (▽), 1.97 umole/ml (●), and 2.8 umole/ml (▲).
this was increased to 420 umole/mg.min. after a second passage through the column.

B. Kinetics of MAC inhibition of bovine erythrocyte AChE: To investigate the nature of the interaction between MAC and bovine erythrocyte AChE, the kinetics of MAC inhibition were studied. Lineweaver-Burk plots showed that both $K_m(app)$ and $V_{max}$ were altered by MAC (Fig. 7), suggesting mixed (competitive/uncompetitive) inhibition. Replots of the slopes and $1/v$-intercepts against MAC concentration (101) yielded a $K_i$ value of 1.0uM for the competitive component and a $K_i'$ value of 2.0uM for the uncompetitive component (Fig. 8).

The effect of Ca$^{+2}$ on the inhibition of bovine erythrocyte AChE by MAC in the absence of other added ions was also examined. CaCl$_2$ (0.1mM) shifted the inhibition curve ($v/v_o$) to the right, indicating that CaCl$_2$ antagonized the inhibition of the enzyme by MAC (Fig. 9A). This is also shown in Dixon plots (Fig. 9B), where 0.1mM CaCl$_2$ shifted the apparent $K_i$ for MAC inhibition from 0.1 uM (compared to 0.016 uM in similar ionic conditions for eel AChE (78)) to 0.22 uM. The antagonism by CaCl$_2$ was found only in the absence of added ions. In the presence of 40mM sodium chloride, 0.1mM CaCl$_2$ had little effect on the inhibition by MAC (Fig. 10).

C. Role of tightly bound cardiolipin in MAC affinity: The affinity of bovine erythrocyte AChE for MAC was 13 fold or more lower than that of the AChE from electric organ of eel, limiting the usefulness of this method for the
Fig. 7. MAC inhibition of bovine erythrocyte AChE. Acetylthiocholine was assayed by spectrophotometric method (see Methods). The MAC concentration was Zero (▼), 1.15 uM (□), 1.84 uM (▲), 2.76 uM (○), and 3.68 uM (■).
Fig. 8. Slope and intercept replots from Lineweaver-Burk plots of MAC inhibition of bovine erythrocyte AChE. The slopes ($K_m/V$) (☐) and intercepts ($1/v$) (▲) were obtained from Fig. 7.
Fig. 9. Effect of CaCl\textsubscript{2} on the inhibition of bovine erythrocyte AChE by MAC. A titrimetric assay was used (see Methods) in the absence of added salts (■) and in the presence of 0.1mM CaCl\textsubscript{2} (△). The acetylcholine concentration was 0.4mM. A. \(v/V_0\) is the fraction of remaining enzyme activity, where \(v\) and \(V_0\) are the velocities in the presence and in the absence of inhibitor. \(v\) was 0.508 umole/min in the absence of CaCl\textsubscript{2} and 0.720 umole/min in the presence of 0.1mM CaCl\textsubscript{2}.
Fig 9B  Dixon plot of the data in Fig 9A. In the absence of added salt (■) and in the presence of 0.1 mM Ca$^{2+}$ (□).
Fig. 10. Effect of CaCl$_2$ on the inhibition of bovine erythrocyte AChE assayed in the presence of 40mM NaCl. The acetylcholine concentration was 0.4mM in the absence (■) and in the presence of 0.1mM CaCl$_2$ (□).
purification of the bovine erythrocyte enzyme. A possible role of tightly bound cardiolipin in the difference in the affinity of bovine erythrocyte AChE and eel electric organ AChE for MAC was investigated. The affinity of the enzyme for MAC was determined under two different conditions; i) in the presence of 1.8M sodium chloride, 10mM sodium phosphate pH 7.4 (cardiolipin dissociating conditions) and ii) in the presence of 1.8M sodium chloride alone (cardiolipin-bound to the enzyme) (44). A similar $K_i$ value of 3.1 nM for MAC (Fig. 11) was observed under both the above situations, suggesting that conditions (1.8M sodium chloride, 2mM sodium phosphate) which abolished the break in the Arrhenius plot did not alter the affinity of the MAC for the enzyme.

3. Extraction of AChE from Bovine Erythrocyte Membranes

A. Phospholipid analysis: Butanol treatment of the erythrocyte ghosts was examined as a method for the extraction and purification of AChE from erythrocyte ghosts, based on a preliminary study of Cohen and Warringa (24). The quantity of various phospholipids extracted during butanol treatment was estimated from the phospholipid analysis of the membrane before and after butanol treatment. Thin layer chromatographic separation on pre-coated silica gel plates showed the presence of phosphatidylserine/phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine and an unidentified phospholipid in both the membranes (Fig. 13).
Fig. 11. Effect of endogeneously bound cardiolipin on MAC affinity: The Ellman assay was used to determine the MAC affinity for the enzyme under two different conditions, representing cardiolipin 'associated' and cardiolipin 'dissociated' forms of the enzyme. All solutions were made in 25mM Tris buffer instead of 25mM phosphate (as described in methods); the reaction was carried out in a) 1.8M sodium chloride (♦) and b) 1.8M sodium chloride, 10mM phosphate (△).
Fig. 12. Flow sheet for the purification of bovine erythrocyte AChE: The method is essentially the same as described by Cohen and Warringa (24) with slight modification.
Fig. 13. Thin layer chromatographic analysis of phospholipid composition before and after the butanol treatment of the freeze dried erythrocyte ghosts. PE-phosphatidylethanolamine; PS-phosphatidylserine; PC-phosphatidylcholine; CL-cardiolipin and PI-phosphatidylinositol.
<table>
<thead>
<tr>
<th>Spot</th>
<th>'Pre butanol' membrane phosphate/mg</th>
<th>'Post butanol' membrane phosphate/mg</th>
<th>Percent extracted</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>1.95</td>
<td>0.479</td>
<td>75.4</td>
</tr>
<tr>
<td>b</td>
<td>1.575</td>
<td>0.455</td>
<td>71.1</td>
</tr>
<tr>
<td>c</td>
<td>0.160</td>
<td>0.031</td>
<td>80.6</td>
</tr>
<tr>
<td>d</td>
<td>1.42</td>
<td>0.296</td>
<td>79.6</td>
</tr>
<tr>
<td>e</td>
<td>0.019</td>
<td>0.000</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Table II  Quantitative estimation of the phospholipid content of the various phospholipid spots obtained on thin layer chromatography (Fig. 13).
Phosphate determination of the phospholipids on TLC plates indicated that 70-80% of various phospholipids were extracted by butanol (Table 2). This is in agreement with previous findings that 70-80% of membrane lipids are mobile (72,73), while the remaining lipids are immobilised due to protein binding.

B. Release of AChE from butanol treated bovine erythrocyte membranes: Various conditions for the release of AChE from butanol treated freeze dried erythrocyte ghosts were examined, because 10mM phosphate (pH 8.0) used in the original study (24), extracted only 40% of the enzyme. The amount of enzyme released was increased to 60-70% by increasing the ionic concentration by addition of sodium chloride (final concentration 0.5M) or sodium phosphate buffer (final concentration 100mM). A further increase in the amount of enzyme released was achieved by adding EDTA (to a final concentration of 0.2mM) to the extraction medium (Table 3). The increased release of the enzyme due to addition of sodium chloride and EDTA were consistent with previous reports in the extraction of AChE from erythrocyte ghosts (12,129).

C. Affinity purification of AChE: The AChE extracted with 0.1M NaCl, 0.2mM EDTA in 10mM sodium phosphate, pH 8.0 from butanol-treated freeze dried erythrocyte ghosts, was applied to an affinity column with PTA as the affinity ligand (Fig 14). Though complete retention of the enzyme was
<table>
<thead>
<tr>
<th>System</th>
<th>Percent extracted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>44</td>
</tr>
<tr>
<td>10mM sodium phosphate pH 8.0</td>
<td>41</td>
</tr>
<tr>
<td>0.1M sodium chloride in 10mM sodium phosphate pH 8.0</td>
<td>41</td>
</tr>
<tr>
<td>100mM sodium phosphate pH 8.0</td>
<td>61</td>
</tr>
<tr>
<td>0.5M sodium chloride in 10mM sodium phosphate pH 8.0</td>
<td>69</td>
</tr>
<tr>
<td>0.1M sodium chloride, 0.2mM EDTA in 10mM sodium phosphate pH 8.0</td>
<td>79</td>
</tr>
</tbody>
</table>

*Table III: Extraction of AChE from butanol treated erythrocyte ghosts with different ionic strength media.*
Fig. 14. Affinity purification of AChE extracted from butanol treated erythrocytes ghosts with 0.1M NaCl, 0.2mM EDTA, 10mM sodium phosphate pH 8.0, on a PTA affinity column. The column was eluted with different solutions as described under methods.
achieved during column loading, only 20 and 8 percent of
the loaded enzyme were eluted with 20mM decamethonium in
buffer (L) and 1% Triton X-100 in buffer (L), respectively.
The remaining 72% of the enzyme could not be eluted, presumably
because it was tightly bound to the affinity resin.

D. Sucrose density sedimentation: The nature of the
molecular forms of the AChE present under low and high ionic
strength conditions was determined from the sucrose density
centrifugation profile of the butanol extracted enzyme.
Under low ionic strength conditions four different forms of
the enzyme with sedimentation coefficients ranging from
7S to 20S were obtained (Fig. 15). This may be the result of
aggregation which has been reported to occur at low ionic
strength conditions (26). In the presence of 0.1M NaCl,
20mM sodium phosphate pH 7.4, 70-80 percent of the enzyme
appeared in a peak with a sedimentation coefficient of 11S
(Fig. 16). The presence of aggregation was noted even at
this ionic strength.
Fig 15. Sedimentation profile of AChE under low ionic strength conditions. The enzyme was loaded on a 5-20% continuous sucrose gradient with catalase and bovine serum albumin as markers. Gradient was centrifuged at 90,000g for 66 h. Enzyme and markers were estimated as described under methods.
Fig. 16. Sedimentation profile of AChE in 0.1M sodium chloride, 20mM sodium phosphate. Enzyme was loaded with marker enzymes —galactosidase, catalase and bovine serum albumin on an isokinetic sucrose gradient prepared in 0.1M sodium chloride, 20mM sodium phosphate. The gradient was centrifuged at 100,000g for 9 h. Enzyme and markers were estimated as described under methods.
DISCUSSION

Cardiolipin Modulation Of AChE Catalysis

Many proteins have been shown to require lipids for their optimum activity (13,14,59,60,61,62). One of the most common methods for studying lipid protein interactions is to observe the effect of various lipids on a "lipid free protein" system (13,88). Detergent extraction most commonly used for lipid displacement suffers from certain drawbacks. Ionic detergents often denature enzymes (12,130). While many non ionic detergents preserve enzyme activities, it is often difficult to obtain a "detergent free" form of the enzyme, because of the tight binding of the detergent to the protein molecule.

Two enzymes which have been shown to posses tightly bound cardilipin are cytochrome oxidase (67) and AChE (14,15). Contrary to what was believed earlier, Marsh et al. (89) have recently shown that cardiolipin is not essential for cytochrome oxidase activity.

In AChE the involvement of cardiolipin in the activity of AChE is based on the interpretation of Arrhenius plots. Beauregard and Roufogalis (15) obtained soluble and particulate forms of the enzyme on hypo-osmotic shock of the bovine erythrocyte ghosts. The particulate form and a Lubrol WX
solubilised preparation of the particulate form gave a biphasic Arrhenius plot, while the soluble form of the enzyme gave a linear Arrhenius plot. Enzyme preparations showing a non-linear Arrhenius plot, yielded a linear Arrhenius plot after treatment with 1.8M sodium chloride, 2mM sodium phosphate (pH 7.4) or 1M sodium bicarbonate (pH 8.0-10) or 0.7M sodium phosphate (pH 7.4). Under conditions where the break in the Arrhenius plot was abolished it was possible to extract the phospholipid, cardiolipin, by chloroform-methanol, whereas cardiolipin could not be extracted before the "high salt" treatments (14,15). From the study of the effect of "high salt" treatment on the various steps of acetylcholine hydrolysis (43), it was postulated that cardiolipin modulated a rate determining conformational change in the enzyme-substrate complex.

In order to obtain direct evidence for the involvement of cardiolipin in AChE catalysis, it is essential to have a cardiolipin-free AChE. The method previously used to remove cardiolipin involved "high salt" treatment followed by chloroform-methanol extraction, causing denaturation of the enzyme. Use of detergents for the extraction of a fraction of tightly bound cardiolipin has been unsuccessful in the case of cytochrome oxidase (90) and AChE (15). Two physical methods for the removal of cardiolipin have been investigated. Sephadex-gel filtration has been very often used for the separation of excess detergent and lipids from protein-detergent complexes (131). It was hypothesized that if under "high salt"
conditions cardiolipin dissociated from the enzyme, as suggested by Beauregard & Roufogalis (14), it should be possible to separate it from AChE by Sephadex gel chromatography, because of the difference in size between cardiolipin and AChE. The partial specific volume of 0.81 ml/g³ (Fig. 3) obtained for AChE following treatment with "high salt" and gel filtration on Sephadex G-200 was larger than 0.72 ml/g calculated from the amino acid composition of the pure enzyme (7), suggesting that bound lipid was present in the enzyme even after "high salt" treatment and gel chromatography.

A greater understanding of the nature of the AChE-cardiolipin interaction was obtained from a sucrose gradient "flotation" experiment. The attempt to separate cardiolipin from AChE by "flotation" in a sucrose gradient was based on the expectation that if "high salt" treatment caused separation of cardiolipin from the enzyme, the less dense cardiolipin should float to the top of the gradient, while the AChE would penetrate the denser sucrose layer. The fraction containing AChE activity was assayed after various times following recovery of the enzyme from the sucrose gradient used in the flotation experiment. A linear Arrhenius plot was seen after 24 h (Fig. 4A). However, with time a break in the Arrhenius plot was recovered, which become very distinct in 5-8 days (Fig. 4C&4D). In between the two extremes a degree of scatter was observed in the Arrhenius plots, which made it difficult to decide between either a single straight line or two lines with a break. Repeating the
above experiment in the presence of 0.09% Triton X-100, to solubilise any cardiolipin released by the high salt treatment, did not prevent the regeneration of the break (Fig. 5). If anything, the regeneration seemed to occur faster. From these experiments it is clear that the break abolished by "high salt" treatment reappears with time. This suggests that the ionic disruption brought about by "high salt" treatment was reversible, as it underwent a time dependent reassociation.

Beauregard and Roufogalis (44) found that the addition of Ca\(^{+2}\) with mild homogenization to "high salt" treated AChE preparations regenerated the Arrhenius plot break. However, as the "high salt" treated enzyme retained endogeneous cardiolipin, two mechanisms of AChE modulation were possible. In the first mechanism both Ca\(^{+2}\) and cardiolipin cooperate to regulate the temperature dependent modulation of AChE, while in the second alternative Ca\(^{+2}\) alone may be sufficient for the modulation process. If the second alternative is correct, the ability of "high salt" in Ca\(^{+2}\) chelating conditions to increase the extractability of cardiolipin from the enzyme by chloroform-methanol could be coincidental and unrelated to the modulation mechanism. The following evidence supports the former mechanism. The present observation of the reappearance of the Arrhenius plot break with time can be best explained by the simultaneous involvement of Ca\(^{+2}\) and cardiolipin rather than calcium alone and suggests the following model for the lipid-protein interactions in AChE. The inaccessibility of cardiolipin and calcium to phospholipases
and C and EDTA, respectively, suggested that Ca\(^{+2}\) and cardiolipin may be buried within the AChE dimer (44). It is now proposed that cardiolipin may be bound to the core of the protein by hydrophobic as well as ionic interactions. The "high salt" conditions may disrupt the ionic interactions, thereby causing a functional dissociation of the enzyme-phospholipid complex, which may result in abolition of the Arrhenius plot break. At the same time the high ionic strength treatment will strengthen the hydrophobic interaction; which facilitates the regeneration of the ionic protein-lipid interaction with time. The hydrophobic interaction and its strengthening in "high salt" conditions may also explain the lack of "physical separation" of the cardiolipin from AChE, under conditions where "functional dissociation" has occurred.

There are other examples where Arrhenius plot breaks in the membrane bound enzymatic activity is attributed to the direct involvement of phospholipid (61, 62, 66). Recently, some of these reports have been disputed. In (Mg\(^{+2}\)-Ca\(^{+2}\))ATPase (63) and 5'-nucotidase (116) the Arrhenius plot break were attributed to phospholipid independent protein conformational change. In cases where phospholipids are implicated in modulating the Arrhenius plot behaviour various mechanisms have been proposed; transition of the lipid from gel to liquid crystalline state (62, 80), lipid cluster or related phase changes (17, 55), or altered temperature dependent protein-lipid interactions with tightly associated
lipids (9,66,90). In bovine erythrocyte AChE, simultaneous involvement of both Ca\(^{+2}\) and cardiolipin, along with their presence inside the protein molecule, may suggest the following possible mode of modulation; Ca\(^{+2}\) may form a salt bridge between the anionic sites of the enzyme and the phosphate group of cardiolipin or it may be required to maintain the cardiolipin in a particular phase compatible with optimum enzyme activity. Calcium cardiolipin exists in a cylindrical hexagonal (H\(_{II}\)) phase (17) in a 1:1 stoichiometric ratio of Ca\(^{+2}\) to cardiolipin. On the other hand the sodium-cardiolipin complex, which may be produced after treatment of AChE in the presence of high concentrations of sodium salts, exists predominantly in the bilayer configuration (92). Changes in temperature may alter the local transition between phases of the Ca\(^{+2}\)-cardiolipin complex or the extent of protein-lipid phase interaction resulting in changes in the protein conformation, reflected by changes in the activation energy of the enzyme activity. The exact nature of the Ca\(^{+2}\) dependent cardiolipin-enzyme changes that occur at the transition in the enzyme activity at 20\(^\circ\)C is unknown at present. Acyl chains of cardiolipin do not appear to undergo fluidity changes between 4\(^\circ\)C and 40\(^\circ\)C, when cardiolipin is in hexagonal phase (92), although the additional influence of adjacent proteins or other lipids (65) is unknown. Cardiolipin undergoes cluster formation around 20\(^\circ\)C (93) but there is no evidence of the biological significance of this transition. Further studies are required to confirm exactly the role of cardiolipin in AChE as well as the mechanism of modulation.
Affinity Chromatography On MAC Affinity Column

The purpose of affinity chromatography of AChE in this study was two fold a) purification and b) cardiolipin separation. To separate cardiolipin from AChE, an affinity ligand was needed which could retain the enzyme in the presence of high ionic strength conditions such as 1M sodium chloride. The PTA ligand used previously for the purification of eel (25) and erythrocyte AChE (25) exhibits a low affinity for the enzyme under high ionic strength. In the case of eel AChE, this problem of low affinity has been recently overcome by the use of N-methylacridinium (MAC) as the affinity ligand (28). MAC has also been found to be the most suitable ligand for the purification of pig brain AChE (29). The abovementioned factors, along with the potential use of MAC as a fluorescent probe (95,96), has led to the study of MAC as an affinity ligand in this study.

Comparison of the elution profile of bovine erythrocyte AChE with that of the eel AChE (28), at different ligand concentrations of MAC, indicated that they behaved differently on the MAC affinity column. A high salt extract of eel AChE is optimally retained by the affinity column at a ligand concentration of 0.44-0.98 umole/ml gel (28). By contrast, retention of the erythrocyte enzyme required a ligand concentration of 2.0-2.8 umole/ml gel. Under these conditions the eel enzyme is very tightly retained, and is eluted in only poor or zero yield by decamethonium (28). Various possible reason for the difference in behaviour between the two enzymes
have been investigated.

**Effect of structure:** It was shown previously that differences in the affinity chromatographic retention of various molecular forms of eel AChE was due to simultaneous multiple ligand interactions with the larger oligomeric forms (28). The "multisite interaction" proposed above cannot account for the retention difference seen between the dimeric bovine enzyme and tetrameric eel enzyme. This was suggested by the following observation. The 8S dimeric form of the eel enzyme obtained from the sonication of 14 and 18S forms in the presence of guanidine, could be optimally retained at a MAC concentration of 0.44 umole/ml gel (94). Therefore, it is concluded that the fact that bovine erythrocyte AChE is a dimer, in contrast to the 11S eel AChE which is a tetramer, cannot account for the low retention of the erythrocyte enzyme relative to the eel enzyme.

**Affinity for ligand:** The inhibition constant, $K_i$, for the competitive component of MAC inhibition of bovine erythrocyte AChE (1.0 uM) is 13-fold (95) or more (96) higher than for eel AChE at a similar ionic strength. A ten-fold decrease in binding affinity of the aminohexanoyl derivative of PTA with increasing ionic strength correlates with the marked ionic strength dependence of AChE retention in affinity columns containing this ligand (28,97-99). The difference in MAC binding affinity between eel and erythrocyte AChE is entirely adequate, therefore, to account for the observed differences in affinity chromatographic behaviour between the two enzymes on MAC affinity columns.
Site of inhibition: The mixed competitive/uncompetitive inhibition of bovine erythrocyte AChE by MAC was also found in the inhibition of eel AChE (95), suggesting that MAC reacts with both enzymes at similar sites. The uncompetitive component has been attributed to binding of MAC to the acetylated-enzyme intermediate of AChE (95). Alternatively, mixed inhibition could occur as a result of binding of MAC to the peripheral anionic site on AChE (100). In a previous study (100) it was shown that the influence of Ca$^{+2}$ on the inhibition of AChE is diagnostic of the relative affinity of a ligand for the catalytic and peripheral anionic sites. Low Ca$^{+2}$ concentrations enhance the inhibition of the enzyme when the ligand-affinity for the peripheral site is high, while Ca$^{+2}$ antagonizes the inhibition when the peripheral site-affinity is low (100). The antagonism by Ca$^{+2}$ at low ionic strength, and the loss of antagonism in 40mM NaCl in the present results, suggest that MAC inhibits bovine erythrocyte AChE by binding predominantly at the catalytic anionic site; the linear slope replot of the Lineweaver-Burk plots (101) and the linear Dixon plot (102) support this conclusion. Although a similar kinetic study was not made with eel AChE, fluorescent measurements show that MAC also binds selectively to the catalytic anionic site on this enzyme (95,96). Thus the different behaviour of the erythrocyte and eel AChE on MAC affinity columns cannot be accounted for by a different site of interaction of the ligand on the side-arm with the two enzymes.

In summary, it appears that the lower retention of bovine erythrocyte AChE on MAC-affinity columns is adequately
explained by a 13-fold or more lower affinity of this enzyme, compared to the eel enzyme, for the side-arm ligand in the chromatographic conditions. The lower affinity of MAC for bovine erythrocyte AChE also limits the usefulness of MAC as a fluorescent probe for this enzyme, while it has been used successfully with the eel AChE.(95,96).

The difference in affinity between the bovine and eel AChE for MAC could possibly be due to any of the following reasons: a) The tryptophan moiety at the anionic site of the eel AChE, which has been shown to bind organic cations (103), is either lacking or in a different spatial arrangement with respect to the anionic site in the bovine erythrocyte AChE or b) the presence of a tightly bound phospholipid, cardiolipin, which has been shown to be present only in the erythrocyte enzyme. A possible phospholipid regulation of the properties of cholinceptive proteins has been suggested by Changeux (128).

Preliminary experiments suggested that the conditions which abolished the break in the Arrhenius plot did not alter the affinity of the enzyme for MAC. However, in order to verify the contribution of cardiolipin to MAC affinity, a cardiolipin free enzyme will be required.

Isolation And Purification Of Bovine Erythrocyte AChE

The primary requirement for the use of the detergent exchange method (89) in the substitution of the endogeneous cardiolipin from AChE is the availability of large quantities of pure AChE. The commercially obtained enzyme from Sigma
contained two other proteins with large quantities of inorganic salt, thereby making it an uneconomical source for obtaining large quantities of pure AChE.

Most methods reported for the purification of bovine or human erythrocyte AChE use either the commercially obtained soluble form of the enzyme or detergent solubilised enzyme for affinity chromatography. Detergents such as Triton X-100 are capable of dissociating multiple oligomeric forms in vitro. Presuming that such a phenomenon also exists in vivo, the detergent solubilised form may not represent the native form of the enzyme. This is contrary to the alternative belief that Triton X-100 may substitute for the native hydrophobic environment of the enzyme, thereby helping to maintain the native intact structure of the enzyme.

A very small percent of AChE can be obtained in the soluble form by hemolysis of bovine erythrocyte ghost in hypo-osmotic phosphate buffer, while the rest of it is released in the particulate form. In a preliminary communication in 1953, Cohen and Warringa have described a non detergent method for the purification of AChE from ox erythrocytes. In the present work, various steps involved in the purification of bovine erythrocyte AChE by the above method have been characterised.

Butanol treatment has been previously reported to extract phospholipids and thereby facilitate protein solubilisation. In our studies, by quantifying the phospholipids
before and after the butanol extraction of the membrane, it was found that butanol treatment extracts 70-80% of various membrane phospholipids such as phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol (Table 2). This is in agreement with findings on various other membranes (72,73), where it was found that 70-80% of phospholipids are mobile and could be extracted while the rest of the 20-30% of the phospholipids are immobilised due to protein binding. Butanol extraction of the mobile phospholipids somehow alters the membrane organisation such that the extractability of AChE increases from 20 to 80% after butanol treatment. Butanol treatment of the freeze dried electric organ of eel has been reported to effect the release of AChE (125).

In the original communication (24), 10mM sodium phosphate pH 8.0 was used for the extraction of the enzyme from the butanol treated membrane. As shown in table 3 only 40% of the enzyme was extracted under these conditions. The extraction could be increased to 80% by the incorporation of 0.1M NaCl, 0.2mM EDTA. The increased solubilisation could be attributed to the disruption of ionic interactions by 0.1M NaCl (129) and calcium chelation by 0.2mM EDTA (12). The addition of calcium to the extraction medium decreases the extractability of the enzyme (12). Purification of the solubilised enzyme by ammonium sulphate precipitation gave a very poor yield (25%). Affinity purification by passage of the enzyme through a PTA affinity column, at a ligand concentration of 4.4 umole/ml gel, resulted in 100% retention of the enzyme, but only
20% and 8% were released during elution with 20mM decamethonium
and 1% Triton X-100, respectively. The very low yield could
be attributed to the tight binding of the enzyme to the column
or to the difference in the binding characteristics of the
enzyme obtained by the above method. At present, investigations
are being carried out to optimize the conditions for the
affinity purification of this enzyme.

The column-purified butanol-extracted enzyme gave
a sedimentation coefficient of 11S in 0.1M sodium chloride,
20mM sodium phosphate, with small amounts of other aggregated
forms of the enzyme. It is not clear whether the 11S form
represents the native form of the enzyme or an artifact due
to aggregation.
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