

STUDIES ON THE EFFICIENCY OF SEVERAL SELECTED NONIONIC
DETERGENTS IN SOLUBILIZING ACETYLCHOLINESTERASE
FROM BOVINE ERYTHROCYTE MEMBRANE

by

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ABSTRACT

In the present report, several nonionic and zwitterionic detergents have been examined to determine their efficiency in solubilizing acetylcholinesterase (EC 3.1.1.7) (AChE) from bovine erythrocyte membrane. The objective of this study ultimately was to choose one or more detergents that would be suitable for purifying the enzyme from the erythrocyte membrane in a form representing the enzyme in its native environment.

In the present study, AChE activity was retained after solubilization of bovine erythrocytes with octyl- β -D-glucoside (30-100 mg/ml). The most effective solubilization occurred at detergent concentrations more than ten times higher than the critical micelle concentration (cmc). Enzyme inactivation was apparent at a still higher concentration (250 mg/ml). The octylglucoside-solubilized enzyme lost all its activity when it was passed through a hydrophobic polystyrene resin, Bio Beads SM-2.

Tweens were found to be generally not very effective in solubilizing bovine erythrocyte AChE, requiring concentrations some 17,000 to 77,000 times greater than their cmc for maximum effect. At a fixed concentration of 1000 mg/ml, Tween 20 solubilized more AChE than Tween 40, which in turn solubilized more AChE than Tween 60. Thus, as the saturated alkyl chain length of the acid portion of the molecule is increased, there is a decrease in AChE solubilizing capacity. Tween 80, whose hydrophobicity is less than that of Tween 60 due to the presence of the unsaturated double bond, has a solubilizing capacity similar to that of Tween 20. Almost all detergents that solubilize membranes efficiently have a HLB ranging from 12.5 to 14.5. In the present study, Tween 20, with the highest HLB of 16.7, was found to be the most efficient solubilizer, while Tween 60, with the lowest HLB of 14.9, was found to be the least efficient solubilizer. Tween 80, with an HLB of 15.0, appears anomalous. This may be because Tween 80 contains a more fluid unsaturated chain, in contrast to the other Tweens.

Increase in micelle size occurs with increasing hydrocarbon chain length. The solubilizing ability of nonionic detergents generally decreases as the saturated alkyl chain length is increased above 16 carbon atoms. In the study on the Zwittergent series of detergents, as the cmc of the detergent increases from the least polar Zwittergent 316 to Zwittergent 310, a decrease in enzyme activity was detected in the supernatant. This behavior is in contrast to the Tween series. Thus, the longer chain length compound, Zwittergent 316, was the most efficient detergent in the series for the recovery of active enzyme activity in the supernatant. The amount of enzyme activity solubilized by Z 316 increased with increasing concentration up to a 300:1 ratio of detergent concentration to cmc. By contrast, the shorter chain Zwittergents were most effective at concentrations at or below their cmc, suggesting that solubilization occurred by a different mechanism to Z 316.

The relationship between chain length and solubilization of AChE in an active form could also be examined in the Lubrol class of detergents. Lubrol PX has a shorter polyoxyethylene chain (9-10) than Lubrol WX, which averages 17 polyoxyethylene chains. While both detergents solubilize similar amounts of protein from bovine erythrocytes, both at 100 mg/ml and 500 mg/ml, Lubrol PX was more effective than Lubrol WX in maintaining the AChE active in the 100,000x g-soluble form. Higher concentrations of both Lubrol WX and Lubrol PX (500 mg/ml) decreased the overall enzyme recovery.

Triton X-100 was found to be one of the most efficient of the detergents examined. The high efficiency is consistent with its low cmc (0.16 mg/ml), its large micelle size and the favorable HLB. However, Triton X-100 was not one of the most effective detergents for the purification of the enzyme, as considerably higher specific activities, after affinity chromatography, were obtained with Lubrol PX and Tween 20, while Z 316 was the least effective of the compounds studied.

Determination of the sedimentation coefficient of bovine erythrocyte AChE purified by affinity chromatography following solubilization by various detergents was used as an indication of the molecular structure of the enzyme solubilized by each detergent. Solubilization with Lubrol PX, Tween 20 and Triton X-100 yielded AChE corresponding to *S* values of 6.8 *S* to 7.4 *S*, corresponding to enzyme dimers. AChE solubilized and purified following Zwittergent 316 behaved differently to the enzyme solubilized in the other detergents. In addition to the dimeric 7.1 *S* form, a large amount of enzyme activity appeared in an aggregated form.

Functional characteristics of the various detergent-purified AChE preparations were assessed by Arrhenius plots. A break in the Arrhenius plot at around 16°C was observed in AChE purified with Tween 20 and Lubrol PX. Furthermore, the break was abolished after high salt treatment (1.8 M NaCl). From previous work high salt treatment is known to cause functional but not physical dissociation of a tightly bound fraction of lipid, probably cardiolipin, from the enzyme, as the resulting enzyme behaves as a monomer instead of a dimer by irradiation inactivation or target size analysis. Thus, the non-linear Arrhenius plot appears to be independent of the detergent used for extracting the enzyme species, and therefore represents an intrinsic property of the enzyme itself.

Basil D. Roufogalis, Ph.D.

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LIST OF ABBREVIATIONS

AChE	acetylcholinesterase
ASCh	acetylthiocholine
BSA	bovine serum albumin
°C	degree Celsius
cmc	critical micelle concentration
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EDTA	ethylene diaminetetraacetic acid
HLB	hydrophilic-lipophilic balance
°K	degree Kelvin
K_m	Michaelis-Menten constant
$K_{m(app)}$	Michaelis-Menten constant (apparent)
g	gram
\bar{g}	gravity
l	liter
min	minute
ml	milliliter
M	molar
MW	molecular weight
nm	nanometer
O/W	oil in water
PTA	phenyl- <u>m</u> -trimethylammonium
rpm	revolution per minute
SDS	sodium dodecyl sulfate
sec	second
V_{max}	maximum velocity

v/v	volume/volume
W/O	water in oil
w/v	weight/volume
w/w	weight/weight

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DEDICATION

To my wife.

INTRODUCTION

I. Solubilization of membrane proteins

Solubilization in an aqueous system is the spontaneous dissolving of a normally insoluble substance by a relatively dilute aqueous solution of a surfactant. The solubilized material is incorporated directly into (or around) the surfactant micelles so that, unless the solubilizate is colored, the system resembles the surfactant itself and is thermodynamically stable.

Surfactant molecules form micelles in aqueous solution when the concentration exceeds a definitive value. This concentration is generally called the critical micelle concentration (cmc). In the process of solubilization, little or no uptake of solubilizate is generally found until the cmc is reached, which is an indication that the solubilizate molecules are taken up in some way by the micelles.

Three modes of solubilization have been proposed for ionic surfactants. Non-polar molecules are dissolved in the micellar core, where the hydrocarbon tails of the aggregate molecules serve as the solvent. Polar molecules consisting of a hydrocarbon chain and a weakly hydrophilic group, such as long chain alcohols or amines, are incorporated into the micelle with the hydrocarbon tails in the palisade layer and the hydrophilic heads protruding into the aqueous medium on the micellar periphery. Some solubilizates that are insoluble in hydrocarbon or in water are believed to be adsorbed on the surface of the micelle. These three modes of solubilization are probably similar to those found with nonionic surfactants (Harkins et al. 1949).

Another mode of solubilization can be suggested for nonionic surfactants possessing polyoxyethylene groups. For these surfactants containing polyoxyethylene chains the micelle may be considered to consist of two parts, an inner core of hydrocarbon tails and an outer shell of hydrated polyoxyethylene. In the latter

moiety the concentration of polyoxyethylene groups must be considerably higher than the overall concentration. Organic compounds having a strong affinity for a polyoxyethylene group may be incorporated into this region. This type of solubilization is classified as inclusion into the polyoxyethylene exterior of the micelle rather than adsorption on the surface of the micelle (Nakagawa 1966).

In concentrated solutions the situation must be somewhat different, since the solution can no longer be considered a system in which globular micelles having liquid-like inner structure and mono-molecularly dispersed surfactant molecules (and few solubilize molecules) are randomly distributed in an aqueous medium. We need to introduce the R theory (Winsor 1954; Winsor 1960). Let us consider a ternary mixture composed of water, a lipophilic substance, and an amphiphilic compound. The amphiphilic compound is defined as a compound that possesses both hydrophilic and hydrophobic groups. Surfactants, in general, are typical amphiphilic compounds. In this system, water molecules tend to associate together and to reject the lipophilic substance. The alternative is also true. The lipophilic molecules associate together and expel the water. In this view, the amphiphilic compound serves as a monolayer between the aqueous and the organic environments. Provided there is no lipophilic substance, the organic environment may consist of the lipophilic part of the amphiphilic compound itself. The whole system is now considered a structure composed of three different materials: the aqueous environment W, the amphiphilic monolayer C, and the organic environment O. If C is convex toward W, the system will form an oil in water emulsion. On the contrary, a water in oil emulsion will result if C is convex toward O. Provided C shows no tendency to become either concave or convex toward W or O, the layers W, C, and O tend to orient in a parallel and repeated manner, thus resulting in a liquid crystalline structure. If we consider the ratio

$$R = \frac{\text{tendency of C region to become convex toward O}}{\text{tendency of C region to become convex toward W}}$$

it follows that in the liquid crystalline lamellar phase R will be equal to one.

Many compounds have been used to solubilize proteins from membranes. These include phenol (Kathan et al. 1961), methanol-chloroform (Hamagushi and Cleve 1972), pyridine (Blumenfeld 1968), guanidine hydrochloride (Jansons and Burger 1973), and detergents. Detergents are the class of compounds used most frequently. Complete solubilization of biomembranes into subunits is possible by the use of sodium dodecyl sulfate (SDS) (1.4 g/g protein), an anionic detergent, in the condition described by Tanford (1972), where the protein subunit becomes a rod-shaped complex. According to Ballestrin et al. (1980), neither ionic (sodium deoxycholate and SDS) nor nonionic (Tween 80 and Triton X-100) detergents could, in their experimental conditions, completely solubilize human erythrocyte fragmented membranes (which had previously been washed in EDTA buffers). The protein mixture extracted by the ionic detergents sodium deoxycholate and SDS qualitatively reflects the protein composition of the membranes. Among the non-ionic detergents, Triton X-100 appeared to be able to extract mainly one band (most probably the band 3 zone), while Tween 80 apparently did not extract any of the membrane proteins. Mild detergents such as digitonin have been used to disperse membranes, but there is no separation of components, i.e. true solubilization, by digitonin (Hoppel and Copper 1968). Triton X-100 and other polyoxyethylene non-ionic detergents are frequently inefficient in breaking protein-protein interaction and can, in fact, cause artifactual aggregation of proteins (Liscia et al. 1982).

For ionic surfactants, increasing the hydrocarbon chain length, with the attendant increase in micellar size, is the principal method of increasing the amount of material solubilized. In general, ionic surfactants with hydrocarbon chain lengths of C₁₆ or greater are insoluble at 298°K. The introduction of branches lowers

both the melting point and Krafft point. Presumably this behaviour occurs because the hydrocarbon chains need to be in the liquid state for normal micellization to occur (Arnarson and Elworthy 1980).

The addition of salts can have two effects on nonionic surfactants. The salt may reduce the critical micelle concentration, lower the cloud point and possibly increase micellar weight. Conversely, the reverse effects may be seen when salt concentrations are lowered. In solubilized systems, the increase in micelle size would bring about an increase in the amount of solutes dissolved. In the case of non-electrolytes, salts may either increase or decrease the solubilities of non-electrolytes in water, i.e. salt-in or salt-out. In a 1% w/v aqueous solution of cetomacrogol 1000, B.P.C. (cloud point 120°C), sodium chloride, magnesium sulfate, and sodium nitrate lowered the cloud point while magnesium nitrate raised it emphatically (McDonald and Richardson 1981). The total amount of solute present in a solubilized system is composed of free solute and solute found within the micelle. It is suggested that partition coefficient values are dependent primarily upon changes in micellar size. A decrease in cloud point and cmc leads to increases in the total concentrations of micelles and in micellar sizes. The result is more solutes being bound within the micelle, leading to increases in partition coefficient values. A drop in partition coefficient values occurs when cloud points and cmc are little affected.

One of the more important aspects of the purification of membrane proteins is the choice of a suitable detergent. A useful detergent should be capable of breaking artifactual aggregation of proteins to give maximally disaggregated species in solution. Nonionic detergents are generally less efficient in this respect than are ionic detergents or bile salt anions. The detergent should not affect the charge properties of solubilized proteins to a considerable extent. The new detergent synthesized by Hjelmeland (1980), 3-((3-cholamidopropyl)dimethylammonio)-1-

propanesulfonate, abbreviated as CHAPS, combines the useful features of both the bile salts and the N-alkyl sulfobetaines. Like the zwitterionic sulfobetaines, CHAPS proves to be somewhat better at solubilizing proteins than structurally related carboxylic acid anions. It is much more effective at breaking protein-protein interactions than are either sodium cholate or Triton X-100. The increased capacity of CHAPS to solubilize protein and disaggregate complexes is not gained at the expense of increased denaturing properties. This is another important consideration in the choice of suitable detergents for protein solubilization. For instance, two zwitterionic detergents, CHAPS and CHAPSO, 3-((3-cholamidopropyl)-dimethylammonio)-2-hydroxy-1-propanesulfonate, have been utilized in the solubilization of active prolactin receptors from lactating mouse liver membranes. Concentrations as high as 0.5% of the detergent did not alter the affinity and specificity of this particulate receptor (Liscia et al. 1982).

Detergents have been used for solubilizing membranes for many years. If further characterization of a protein is desired after it has been solubilized, it is usually necessary to remove the detergent quantitatively. However, the physical properties of detergents which make them useful for solubilization of proteins are the same properties that make them difficult to remove from proteins. The hydrophobic tail of the detergent will form stable complexes with hydrophobic regions of proteins (Epstein et al. 1982). The strength of this interaction can be enhanced by ionic bonding between the ionic head of the detergent and ionic amino acids. The polar head group of nonionic detergents may also interact strongly with polar regions of polypeptides. For ease of removal, the most important detergent properties are cmc, aggregation number and hydrophilic /lipophilic balance (HLB) (Furth 1980). Critical micelle concentration (cmc) determines the concentration of free monomer and hence, for example, the feasibility of removing the detergent by dialysis. The cmc should be high (in terms of mM) in order to prevent its tight

binding to the membrane.

A rapid method of estimation of cmc of surfactants by means of soluble fluorescent probes, anionic 8-anilino-1-naphthalenesulfonic acid magnesium salt (ANS) or cationic rhodamine 6G, was presented by Vendittis et al. (1981). When the probe and the surfactant carried charges of the same sign, the fluorimetric titration curves showed two straight lines whose intersection gave cmc value. In contrast, when the probe and the surfactant bore opposite charges, complex fluorimetric titration curves were obtained which were not readily amenable to cmc evaluation. With nonionic and zwitterionic surfactants, both probes proved to be equally adequate for evaluating the cmc. The method was capable of detecting the cmc of surfactants in a wide range of concentrations, from 10^{-5} to 10^{-2} M. Care must be taken if, upon proper dilution of a concentrated solution of detergent, the dissociation of the micelles into monomers occurs at a rather slow rate. In addition, the cmc of detergents changes with salt concentrations. Aggregation number governs micelle size and micelle weight (aggregation number x MW of the detergent), and hence the ease with which detergent may be removed by sucrose density gradient centrifugation or gel filtration. It should be small. The HLB is an inverse measure of hydrophobicity, and is relevant to hydrophobic adsorption and phase partition methods. Values over seven indicate a greater solubility in water than in oil. It is defined by the equation

$$\text{HLB} = 0.36 \ln(C_o/C_w) + 7$$

where C_o/C_w is the distribution ratio of the detergent molecule between hydrocarbon oil and water. C denotes the concentration, and the subscripts o and w refer to oil and water.

Triton X-100, with its comparatively low HLB value, is readily removed from most hydrophobic proteins by phase partition methods. These exploit hydrophobicity differences between protein and detergent by partitioning between two mobile phases, one polar (usually aqueous) and one nonpolar. Detergent transfers to the nonpolar phase leaving protein to precipitate at the interface. A popular method of removing Triton is by hydrophobic interaction with polystyrene resins. Bio Beads SM-2 may be replaced with the cheaper version, Amberlite XAD-2. Neither pH nor ionic strength has any effect on this predominantly hydrophobic interaction, but temperature increases in the range 4° - 65°C improve adsorption of the detergent.

In ion-exchange chromatography for removing nonionic detergents, protein rather than detergent is adsorbed to the resin. Adsorbed protein is washed extensively with detergent-free buffer. The adsorbed protein is eluted by raising the ionic strength or by adding an exchange detergent. Only unbound detergent is removed. In gel chromatography, ionic detergents can be readily separated from protein-containing micelles by using Sephadex G-25 or G-75. Nonionic detergents may require Sephadex G-100 or even Sepharose 4B. Perhaps the simplest way of removing unbound detergent is by dialysis, which exploits the size differences between detergent monomer and micelle. Clearly, it is less suitable for nonionic detergents, with their low cmc values and high micellar molecular weights, than for ionic detergents, whose monomers may diffuse rapidly through a small pore dialysis membrane (Furth 1980).

One way to overcome the difficulties brought about by the physical and chemical properties of detergents would be to sever the hydrophobic from the hydrophilic portion of the detergent by the introduction of a photolabile linkage in the detergent and to cleave the detergent with light. Epstein et al. (1982) found that 4-(3,3-dimethyl-1-oxotridecyl)-benzenesulfonate sodium, an ionic detergent, would be such a detergent. Its cmc is found to be $8 \times 10^{-5}\text{M}$. It behaves like

SDS in polyacrylamide gel electrophoresis and is suitable for separation of peptides under denaturing conditions, where migration is proportional to molecular weight.

Ionic detergents have low aggregation numbers and high cmc values, with the latter strongly influenced by pH and ionic strength (Furth 1980). Nonionic detergents are considered to be mild yet sufficiently efficient in extracting membrane proteins. They have high aggregation numbers and low cmc values, with the latter being temperature dependent (Furth 1980).

Nonionic detergents have been found to be particularly useful for protein solubilization. According to Pappas (1982), of the nonionic detergents tested, Triton X-100 was the most effective agent for solubilizing the membrane-bound enzymes of the brush-border plasma membrane of Hymenolepis diminuta. Maximum solubilization was achieved with 1% v/v Triton X-100, with solubilization decreasing significantly at lower detergent concentrations. Sixty-nine percent of APase (alkaline phosphohydrolase, E.C. 3.1.3.1) was solubilized at 0.1% v/v Triton X-100. Lubrol PX (2% v/v), 30 mM octylglucoside, and 5% v/v Tween 80 solubilized about 60% of APase. No significant APase activity was recovered from 5% v/v Brij 35, 5% v/v W-1, and 2% w/v Lubrol WX.

Nonionic detergents usually do not cause denaturation of the enzymes extracted. Yet in one report, Lubrol WX in concentrations of 0.5% to 6% inhibited sodium-potassium transport adenosinetriphosphatase activity. In this case, the optimum concentration for extraction was 4%, which yielded 40% - 45% of enzyme from beef brain microsomal suspension (Kahlenberg et al. 1969).

Nonionic surfactants with an appropriate polyoxyethylene chain (at least 8) seem to be the most potent hemolytic agents when comparing compounds with the same hydrophobic fragment size. The cmc of an ionic surfactant may be considered to reflect the relative hydrophobicity of the surfactant. The relative hydrophobicity of the surfactant appears to affect its affinity for the membrane

but not its lytic activity. The hemolytic potency should be considered as the result of two distinct and independent properties of the lytic agent: the degree to which the agent is taken up from solution by the cells, i.e. the affinity for the cell membrane, and the mechanism by which the uptake causes lysis. The affinity is essentially determined by the agent's relative hydrophobic character (Zaslavsky et al. 1978).

Detergents can also be used to gently remove unwanted membrane components during purification. Higher concentrations of detergents will disrupt natural membranes more completely, resulting in a mixture of predominantly lipid-containing micelles and predominantly protein-containing micelles, thereby allowing the proteins to be fractionated. In the presence of detergents, membrane proteins can also be re-associated with chosen lipids. It is shown that substantial effects on membranes can be measured at detergent concentrations less than one tenth their cmc. Bangham and Lea (1978) concluded that detergents affect bilayer lipid membrane conductivity. Specific conductance depends on the fourth or more power of Triton X-100 concentration. For dodecyl sulfate, sodium deoxycholate, and sodium cholate, the dependence is on a power of 1 to 2 of the detergent concentration. Bilayer lipid membrane conductivity measured at constant detergent concentration increases with the conductivity of the bathing salt solution. Consequently, nonionic detergents appear to be essential tools for the separation of both extrinsic and intrinsic membrane proteins from their associated lipids. In concentrations below their cmc, detergent molecules interact with bilayer lipid membranes or liposomes in such a way to increase conductivity to ions and non-electrolytes.

To extract acetylcholinesterase (AChE) from membranes, whether from eel electric organ, bovine erythrocytes, or human erythrocytes, most researchers used 1% Triton X-100, although 5% Triton X-100 (Niday et al. 1977) and Tween 20

(Zittle et al. 1954) have been used. Octyl- β -D-glucoside has recently been found to be an effective solubilizing agent for a number of membrane proteins. It is a small molecule of simple, defined structure (see Appendix 1) which has a high cmc (5.8 mg/ml) that permits easy removal by dialysis. The disaccharide detergent, lauryl maltoside, was found to be superior to other alkyl glucosides and to commercially available nonionic detergents, with respect to its physical properties (small, uniform micelles) and its ability to enhance the activity of cytochrome c oxidase (Rosevear et al. 1980). Characterization of the physical properties of solubilized membrane proteins is facilitated by the use of detergents with micelles of a uniform, small size. Lauryl maltoside and octylglucoside should be valuable for such studies since they form monodisperse populations of micelles of approximately 50,000 and 8,000 molecular weight, respectively. These are unusually small micelles compared to most nonionic detergents and will be advantageous for gel filtration purification as well as for physical studies.

Recent studies on detergent-protein interactions indicate that both the ionic bile salts and nonionic polyoxyethylene detergents bind to intrinsic membrane proteins and are equally effective in maintaining the native conformation of these proteins in aqueous solution. It has been suggested that detergents, in general, form micelles around the exposed hydrophobic surface of membrane proteins. They bind to enzymes above their critical micelle concentrations. Robinson and Capaldi (1977) concluded that "non-denaturing" detergents are not all equivalent and fall into two broad classes: (1) nonionic detergents which are mild and less effective in disrupting protein-protein interactions and are, therefore, useful in maintaining active membrane complexes; (2) ionic bile salts (cholate and deoxycholate) which are stronger detergents, more effective in disrupting protein-protein interactions, and can, therefore, destroy activity that is dependent on multisubunit associations. They are considered to be non-denaturing since they do not cause unfolding and denaturation of individual polypeptide chains.

II. Structure of acetylcholinesterase

Acetylcholinesterase (AChE) of electric eel is present as three main components, which can be distinguished by their sedimentation coefficients (about 18, 14, and 8 S) on sucrose gradient centrifugation at high ionic strength. The 11S AChE is a degradation product, formed from the native form of the enzyme. Electron micrographs reveal that the 18S and 14S forms are asymmetric, composed of a head, containing a large number of subunits, and an elongated tail. The 11S form of AChE is apparently a tetrameric structure devoid of the tail (Dudai et al. 1973). It is arranged as a dimer of dimers, with each subunit containing one active site. The molecular weight of the subunit is estimated to be 70,000 (Rosenberry et al. 1974). Active site numbers of 8.3 and 11.4 were determined for the 14 and 18 S forms, respectively. These results are in accord with a proposed model of two and three tetrameric structures comprising the head groups of the 14 and 18 S forms of electric tissue AChE, respectively (Parker et al. 1978). The mass of the tail is approximated at 100,000 daltons. It is thought to consist of three 30,000 dalton strands (Bon et al. 1976).

The asymmetric forms (17S and 13S) of AChE isolated from Torpedo californica electric organs contain the catalytic subunits, a structural subunit sensitive to trypsin, and a collagen-like tail unit. At 37°C, collagenase digestion of the 17S species, followed by disulfide bond reduction, leads to formation of an 11S species not containing the tail unit. The catalytic subunit is of 68,000 daltons and the structural subunit is of 100,000 daltons. Upon treatment of the 11S species with trypsin, the 100,000 dalton structural subunit is converted to an 85,000 dalton fragment, together with conversion of the larger polypeptides to monomers and oligomers of the catalytic subunit. It appears that the 100,000 dalton peptide links the tail unit with disulfide bonds and is in close proximity to the catalytic subunit (Lee et al. 1982; Lee and Taylor 1982).

Human erythrocyte AChE preparations which vary in lipid content, from lipid-rich to lipid-poor, have been successfully prepared using deoxycholate. It was found that the activity of such preparations was highly dependent on their phospholipid contents. Maximum specific activity was associated with a fixed phospholipid content. The lipid-poor enzyme was highly activated by addition of either endogeneous (membrane) or exogenous lipids (Sihotang 1976).

III. Allosteric properties of acetylcholinesterase

AChE has been shown to possess allosteric properties. It has been shown that atropine abolishes substrate inhibition of AChE at saturating substrate concentrations (Kato et al. 1972). It activates by protecting the enzyme against substrate inhibition rather than accelerating hydrolysis. By using the model described by Monod et al. (1963), one can explain the above experimental observations. The enzyme exists in two states, R and T; R state binds the substrate and T state binds the inhibitor. At saturating concentrations of substrate, substrate binds to the regulatory site and converts the enzyme to the T state, which has reduced catalytic activity, and also binds inhibitors. At high concentrations of substrate, the inhibitor (in this case atropine) competes with the substrate at the regulatory site and prevents inhibition due to excess substrate.

The allosteric model of AChE regulation proposed by Roufogalis and Quist (1972) contained one catalytic anionic site (alpha) and two peripheral anionic sites (beta and gamma). Low concentrations of calcium (less than 0.1 mM) bind to the beta anionic site; TMA (tetramethylammonium) binds to the alpha anionic site; galamine binds to the gamma site, because its inhibition cannot be antagonized by alpha or beta-site binding agents. Bis-quaternary ammonium compounds, such as decamethonium, bind across the alpha and beta sites.

A certain phospholipid, cardiolipin, has been found to be closely associated with AChE from bovine erythrocytes (Roufogalis and Beauregard 1979). Evidence is presented that the cardiolipin-associated enzyme is modulated by a temperature-dependent conformational rearrangement of the active site induced by the binding of substrate and inhibitor to the enzyme. Beauregard and Roufogalis (1977) observed a biphasic Arrhenius plot at low substrate concentrations, with a break around 20°C. The break in the Arrhenius plot of acetylcholine hydrolysis appeared to parallel the non-linear temperature dependence of $V_{\max}/K_m(\text{app})$, which reflects the conformational rearrangement of the enzyme-substrate complex, the rate limiting step at subsaturating substrate concentrations. Treatment of enzyme with 1.8 M NaCl in phosphate buffer abolished the break. At the same time, the phospholipid cardiolipin can be removed by chloroform-methanol extraction, which was not possible before the high salt treatment. However, physical methods, such as sucrose density gradient centrifugation and Sephadex gel chromatography, were not able to separate cardiolipin from the enzyme even after high salt treatment. Cardiolipin is suggested to be "sandwiched" between the two subunits of AChE and was postulated to be attached to the protein through a "calcium bridge", as well as by hydrophobic interaction of the fatty acid acyl chains with the hydrophobic regions in the protein (Beauregard and Roufogalis 1979). High salt treatment (1.8 M NaCl) thus causes functional but not physical dissociation of cardiolipin from the enzyme. The resulting enzyme behaves as a monomer by irradiation inactivation or target size analysis, in contrast to the dimeric functional state observed by the same technique prior to salt treatment.

IV. Aims of the present study

In the present report, several nonionic and zwitterionic detergents have been examined to determine their efficiency in solubilizing AChE from bovine eryth-

rocyte membrane. The objective of this study ultimately was to choose one or more detergents that would be suitable for purifying the enzyme from the erythrocyte membrane in a form representing the enzyme in its native environment. The detergents chosen varied in their overall structure, HLB values and cmc values. The solubilization of the enzyme was assessed in terms of the yield of solubilized enzyme and the preservation of its activity per weight of solubilized protein in the soluble state. In addition, the molecular state of the enzyme extracted by different detergents was assessed by sucrose gradient centrifugation. Solubilization efficiency was determined as a function of detergent concentration in each case. Analysis has been carried out of the relationship between efficiency of solubilization of AChE from bovine erythrocytes and various properties of the detergent used, including its hydrocarbon and head group structure, cmc and HLB value.

MATERIALS AND METHODS

I. Materials

The chemicals and/or proteins were purchased from the following sources:

1. Sigma Chemical Company

Acetylthiocholine

Aldolase (rabbit muscle)

Bovine serum albumin (essentially fatty acid free)

Catalase (bovine liver)

Decamethonium bromide

DTNB

Lubrol PX

Lubrol WX

SDS

Sodium deoxycholate

Triton X-100

Trizma Base

Tween 20

Tween 40

Tween 60

Tween 80

2. J.T. Baker Chemical Company

Sodium chloride

Sodium hydroxide

Sodium phosphate dibasic, 7-hydrate ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$)

Sodium phosphate monobasic, monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$)

Trichloroacetic acid

3. Calbiochem-Behring Corporation

β -D-octylglucoside

Zwittergent 310

Zwittergent 312

Zwittergent 314

Zwittergent 316

4. BDH Chemicals Ltd.

Citric acid monohydrate

Cupric sulfate

Potassium hydrogen tartrate

Tri-sodium citrate

5. Bio Rad Laboratories

Bio Beads SM-2

6. Fisher Scientific Company

Carbowax PEG 20,000

Dextrose

Phenol reagent, 2N

Sodium carbonate

Sucrose

7. Aldrich Chemical Company

Iodomethane

8. Pharmacia Fine Chemicals

DEAE-Sephadex A-50

II. Methods

1. Preparation of membranes from bovine erythrocytes

Blood was obtained from a local slaughterhouse, and prevented from coagulating with anhydrous citric acid, 0.44 g, sodium citrate dihydrate, 1.32 g, dextrose, 1.47 g per liter of blood. It was centrifuged for 10 min at 5000 rpm in an IEC B-20A centrifuge. The supernatant and buffy coat were discarded. The packed red cells were washed with 0.9% NaCl twice, each time discarding the supernatant after centrifuging at 5000 rpm for 10 min in the above centrifuge. The packed cells were hemolyzed with 8.93 mM sodium phosphate buffer, pH 7.40 in 7 volumes of the buffer for 10 min. The solution was centrifuged at 12,000 rpm for 10 min in the above centrifuge. The supernatant was discarded. The above procedure was repeated; this time the supernatant and the small solid pellet were discarded. The membrane suspension just above the pellet was saved. The above procedures were done at 4°C.

2. Solubilization of membranes

To one ml of membrane suspension, a required amount of detergent in solid or liquid state was added. The solution was incubated at the temperature specified for the required period with gentle mechanical shaking. Three ml of 0.1 M NaCl in 20 mM sodium phosphate buffer, pH 7.40 was added to the solution. The solution was centrifuged at 40,000 rpm for 60 min in a Beckman L2-65B ultracentrifuge using a type 65 rotor at 4°C. The pellet was resuspended into solu-

tion by adding 4 ml of the above buffer. The AChE activity in both the supernatant and pellet was determined by the method of Ellman et al. (1961), using a substrate concentration of 0.8 mM and a final volume of 2 ml.

3. Purification of acetylcholinesterase

DEAE-Sephadex (5 g) was allowed to swell in 400 ml of water heated by immersion in boiling water for 2 hours. After the suspension had been chilled, the supernatant from the last ultracentrifugation step (see above) was added to it and the mixture stirred for 20 to 30 min in the cold. The solution was filtered with a Buchner funnel using Whatman filter paper #1. The residue was washed with 500 ml of cold water, then the enzyme eluted with 500 ml of 0.5 M NaCl in 2 mM sodium phosphate buffer, pH 7.40. The eluent was concentrated down to about 50 ml using an Amicon ultrafiltration kit with a PM-10 membrane (molecular weight cut-off 10,000) filter at 4°C. The concentrated solution was loaded onto an affinity column at the rate of 2 ml per hour. The affinity column consisted of trimethyl (m-aminophenyl) coupled to Sepharose 2B, with one amino caproyl group as a spacer arm (Dudai et al. 1972) (see Appendix 4). The column was washed with 60 ml of 0.1 M NaCl in 20 mM sodium phosphate buffer, pH 7.40. The enzyme was eluted from the column with 60 ml of 20 mM decamethonium bromide in the same buffer in fractions of 4 ml each at a rate of 4 ml per hour. The fractions were dialyzed against 3500 ml of the same buffer for 16 hours with one change of buffer after 3 to 4 hours. Fractions containing high enzyme activity were pooled and concentrated down to appropriate volume by means of polyethyleneglycol (PEG) 20,000 through Spectrapor #1 membrane tubing.

4. Removal of detergent

Bio Beads SM-2, soaked with methanol and washed with water, were equilibrated with the above buffer in a Bio Rad Econo column at 4°C. About 3 g of Bio Beads were used for 1 ml of concentrated enzyme solution. Eluent from

the column was collected in fractions of approximately 2 ml each and assayed for enzyme activity. Fractions with significant amounts of enzyme activity were pooled and concentrated by means of PEG 20,000 through Spectrapor #1 membrane tubing.

5. Determination of S Value (sedimentation coefficient)

A 5 to 20% continuous sucrose density gradient was prepared by means of the Searle Isograd Equipment in 20 mM sodium phosphate buffer, pH 7.40 with 0.1 M NaCl. A 200 μ l sample was layered on top of the gradient and centrifuged for 17 hours at 40,000 rpm (200,000 \times g) in a Beckman L5-50 ultracentrifuge using an SW 40 rotor at 4°C. Fractions of 0.4 ml each were collected, starting from the bottom of the tube. Each fraction was assayed for enzyme activity. The marker proteins used were bovine serum albumin, catalase and aldolase. They were detected by reading absorbance at 280 nm for BSA and aldolase and at 405 nm for catalase.

6. Arrhenius plot determination

A solution consisting of 500 μ l of 0.2 M sodium phosphate buffer, pH 7.40, 300 μ l of 10 mM DTNB, 1.1 ml of water, and 20 μ l of enzyme solution was equilibrated to the desired temperature in a water-methanol bath. The reaction was started by adding to it 80 μ l of 10 mM ASCh previously equilibrated to the same temperature. The absorbance was measured immediately over a period of at least 4 min, with a Beckman Model 25 spectrophotometer at 412 nm. The change in absorbance of a similarly treated solution without the enzyme was also recorded. The rate was calculated as the net absorbance change per min. For high salt treatment, equal volumes of enzyme solution and 4 mM sodium phosphate buffer, pH 7.40, containing 3.6 M NaCl, were mixed. After 30 min, the solution was dialyzed against 1000 ml of distilled water at 4°C using Spectrapor #1 membrane tubing for 16 hours with one change of water after 3 to 4 hours.

7. Specific activity determination

In separate experiments, ASCh at a concentration of 0.8 mM was found to give maximum enzyme activity without showing substrate inhibition. So this concentration was chosen in determining the specific activity of AChE. The method was adapted from that of Ellman et al. (1961). The assay medium consisted of 1 ml of 0.2 M sodium phosphate buffer, pH 7.40, 160 μ l of 10 mM ASCh, 600 μ l of 10 mM DTNB, 20 μ l of enzyme solution, and 220 μ l of water to make a final volume of 2 ml.

For protein determination, the method of Peterson (1977) was used. Appropriate amounts of protein (5 to 100 μ g) were dissolved in a total volume of 1 ml of water. To the solution, 0.1 ml of 0.15% sodium deoxycholate was added. After 10 min, 0.1 ml of 72% trichloroacetic acid was added. The solution was centrifuged at 3000 g for 15 minutes. The supernatant was discarded. The pellet was dissolved in 1 ml of water and 1 ml of a mixture of CTC solution-10% SDS-0.8 N NaOH-water (1:1:1:1). The CTC solution contained 0.1% cupric sulfate, 0.2% potassium hydrogen tartrate and 10% sodium carbonate. Ten minutes later, 0.5 ml of a mixture of water-phenol reagent 2N (1:5) was added. The color was read at 660 nm after 30 min at room temperature.

RESULTS

I. Efficiency of acetylcholinesterase solubilization by various detergents

The following detergents have been tested: β -D-octylglucoside, Lubrol WX, Lubrol PX, Tween 20, Tween 40, Tween 60, Tween 80, Zwittergent 310, Zwittergent 312, Zwittergent 314, Zwittergent 316, and Triton X-100. The structures of the various detergents are shown in Appendix 1.

Table I lists the results of solubilization of AChE activity with increasing concentrations of β -D-octylglucoside. The incubation for solubilization was at room temperature for 1 hour. At a concentration of 30 mg/ml, 40% of the enzyme activity was recovered in the supernatant. As concentrations were progressively increased up to 100 mg/ml, increasing amounts of AChE activity were solubilized. Up to 92% of enzyme activity was recovered in the supernatant, although the proportional yield was diminishing. At an even higher concentration (250 mg/ml), only 72% of the enzyme activity could be detected in the supernatant, presumably because of inactivation. At a concentration of 30 mg/ml and 4°C as the incubation temperature, increasing the incubation time with membranes from 1 hour to 17 hours resulted in solubilization of enzyme from only 10% to 20%. From Table 1, the minimum concentration required for over 50% solubilization is 40 mg/ml at room temperature.

Table 2 shows the results of solubilization with several Zwittergents. The membranes and detergent were incubated for 1 hour at room temperature. Zwittergent 310, with the largest cmc among all the Zwittergents tested, was not very effective in solubilizing AChE from erythrocyte membranes. At its cmc, 12 mg/ml, only 2% of enzyme activity could be detected in the supernatant. Higher concentrations of the detergent (5x cmc and 10x cmc) did not increase the solubilization yield, resulting in only 2% of enzyme recovery in the supernatant in both cases. However, at 6.0 mg/ml, 65% of AChE was recovered in the supernatant.

Zwittergent 312 behaved similarly to Zwittergent 310. At 1x cmc concentration (1.2 mg/ml), 35% of enzyme activity was detected in the supernatant. At concentrations up to 3000x cmc, less and less enzyme activity was detected in the supernatant as the concentration increased.

Zwittergent 314 acted slightly differently. A detergent concentration of 10x cmc (1.2 mg/ml) resulted in higher AChE activity being detected in the

Table 1. Efficiency of β -D-octylglucoside in solubilizing bovine erythrocyte membrane acetylcholinesterase. All values reported are percentages of input (i.e. original enzyme activity before addition of detergent). Each ml of membrane suspension contained about 10 mg protein. The data are the average of two separate experiments, each done in duplicate. Figures in brackets indicate standard deviation.

Detergent Concentration Used (mg/ml)	Enzyme Activity in Supernatant (%)	Enzyme Activity in Pellet (%)
30	40 (+12.5)	13 (+3.0)
40	80 (+ 1.0)	20 (+3.1)
50	90 (+ 0.3)	9 (+0.1)
60	86 (+ 3.8)	10 (+0.6)
100	92 (+ 0.4)	9 (+0.3)
250	72 (+ 2.8)	3 (+0.3)

Table 2. Efficiency of several Zwittergents (Z) in solubilizing bovine erythrocyte membrane acetylcholinesterase. All values reported are percentages of input. The cmc for each Zwittergent is shown. Each ml of membrane preparation contained about 10 mg protein. The data are the average of 2 separate experiments, each done in duplicate. Figures in brackets indicate standard deviation.

Detergent	cmc (mg/ml)	Concentration Used (mg/ml)	Enzyme Activity in Supernatant (%)	Enzyme Activity in Pellet (%)
Z 310	12	0.12	4.0 (+0.4)	53.0 (+9.3)
		0.60	4.2 (+0.8)	74.0 (+6.9)
		1.20	6.2 (+1.3)	85.5 (+17.6)
		3.60	44.2 (+7.1)	56.0 (+11.6)
		6.00	64.7 (+17.1)	14.8 (+0.7)
		12.00	2.0 (+0.7)	2.4 (+0.9)
		60.00	2.2 (+0.5)	2.2 (+0.9)
		120.00	2.6 (+0.9)	1.4 (+0.2)
Z 312	1.2	0.12	7.0 (+2.2)	83.7 (+5.4)
		0.60	26.8 (+3.4)	64.0 (+6.4)
		1.20	35.0 (+3.8)	37.7 (+1.4)
		6.00	3.7 (+1.0)	3.0 (+0.7)
		12.00	1.8 (+0.3)	1.6 (+0.2)
		60.00	3.0 (+1.5)	1.0 (+0.3)
		120.00	1.9 (+0.3)	2.1 (+1.0)
		360.00	1.6 (+0.3)	1.9 (+0.3)
Z 314	0.12	0.12	8.6 (+1.9)	82.6 (+6.5)
		1.2	76.4 (+1.5)	16.4 (+1.2)
		6.0	19.6 (+8.5)	5.1 (+2.7)
		12.0	6.9 (+1.9)	1.8 (+0.1)
		36.0	1.8 (+0.6)	1.9 (+0.6)
		60.0	1.1 (+0.7)	1.4 (+0.2)
		120.0	1.5 (+0.4)	1.8 (+0.3)
Z 316	0.012	0.012	5.3 (+0.9)	77.8 (+10.8)
		0.12	19.8 (+1.9)	78.5 (+10.9)
		0.60	41.4 (+7.0)	40.3 (+5.0)
		1.2	69.9 (+7.3)	50.1 (+4.2)
		3.6	96.0 (+16.0)	36.3 (+1.6)
		6.0	68.6 (+6.7)	19.1 (+3.9)
		12.0	81.0 (+4.5)	15.7 (+2.6)

supernatant, 76%, than that obtained at 1x cmc (0.12 mg/ml), 9%. Further increases in detergent concentration resulted in less enzyme activity recovery in the supernatant.

With Zwittergent 316, which has the smallest cmc of all Zwittergents tested, at a concentration of 1x cmc (0.012 mg/ml), most of the enzyme activity (78%) remained in the pellet. At a concentration of 100x cmc, (1.2 mg/ml), however, more than 50% of enzyme activity was extracted from membranes, while at concentration of 300x cmc (3.6 mg/ml) enzyme activation appeared to have taken place, since more than 100% of enzyme activity was recovered from membranes. A further increase in detergent concentration (at 1000x cmc) resulted in a decrease in enzyme activity recovered from membranes.

Table 3 shows the results of solubilization with several Tweens. The membranes were incubated with Tweens at room temperature for one hour. With Tween 40 and Tween 60, concentrations ranging from 1 to 1000 mg/ml were found to be unsuccessful in extracting AChE from membranes. Most of the enzyme activity was detected in the pellet. Tween 20 and 80, at concentrations up to 100 mg/ml, were also ineffective in solubilizing AChE from membranes. However, at a concentration of 1000 mg/ml, 50 to 60% of enzyme was extracted. It seemed possible that increasing the incubation time with membranes might help. With Tween 20, increasing the incubation time to 18 hours at 100 mg/ml concentration resulted in 26% and 24% of enzyme activity in supernatant and pellet, respectively. The corresponding figures for Tween 80 were 12% and 85%. However, at 500 mg/ml concentration, 18 hours of incubation with Tween 20 gave 66% enzyme activity in the supernatant, while Tween 80 gave 63% (results not shown).

Table 4 shows the results obtained when Lubrol WX and Lubrol PX were used as the membrane solubilizing detergent. Lubrol PX, at a concentration of 100 mg/ml, produced slight enzyme activation in the supernatant, after 1 to 18 hours of

Table 3. Efficiency of several Tweens in solubilizing bovine erythrocyte membrane acetylcholinesterase. All values reported are percentages of input. Each ml of membrane preparation contained about 10 mg protein. The data are the average of 2 separate experiments, each done in duplicate. Figures in brackets indicate standard deviation.

Detergent	cmc (mg/ml)	Concentration Used (mg/ml)	Enzyme Activity in Supernatant (%)	Enzyme Activity in Pellet (%)
Tween 20	0.060	1	2.9 (+0.6)	94.0 (+2.8)
		10	4.7 (+0.3)	70.6 (+3.8)
		100	5.6 (+0.4)	76.0 (+3.8)
		1000	62.6 (+1.9)	33.3 (+0.9)
Tween 40	0.029	1	3.7 (+0.7)	85.3 (+1.9)
		10	6.0 (+0.2)	80.6 (+4.7)
		100	4.5 (+0.3)	72.6 (+2.8)
		1000	39.3 (+2.8)	18.0 (+2.8)
Tween 60	0.027	1	2.2 (+0.3)	82.0 (+1.5)
		10	3.2 (+0.2)	81.3 (+1.9)
		100	4.9 (+0.2)	81.3 (+1.9)
		1000	9.0 (+0.5)	47.8 (+1.7)
Tween 80	0.013	1	2.3 (+0.2)	80.8 (+1.2)
		10	2.1 (+0.2)	80.2 (+0.3)
		100	3.5 (+0.2)	81.7 (+0.5)
		1000	48.3 (+0.5)	20.0 (+1.9)

Table 4. Efficiency of Lubrol PX and Lubrol WX in solubilizing bovine erythrocyte membrane acetylcholinesterase. All values reported are percentages of input. Each ml of membrane preparation contained about 10 mg protein. The data are the average of 2 separate experiments, each done in duplicate. Figures in brackets indicate standard deviation.

Detergent	Concentration Used (mg/ml)	Incubation Time (hr)	Enzyme Activity in Supernatant (%)	Enzyme Activity in Pellet (%)
Lubrol PX	100	1	126.5 (+19.6)	5.7 (+2.0)
		2	103.9 (+0.9)	4.3 (+0.3)
		18	106.5 (+1.2)	3.7 (+0.6)
	500	2	70.5 (+1.2)	15.5 (+1.0)
		18	79.3 (+0.9)	8.6 (+0.6)
Lubrol WX	100	1	38.2 (+10.0)	82.1 (+18.8)
		2	39.2 (+2.3)	58.7 (+0.5)
	500			
		2	19.7 (+0.5)	31.6 (+1.7)
		18	11.0 (+0.6)	82.7 (+1.2)

incubation with membranes. At a higher concentration (500 mg/ml), enzyme activation was lost. An incubation time of 2 hours with the membranes produced 71% enzyme activity in the supernatant, while increasing the incubation time to 18 hours only increased enzyme activity in the supernatant to 80%. Lubrol WX did not show promising results in the extraction of AChE from erythrocyte membranes. Neither concentration tested, 100 mg/ml and 500 mg/ml, extracted more than 50% of enzyme activity from the membranes. The incubation time with membranes ranged from 1 to 18 hours.

Triton X-100 was used as a reference only, since most previous studies have used this detergent for enzyme solubilization. At 10 mg/ml (1%) and one hour of incubation with membranes, 104% of enzyme activity was obtained in the supernatant.

II. Efficiency of protein solubilization

From the above experiments, five detergents were selected for further studies: β -D-octylglucoside at 40 mg/ml, Zwittergent 316 at 3.6 mg/ml, Lubrol PX at 100 mg/ml, Tween 20 at 500 mg/ml, and Triton X-100 at 10 mg/ml. The incubation time with membranes was one hour in each case, except with Tween 20, which was incubated with the membranes overnight.

To determine if the low enzyme activity in the supernatant was due to inability of the detergent at the particular concentration to stabilize enzyme activity or due to the inefficiency of protein solubilization, protein determination was carried with some of the detergents (see Table 5). β -D-Octylglucoside was found to efficiently solubilize protein at both the concentrations tested, 30 mg/ml and 40 mg/ml. In both cases, over 80% of the proteins originally present in the membrane were detected in the supernatant. Accompanying the protein a moderate amount of AChE activity was present, 40% at 30 mg/ml and 60% at 40 mg/ml. Both Lubrol

Table 5. Relationship between protein solubilized and enzyme activity for some selected detergents. All values reported are percentages of input. Each ml of membrane preparation contained about 10 mg protein. The data are the average of 2 separate experiments, each done in duplicate. Figures in brackets indicate standard deviation.

Detergent and Concentration Used (mg/ml)	Supernatant		Pellet	
	Enzyme Activity (%)	Protein Amount (%)	Enzyme Activity (%)	Protein Amount (%)
octylglucoside : 30	40 (<u>+14.1</u>)	97 (<u>+2.3</u>)	13 (<u>+4.2</u>)	11 (<u>+1.4</u>)
octylglucoside : 40	60 (<u>+28.3</u>)	83 (<u>+0.4</u>)	20 (<u>+5.0</u>)	8 (<u>+1.4</u>)
Lubrol PX : 100	125 (<u>+17.0</u>)	88 (<u>+1.8</u>)	5 (<u>+4.2</u>)	9 (<u>+0.6</u>)
Lubrol WX : 100	38 (<u>+11.3</u>)	102 (<u>+4.2</u>)	82 (<u>+11.3</u>)	18 (<u>+1.1</u>)
Triton X-100 : 10	104 (<u>+11.3</u>)	93 (<u>+1.8</u>)	20 (<u>+4.2</u>)	12 (<u>+0.3</u>)
Zwittergent 312 : 360	2 (<u>+0.7</u>)	84 (<u>+0.4</u>)	3 (<u>+1.4</u>)	5 (<u>+0.4</u>)
Zwittergent 314 : 36	2 (<u>+0.7</u>)	108 (<u>+20.4</u>)	3 (<u>+1.4</u>)	5 (<u>+0.4</u>)
Zwittergent 316 : 3.6	93 (<u>+8.5</u>)	79 (<u>+12.0</u>)	44 (<u>+5.7</u>)	11 (<u>+0.7</u>)

PX at 100 mg/ml and Triton X-100 at 10 mg/ml solubilized around 90% of proteins into the supernatant. Both showed enzyme activation, resulting in more enzyme activity being detected than the amount of activity originally present. Lubrol WX behaved in a similar way to Lubrol PX as far as protein solubilization was concerned. Considerably more protein was detected in the supernatant than in the pellet, yet twice the enzyme activity was detected in the pellet. This suggests that proteins other than AChE were the ones mainly solubilized by this detergent.

Zwittergent 312 and Zwittergent 314 were efficient protein solubilizers at the concentrations used, 360 mg/ml and 36 mg/ml, respectively, but at these concentrations they failed to fully sustain AChE activity. In both cases only 2% of enzyme activity was detected in the supernatant. If proteins other than AChE were solubilized, most of the AChE would still remain in the pellet. This would give a high AChE activity to the pellet, which was not the case. It would thus appear that AChE was efficiently solubilized by the detergent but during the process of solubilization, much of it was inactivated.

Zwittergent 316 was another of the detergents that showed enzyme activation during solubilization. At 3.6 mg/ml concentration, it activated AChE by 50%. It solubilized 80% of the proteins from the erythrocyte membrane into the supernatant, and in doing so, retained all or most of the AChE activity. The fact that 11% of protein content in the pellet contained 44% of enzyme activity indicated that other proteins may be preferentially or more easily accessible to Zwittergent 316 for solubilization than AChE. These results suggest that at certain concentrations some detergents (e.g. Lubrol WX at 100 mg/ml, Zwittergent 312 at 360 mg/ml, Zwittergent 314 at 36 mg/ml) fail to support enzyme activity, despite their ability to solubilize membrane proteins.

III. Purification of acetylcholinesterase solubilized by various detergents

Acetylcholinesterase was purified from membranes using Lubrol PX at 100 mg/ml, Tween 20 at 500 mg/ml, Triton X-100 at 10 mg/ml, and Zwittergent 316 at 3.6 mg/ml as the solubilizing detergent. Figure 1 shows a typical chromatogram for the PTA affinity purification of bovine erythrocyte AChE. In this particular case, the detergent used was Tween 20. Each fraction was 0.5 ml. As can be seen from the chromatogram, over 95% of the enzyme activity was eluted in two fractions (fraction 26 and 27). In each case, the detergent was depleted by means of a Bio Beads SM-2 column. Figure 2 shows the elution of AChE from a Bio Beads column in the presence of detergent, in this case Tween 20. Each fraction collected was 2 ml. Most of the enzyme eluted in a single fraction. The specific activities of the enzyme species purified have been determined (see Table 6).

When the detergent β -D-octylglucoside was used as the solubilizing detergent, AChE eluted from the affinity chromatography column did not retain its activity. Hence, no further experiments were performed on this preparation. Enzyme species obtained using Lubrol PX and Tween 20 as the solubilizing detergent were more active than the corresponding species using Triton X-100 and Zwittergent 316 as the solubilizing detergent. This was the case both before Bio Beads treatment and after Bio Beads treatment. In all cases, removal of the detergent resulted in considerable decrease in enzyme activity.

Lubrol PX-solubilized AChE was found to be more active than Tween 20-solubilized AChE before Bio Beads treatment. After Bio Beads treatment, the activity was similar in both preparations. A similar situation occurred for the other two preparations of AChE. Triton X-100-solubilized AChE was more active than Zwittergent 316-solubilized AChE before Bio Beads treatment and both preparations lost most of their activity after Bio Beads treatment.

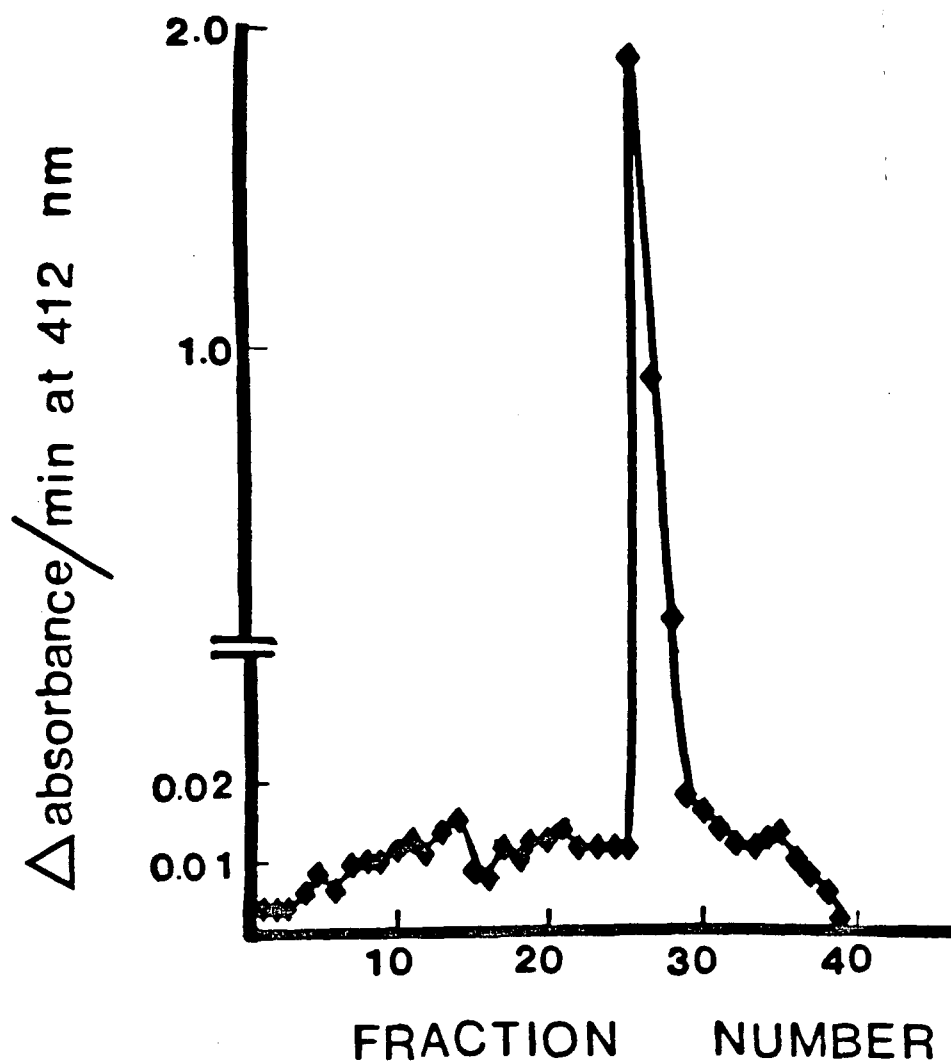


Figure 1. The purification of Tween 20-solubilized bovine erythrocyte membrane acetylcholinesterase by means of PTA affinity chromatography. Loading was carried out during collection of fractions 1 to 4; washing was carried out during collection of fractions 5 to 24; and eluting fractions were 25 to 39. The overall recovery was 30%. Each fraction was 5 ml.

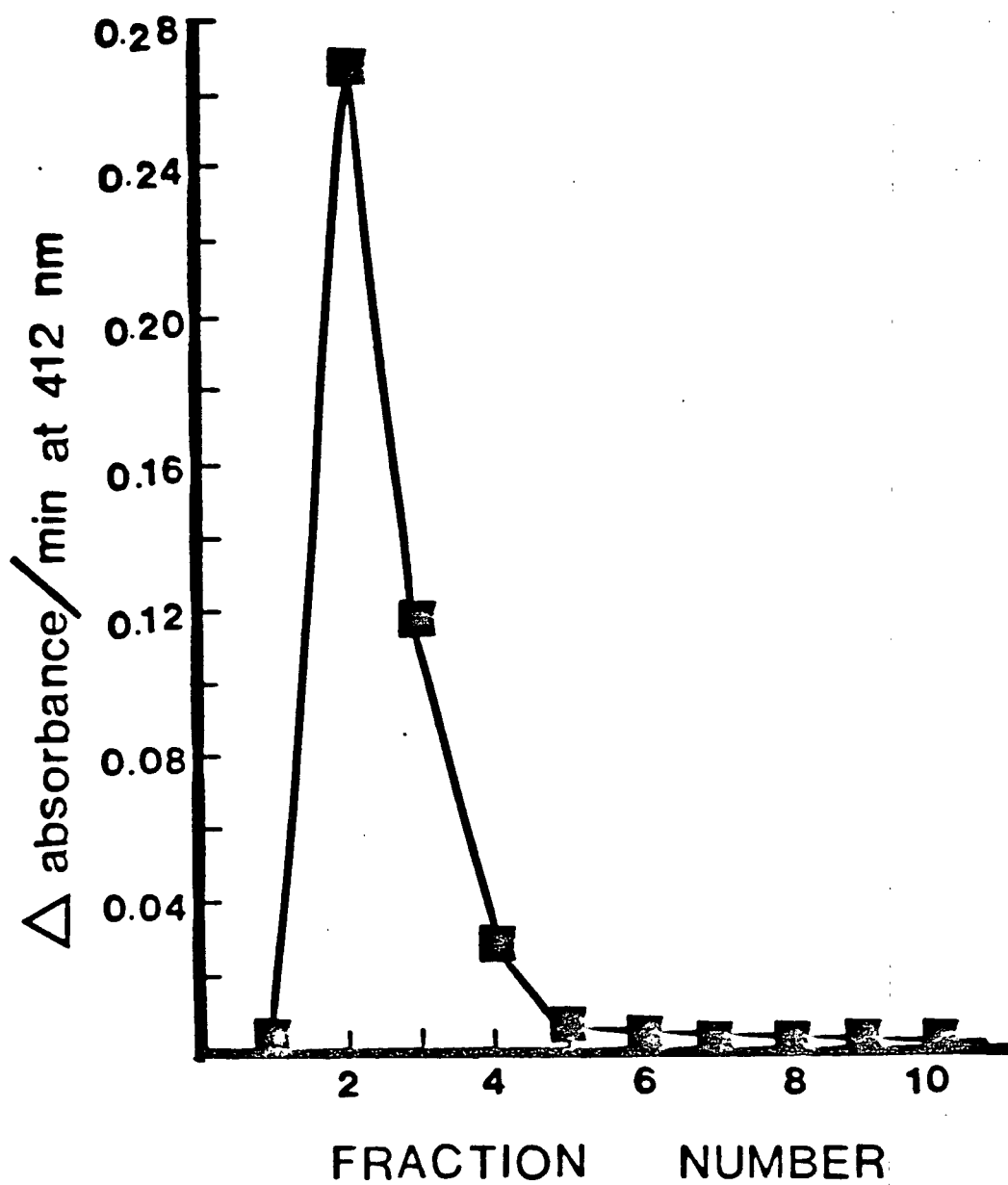


Figure 2. Depletion of detergent Tween 20 from PTA affinity chromatography-purified bovine erythrocyte acetylcholinesterase solubilized with Tween 20, using a Bio Beads SM-2 column.

Table 6. Specific activity (I.U./mg protein) of some acetylcholinesterase preparations. The data are the average of 2 separate experiments, each done in duplicate. Figures in brackets indicate standard deviation.

Membrane Solubilizing Detergent	Before Bio Bead Treatment	After Bio Bead Treatment
Lubrol PX at 100 mg/ml	750 (+106.8)	56 (+16.3)
Tween 20 at 500 mg/ml	275 (+37.4)	55 (+18.5)
Triton X-100 at 10 mg/ml	46 (+7.9)	3 (+0.2)
Zwittergent 316 at 3.6 mg/ml	21 (+2.9)	4 (+0.3)

IV. Characterization of molecular forms of acetylcholinesterase

In order to estimate the nature of the molecular forms of AChE obtained using different detergents, the sedimentation coefficient (S value) of a number of preparations was obtained.

Figures 3 and 4 show the continuous sucrose density gradient centrifugation of AChE solubilized with Lubrol PX before and after Bio Beads treatment, respectively. Before Bio Beads treatment, the enzyme gave a single broad symmetrical peak, with enzyme activity spread among 5 to 6 fractions. The S value can be determined with relative certainty because of the symmetrical shape of the peak. It was found to be 6.8 S. After depletion of the detergent, more than one AChE forms were detected (Figure 4). Peaks were located in fraction 7 and 14, corresponding to S values of 12 and 7.7, respectively. The 7.7 S form was present in greater abundance. However, most of the enzyme activity was found to stay on top of the gradient. This may be explained by AChE being trapped in detergent micelles which are lighter than the sucrose gradient.

Figures 5 and 6 show the continuous sucrose density gradient centrifugation of AChE solubilized with Tween 20 before and after Bio Beads treatment, respectively. In Figure 5, the chromatogram shows a broad peak of AChE activity. The fraction with the most enzyme activity (fraction 14) corresponded to a S value of 7.6. When AChE solubilized with Tween 20 was treated with Bio Beads to deplete the detergent, the chromatogram obtained was similar but broader in shape to the one before detergent removal. In this case, peak enzyme activity appeared to have spread among five fractions, with fraction 27, having the maximum activity, corresponding to a S value of 7.1. A small amount of enzyme floated on top of the gradient, as the last fraction was found to have higher enzyme activity than the second last one.

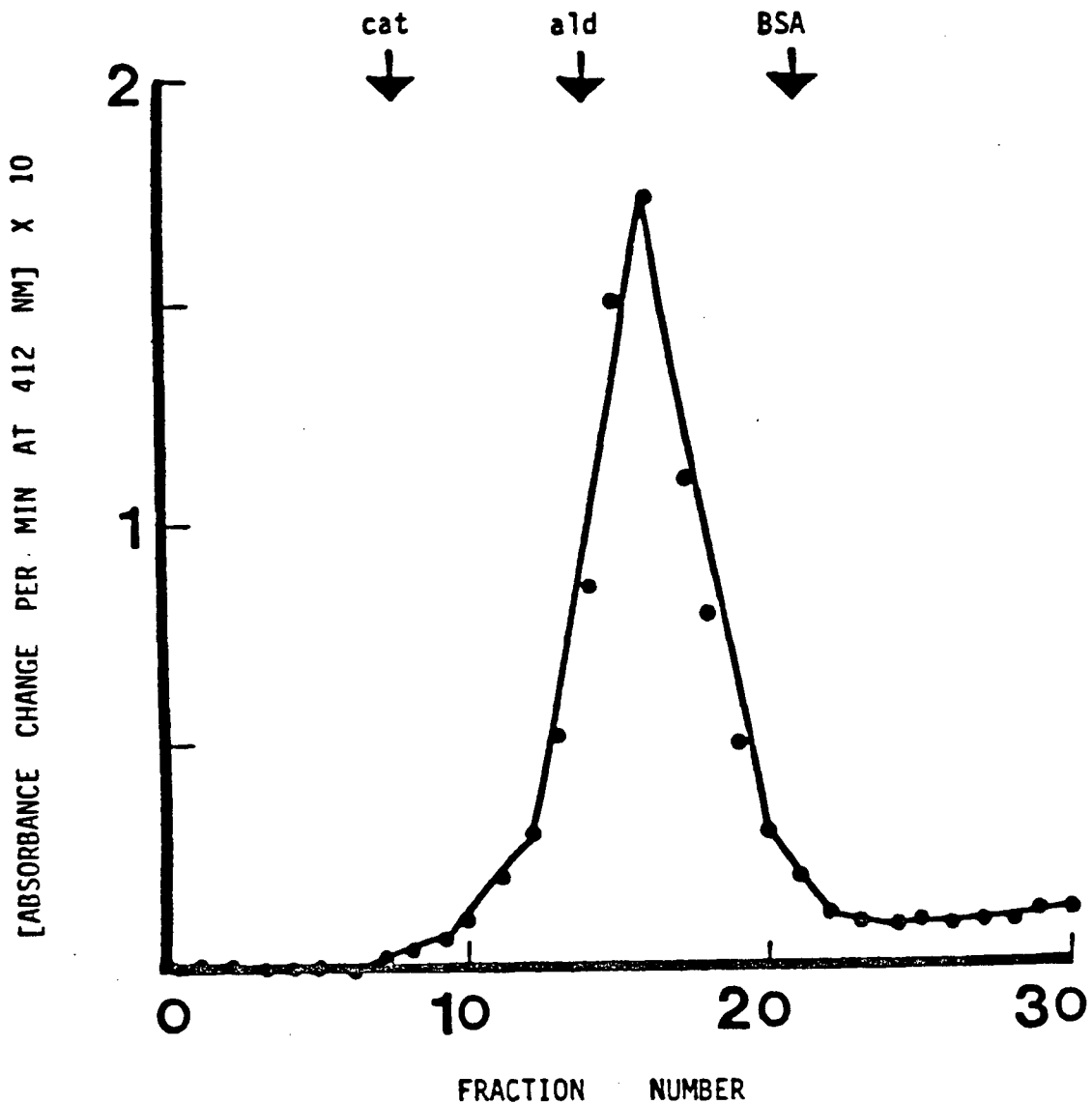


Figure 3. Continuous sucrose density gradient centrifugation of AChE solubilized with Lubrol PX before passing through a Bio Bead column. After centrifugation at $200,000\times g$ and 4°C for 17 hours, 30 fractions were collected from the bottom of the tube. Arrows indicate the positions of the marker proteins: cat is catalase, 11.4 S; ald is aldolase, 7.4 S; and BSA is bovine serum albumin, 4.4 S. Each fraction was 0.38 ml. The graph shown is one of two similar experiments. The S value obtained in this experiment (6.8 S) compares to 6.8 S found in the second experiment.

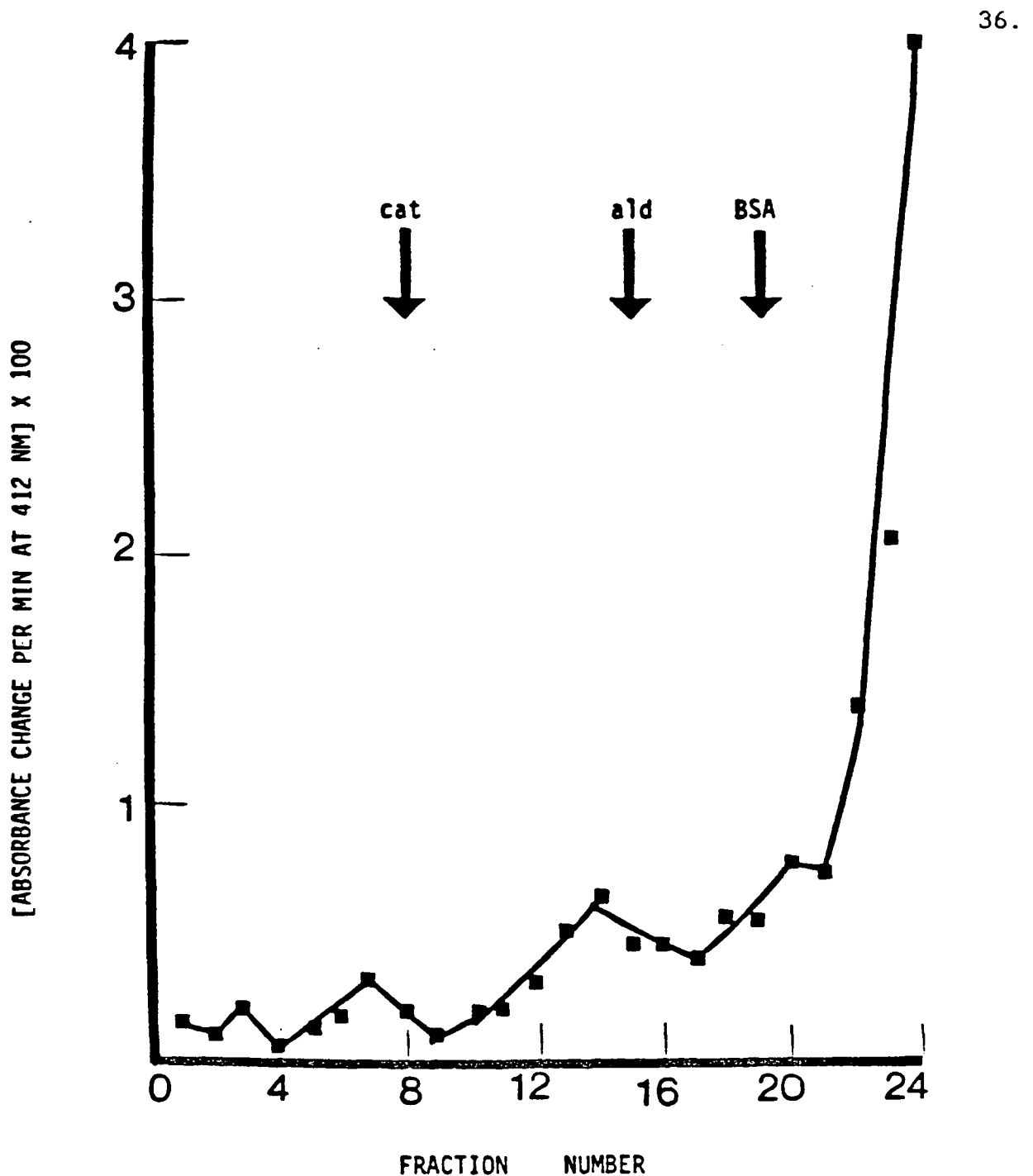


Figure 4. Continuous sucrose density gradient centrifugation of AChE solubilized with Lubrol PX after passing through a Bio Bead column. After centrifugation at $200,000\times g$ and 4°C for 17 hours, 24 fractions were collected from the bottom of the tube. Arrows indicate the positions of the marker proteins: cat is catalase, 11.4 S; ald is aldolase, 7.4 S; and BSA is bovine serum albumin, 4.4 S. Each fraction was 0.48 ml. The graph shown is one of two similar experiments. The S value obtained in this experiment (7.7 S) compares to 7.2 S found in the second experiment.

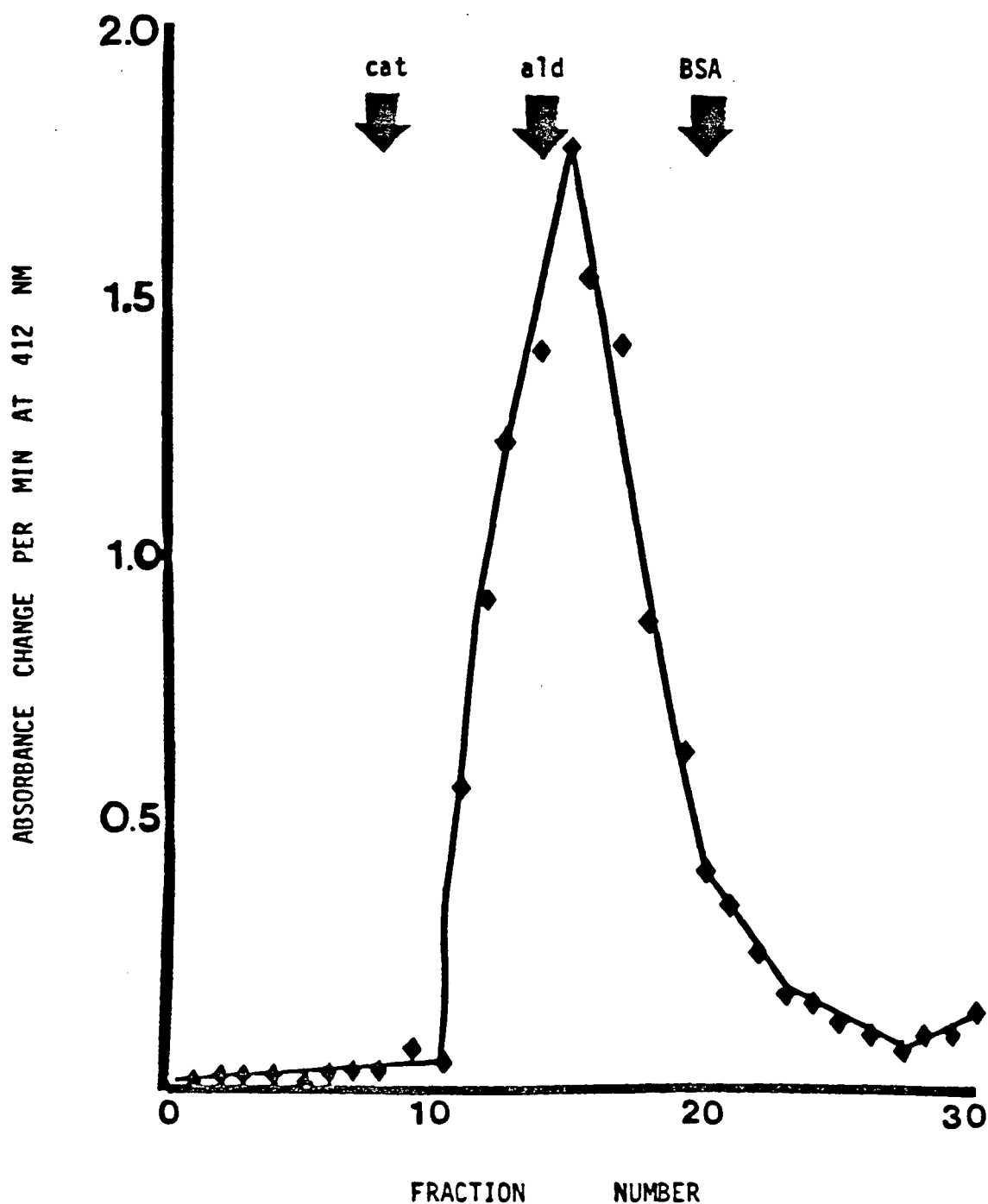


Figure 5. Continuous sucrose density gradient centrifugation of AChE solubilized with Tween 20 before passing through a Bio Bead column. After centrifugation at $200,000\times g$ and 4°C for 17 hours, 29 fractions were collected from the bottom of the tube. Arrows indicate the positions of the marker proteins: cat is catalase, 11.4 S; ald is aldolase, 7.4 S; and BSA is bovine serum albumin, 4.4 S. Each fraction was 0.38 ml. The graph shown is one of two similar experiments. The S value obtained in this experiment (7.4 S) compares to 7.8 S found in the second experiment.

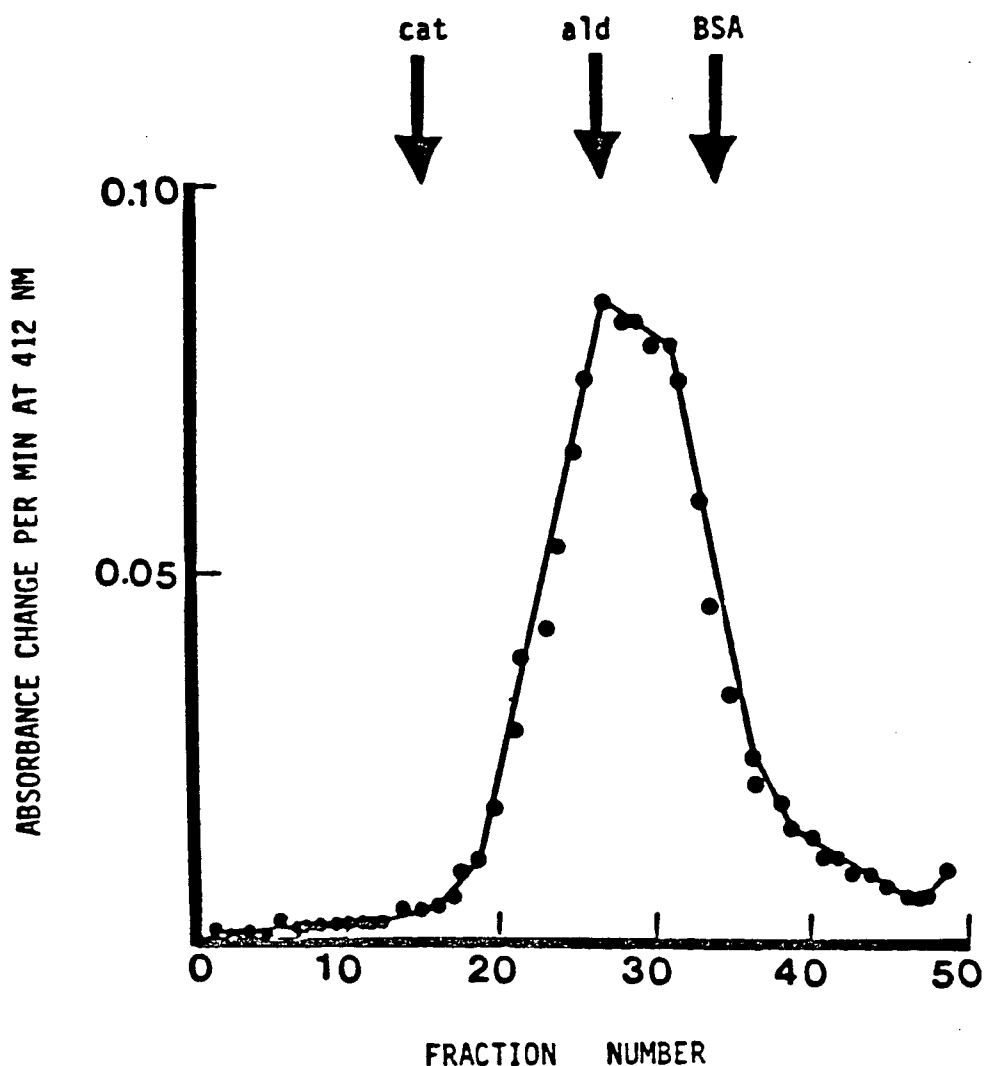


Figure 6. Continuous sucrose density gradient centrifugation of AChE solubilized with Tween 20 after passing through a Bio Bead column. After centrifugation at $200,000\times g$ and 4°C for 17 hours, 49 fractions were collected from the bottom of the tube. Arrows indicate the positions of the marker proteins: cat is catalase, 11.4 S; ald is aldolase, 7.4 S; and BSA is bovine serum albumin, 4.4S. Each fraction was 0.23 ml. The graph shown is one of two similar experiments. The S value obtained in this experiment (7.1 S) compares to 6.8 S found in the second experiment.

Figures 7 and 8 show the continuous sucrose density gradient centrifugation of AChE solubilized with Triton X-100 before and after Bio Beads treatment, respectively. In Figure 7, fraction 15 contained the highest amount of AChE activity, corresponding to a *S* value of 7.4. The amount of enzyme activity present in fraction 16 was only slightly less than that in fraction 15, corresponding to a *S* value of 6.8. Other molecular forms may also be present. However, the enzyme activity detected in the other fractions was too low to indicate the presence of significant amount of other AChE molecular forms. It was noted that enzyme activity was on the rise during the last four fractions, indicating some of the enzyme never sedimented into the gradient. The chromatogram in Figure 8 shows a broad, pointed peak with fraction 11 having the highest enzyme activity, corresponding to a *S* value of 7.1. After depletion of the detergent, some of the enzyme was found to stay at the top of the gradient, as indicated by the presence of small amounts of enzyme activity in the last four fractions.

Figures 9 and 10 show the continuous sucrose density gradient centrifugation of AChE solubilized with Zwittergent 316 before and after Bio Beads treatment, respectively. As shown in Figure 9, more enzyme activity was detected at the bottom of the gradient than elsewhere. This may indicate aggregation of the enzyme detergent complex into a form that sedimented to the bottom of the tube after the indicated period of centrifugation. The *S* value cannot be determined because it is the first fraction in the chromatogram. Other peaks were present in the chromatogram, shared by two fractions, 12 and 13, the peak activity corresponding to a *S* value of 7.1. The chromatogram in Figure 10 indicated multiple AChE forms were present after the detergent was depleted. Fraction 8 contained the highest amount of enzyme activity, with fraction 11 and fraction 16 decreasing in order of enzyme activity. The corresponding *S* values for fraction 8 and 11 were

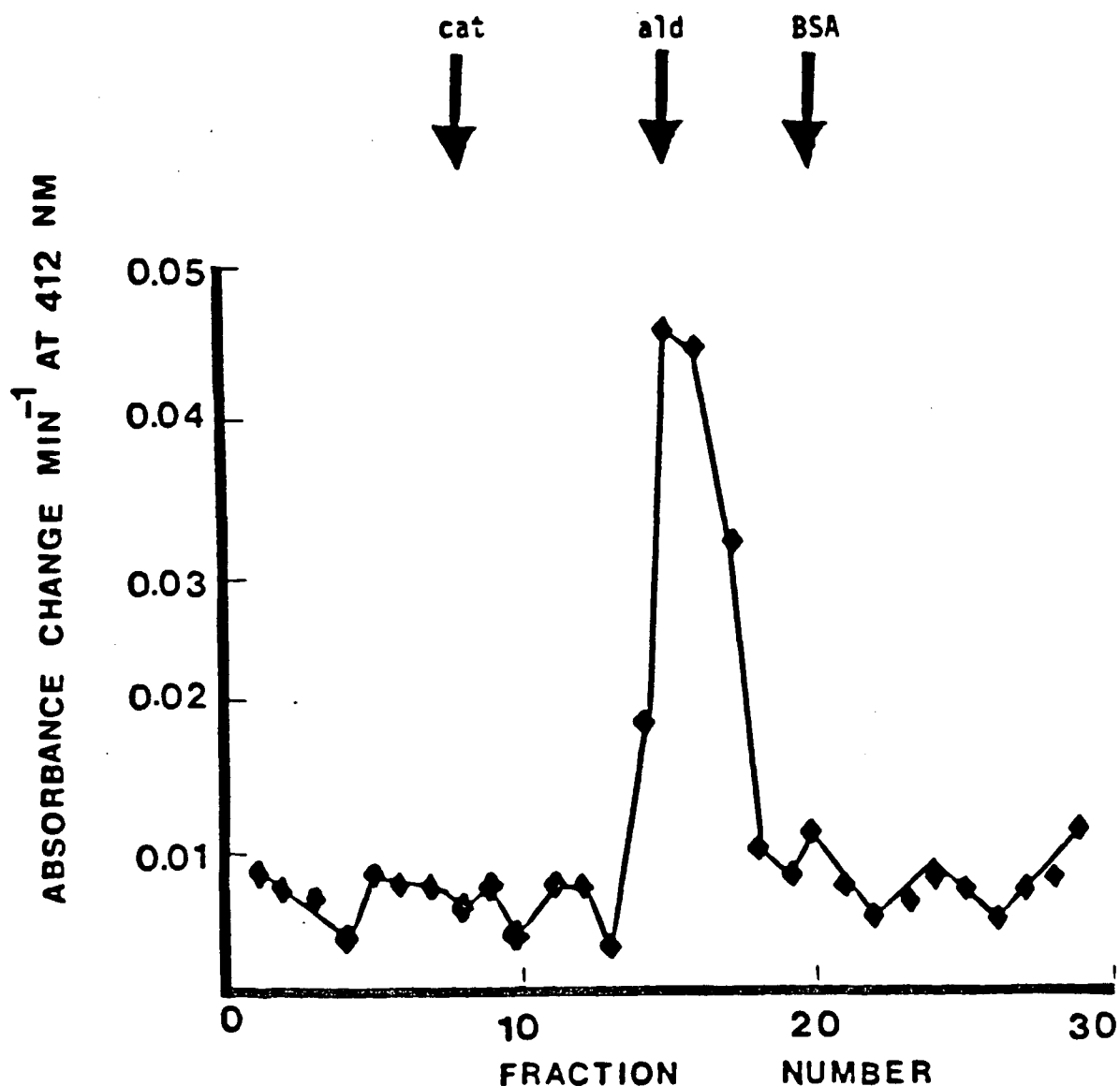


Figure 7. Continuous sucrose density gradient centrifugation of AChE solubilized with Triton X-100 before passing through a Bio Bead column. After centrifugation at $200,000\times g$ and 4°C for 17 hours, 29 fractions were collected from the bottom of the tube. Arrows indicate the positions of the marker proteins: cat is catalase, 11.4 S; ald is aldolase, 7.4 S; and BSA is bovine serum albumin, 4.4 S. Each fraction was 0.39 ml. The graph shown is one of two similar experiments. The S value obtained in this experiment (7.4 S) compares to 7.3 S found in the second experiment.

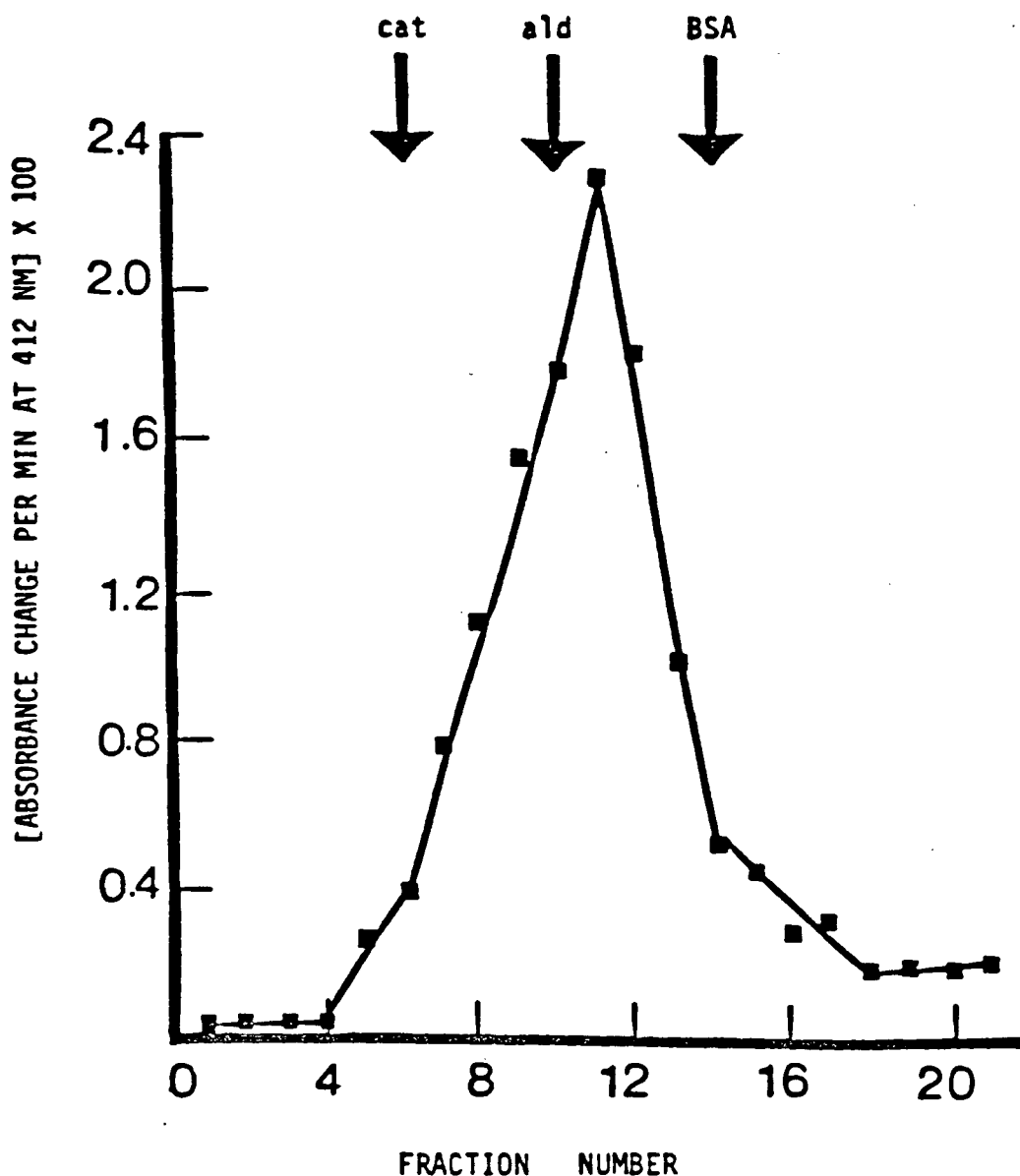


Figure 8. Continuous sucrose density gradient centrifugation of AChE solubilized with Triton X-100 after passing through a Bio Bead column. After centrifugation at $200,000\times g$ and 4°C for 17 hours, 21 fractions were collected from the bottom of the tube. Arrows indicate the positions of the marker proteins: cat is catalase, 11.4 S; ald is aldolase, 7.4 S; and BSA is bovine serum albumin, 4.4 S. Each fraction was 0.54 ml. The graph shown is one of two similar experiments. The S value obtained in this experiment (7.1 S) compares to 6.5 S found in the second experiment.

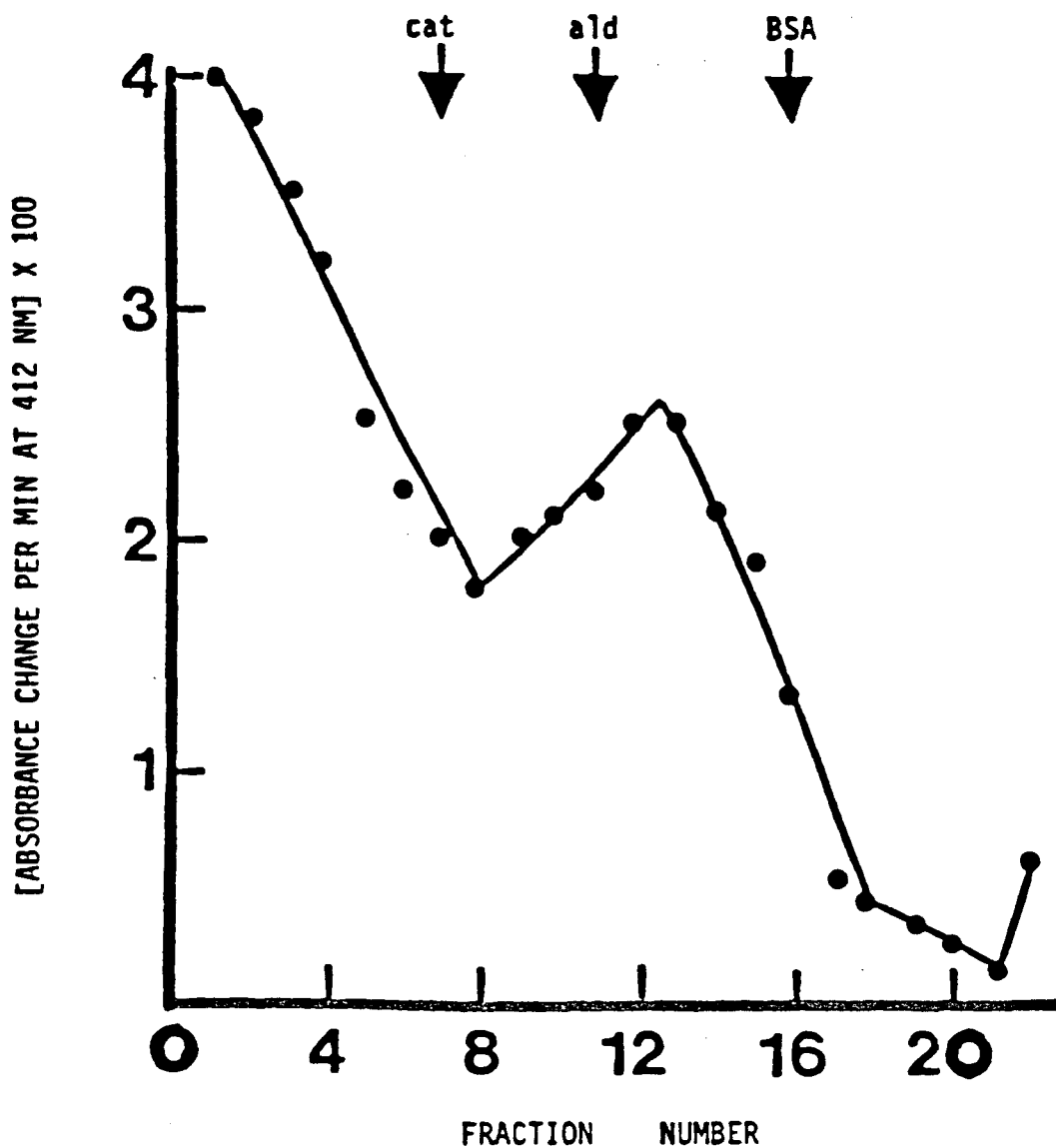


Figure 9. Continuous sucrose density gradient centrifugation of AChE solubilized with Zwittergent 316 before passing through a Bio Bead column. After centrifugation at $200,000\times g$ and 4°C for 17 hours, 22 fractions were collected from the bottom of the tube. Arrows indicate the positions of the marker proteins: cat is catalase, 11.4 S; ald is aldolase, 7.4 S; and BSA is bovine serum albumin, 4.4 S. Each fraction was 0.52 ml. The graph shown is one of two similar experiments. The S value obtained in this experiment (7.1 S) compares to 7.5 S found in the second experiment.

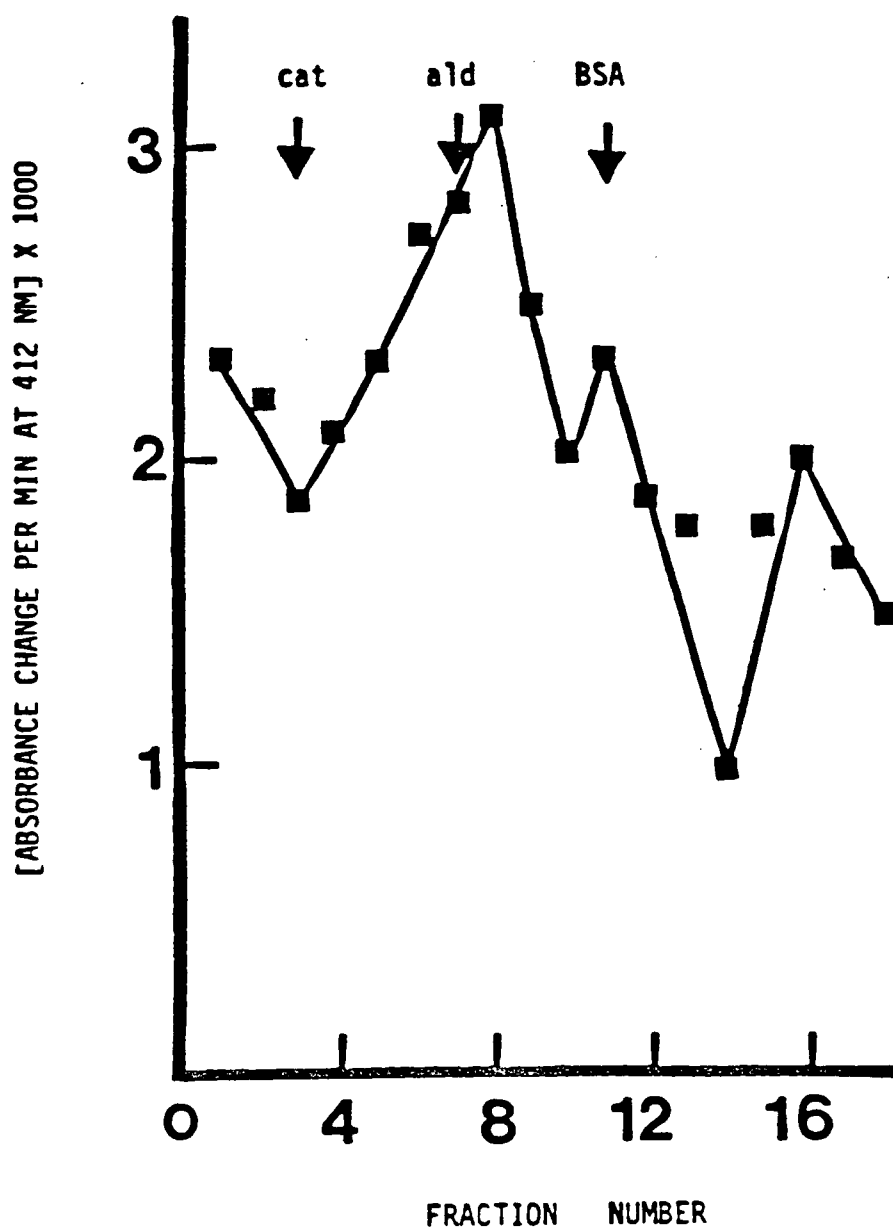


Figure 10. Continuous sucrose density gradient centrifugation of AChE solubilized with Zwittergent 316 after passing through a Bio Bead column. After centrifugation at $200,000 \times g$ and 4°C for 17 hours, 18 fractions were collected from the bottom of the tube. Arrows indicate the positions of the marker proteins: cat is catalase, 11.4 S; ald is aldolase, 7.4 S; and BSA is bovine serum albumin, 4.4 S. Each fraction was 0.63 ml. The graph shown is one of two similar experiments. The S value obtained in this experiment (7.0 S) compares to 6.5 S found in the second experiment.

7.0 and 4.4, respectively. Fraction 16 was too close to the top of the gradient to allow the S value to be accurately determined.

The marker proteins used were catalase (11.4 S), aldolase (7.4 S), and BSA (4.4 S). In all cases the S value of all major enzyme species ranged from 6.8 to 7.6. The results are summarized in Table 7. Using the formula $S_1/S_2 = (MW_1/MW_2)^{2/3}$, with BSA (MW of 66,500 and S value of 4.4) as standard, the molecular weight corresponding to the above S values is found to be 128,000 to 151,000. Since the MW of an acetylcholinesterase monomer is 73,000 (Ott *et al.* 1982), the above figure corresponds to a dimer. An S value of 12 will be equivalent to a MW of 300,000. This would indicate a tetramer, as found for a small component of the Lubrol PX solubilized enzyme following Bio Beads treatment. A larger aggregate is obtained in the presence of Zwittergent 316.

V. Arrhenius plots of acetylcholinesterase preparations

The Arrhenius plots of AChE solubilized with two different detergents (Tween 20 and Lubrol PX) were obtained. In Figure 11, the solubilizing detergent used was Tween 20. The temperature range studied was 4°C to 37°C. The final substrate concentration was 0.4 mM. At this low substrate concentration, a biphasic Arrhenius plot was observed, with a break around 16°C. Treatment of the enzyme with 1.8 M NaCl in phosphate buffer abolished the break.

In Figure 12, the solubilizing detergent used was Lubrol PX. The temperature range was 6°C to 37°C. The final substrate concentration remained unchanged at 0.4 mM. The results obtained were similar to those of Figure 11, i.e. a biphasic Arrhenius plot with a break around 16°C and abolishment of the break after high salt treatment of the enzyme.

Table 7. Sedimentation coefficient (S value) of some acetylcholinesterase preparations. The data are the average of 2 separate experiments, each done in duplicate. S values in brackets indicate minor components.

Membrane Solubilizing Detergent	Before Bio Bead Treatment	After Bio Bead Treatment
Lubrol PX at 100 mg/ml	6.8	7.5 (12.0)
Tween 20 at 500 mg/ml	7.6	6.9
Triton X-100 at 10 mg/ml	7.4 (6.8)	6.8
Zwittergent 316 at 3.6 mg/ml	7.3 and Aggregate	6.8 (4.4)

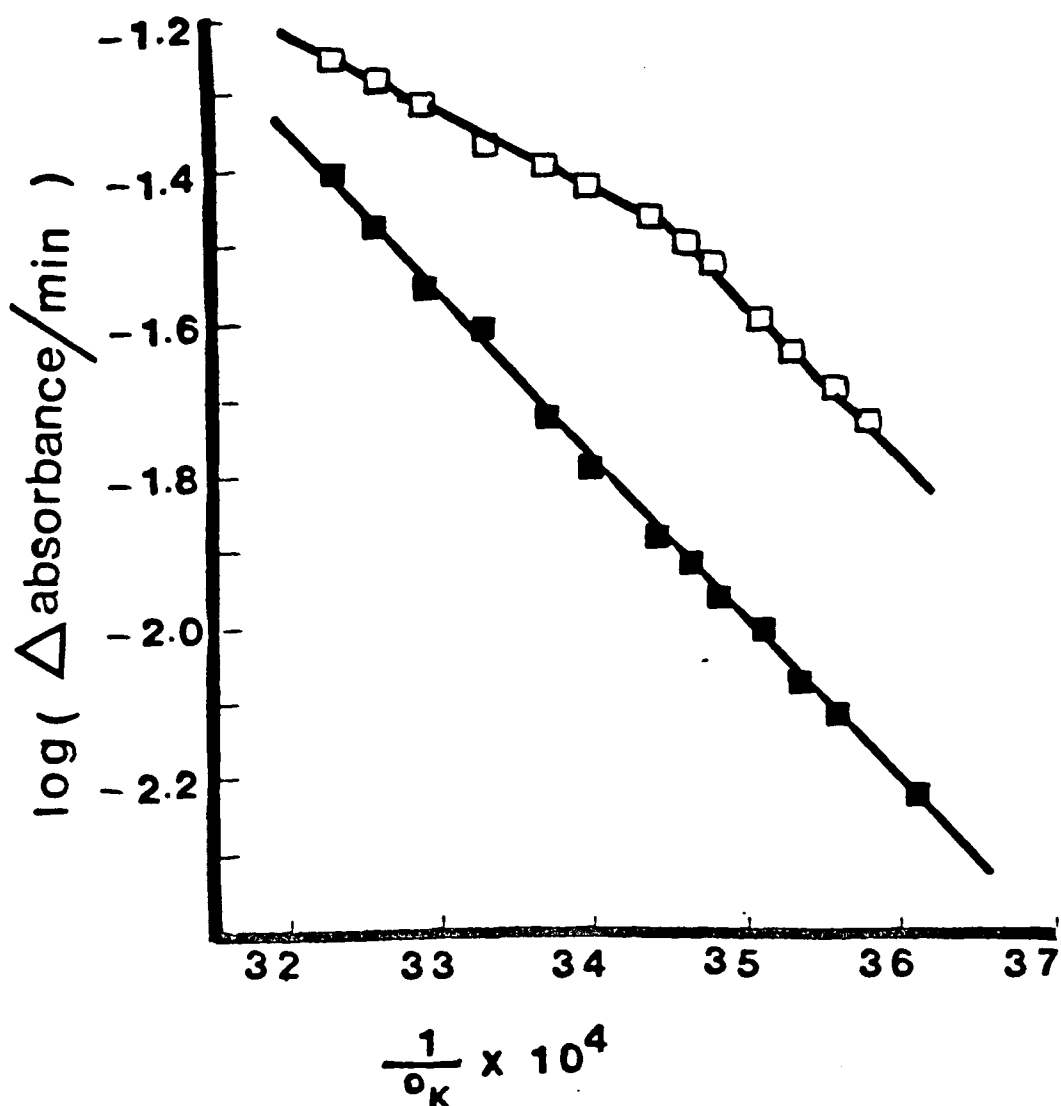


Figure 11. An Arrhenius plot for bovine erythrocyte acetylcholinesterase using Tween 20 as the membrane solubilizing detergent. (\square , control; \blacksquare , high salt-treated enzyme). In the control case, there was a break around 16°C . The enzyme solution contained $4.5 \mu\text{g}$ protein/ml. The graph is the average of two separate experiments, each done in duplicate.

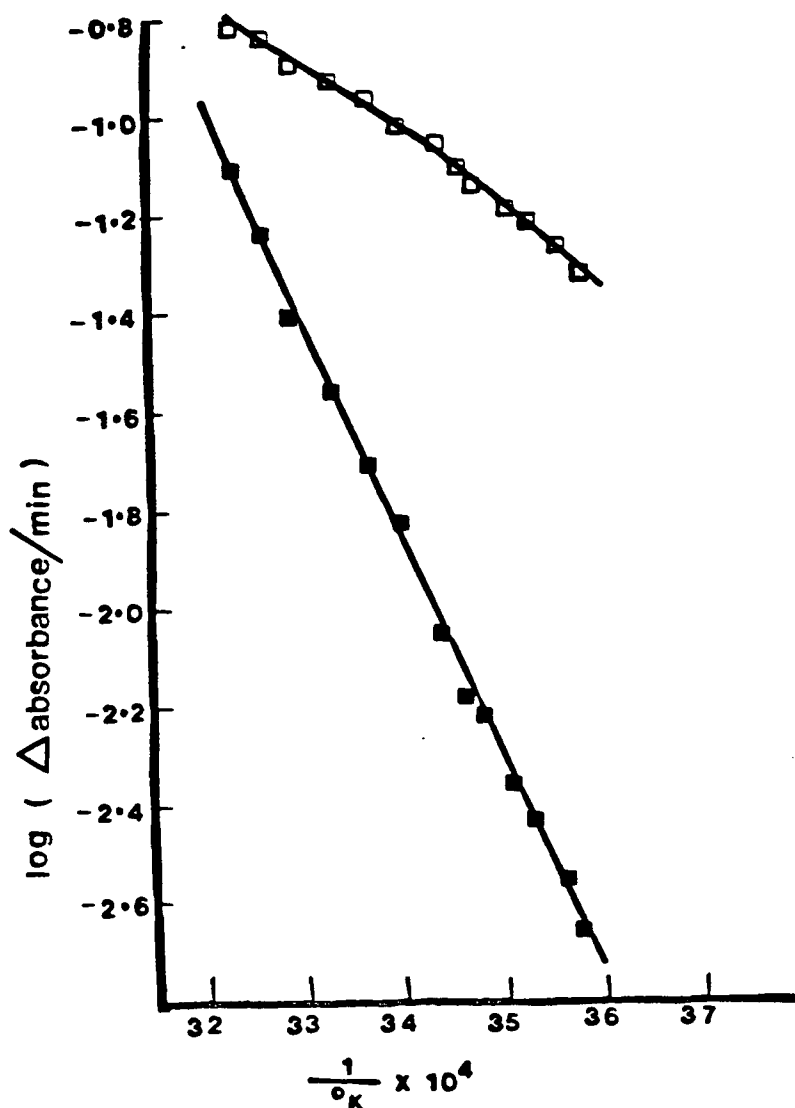


Figure 12. An Arrhenius plot for bovine erythrocyte acetylcholinesterase using Lubrol PX as the membrane solubilizing detergent. (\square , control; \blacksquare , high salt-treated enzyme). In the control case, there was a break around 16°C. The enzyme solution contained 3.2 μg protein/ml.

DISCUSSION

In the present study a number of nonionic detergents and a homologous series of zwitterionic detergents were evaluated for their efficiency in solubilizing acetylcholinesterase (AChE) from bovine erythrocytes. An effective detergent was considered to be one which both released a large portion of the enzyme activity into a form not readily precipitable by centrifugation at 100,000x g and allowed recovery of maximum enzyme activity without denaturation. Another desirable property of the detergent was that it solubilized the AChE in a form that predominates in the native environment of the enzyme. This latter property is difficult to assess, however, as the native enzyme form of AChE in bovine erythrocytes is not known. In this study the acetylcholinesterase solubilized by various detergents was purified by affinity chromatography and its physical state assessed by sucrose gradient centrifugation before and after detergent depletion. The functional properties of the enzyme were also assessed by examination of the Arrhenius plots before and after high salt treatments.

According to Landauer et al. (1982), the purified AChE preparation from brain neuronal membranes is rapidly denaturated at octyl- β -D-glucoside concentrations only slightly higher than the cmc, with nearly total loss of activity within two hours. The denaturation caused by incubation with three octyl-glycosides was demonstrated to be at least partially reversible, by diluting the detergent enzyme mixture into a detergent free assay medium. They found that the activity of bovine brain AChE is not influenced by detergents like Triton X-100, up to concentrations more than 100 times higher than the corresponding cmc, but it is severely diminished in the presence of the three octyl-glycoside detergents studied, at concentrations around the cmc. This decrease of enzyme activity they observed is due apparently to the length of the hydrophobic chain of the detergent and not to the different kinds of hydrophilic groups. In the present study, AChE activity

was retained after solubilization of bovine erythrocytes with β -D-octylglucoside from 30 - 100 mg/ml (Table 1). The high activity recovered in the high speed supernatant was consistent with the efficient solubilization of membrane protein by these concentrations of β -D-octylglucoside (Table 5). The most effective solubilization occurred at detergent concentrations more than ten times higher than the cmc. Enzyme inactivation was apparent at a still higher concentration (250 mg/ml). The octylglucoside-solubilized enzyme lost all its activity when it was passed through a hydrophobic polystyrene resin, Bio Beads SM-2. These results thus differ with those of Landauer et al. (1982) for AChE in bovine nucleus caudatus neuronal membranes. A possible explanation for this is the influence of the different tissues on the enzyme properties or differences in the AChE itself from the two sources.

The maximal reactivation by detergents was shown in general to depend on their relative hydrophobicity. An estimate of the relative hydrophobicity of the detergents used is the HLB number. This empirical measure allows one to compare the relative contribution of the polar head group and the hydrophobic hydrocarbon chain. As the HLB increases, polarity increases, the degree of hydration increases, the cmc increases, the detergent micelle size decreases and the reactivating potency also generally decreases (Mel'gunov and Akimova 1980). In Appendix 2 are shown the HLB values of various Tweens. A low HLB (1 - 10) indicates hydrophobicity. A high HLB indicates hydrophilicity. Effective surfactants for protein solubilization are usually in the 12.5 to 14.5 HLB ranges, i.e. lower than that of the Tweens. Nevertheless, Tweens have found many applications in membrane work. They release mainly peripheral proteins from many membranes, but fail to dissociate the lamellar membrane structure at the concentrations normally used for solubilization (less than 5%). In the present study, Tweens were found to be generally not very effective in solubilizing bovine erythrocyte AChE, at concentrations from 10 to 70,000 times greater than their cmc. This result is con-

sistent with their lack of efficiency in disrupting the lamellar membrane structure. The result also supports the idea that bovine erythrocyte AChE is embedded in the erythrocyte membrane, rather than being attached peripherally to it. Appendix 3 lists HLB values of amphiphilic compounds and properties associated with various HLB ranges. Detergents have HLB values of 13 to 15. Solubilizing agents have HLB values ranging from 10 to 18.

Akimova and Mel'gunov (1981) found that, in the case of isolated calcium-dependent adenosine triphosphatase, the dimensions of the polar segment of the molecule are among the most important factors determining the capacity of detergents to restore the function of the enzyme. For example, the efficiency of the reactivation of enzyme activity increases sharply with a decrease in the length of the polar segment from 20 oxyethylene groups, as in Brij 58, to two, as in Brij 52. At the same time, a variation of 12 to 18 carbon atoms in the length of the hydrophobic component was not found to be critical. In the Tween series, all four detergents have the same polar part, namely, 20 polyoxyethylene units. They only differ in the fatty acid portion of the molecule. Tween 20, Tween 40, and Tween 60 contain a monolaurate (12 C), a monopalmitate (16 C), and a monostearate (18 C), respectively. Tween 80 contains a monooleate (18 C with a double bond). In the present study, at a fixed concentration of 1000 mg/ml, Tween 20 solubilized more AChE than Tween 40, which in turn solubilized more AChE than Tween 60. Thus, as the saturated alkyl chain length of the acid portion of the molecule is increased, there is a decrease in AChE solubilizing capacity. Tween 80, whose hydrophobicity is less than that of Tween 60 due to the presence of the unsaturated double bond, has a solubilizing capacity between that of Tween 20 and Tween 40 (see Table 3). One can also attempt to correlate the solubilizing capacity of the Tweens with their HLB, which permits an evaluation and comparison of the relative dimensions of the polar head group and the hydrophobic

hydrocarbon portion of the molecule. In the literature the curves of the dependence of the solubilizing capacity of a detergent on its HLB are bell-shaped with a clearly pronounced maximum (Houslay and Stanley 1982). Almost all detergents that solubilize membranes efficiently have a HLB ranging from 12.5 to 14.5. In the present study, Tween 20, with the highest HLB of 16.7, was found to be the best solubilizer, while Tween 60, with the lowest HLB of 14.9, was found to be the least efficient solubilizer. Tween 80, with an HLB of 15.0, appears anomalous. This may be because Tween 80 contains an unsaturated chain, in contrast to the other Tweens. Unsaturation has been shown to increase solubilization in alcohol-derived detergents. When erucyl (E) alcohol ($\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_{12}\text{OH}$) was used as the hydrophobe, the micelle size decreased, and the solubilizing capacity increased, compared with the behavior of the saturated surfactant BE_{21} ($\text{CH}_3(\text{CH}_2)_{21}(\text{OCH}_2\text{CH}_2)_{21}\text{OH}$). Solubilization results show that the unsaturated E series surfactants are better solubilizers than the B (behenyl) series (Arnarson and Elworthy 1982). Therefore, within the Tweens with saturated acyl chains, it may be concluded that detergents with higher HLB and higher cmc values are better solubilizers of erythrocyte AChE. However, solubilization by these detergents requires concentrations in large excess of the cmc (Table 3) and is below maximal.

Increase in micelle size occurs with increasing hydrocarbon chain length. The number of carbon atoms in the hydrocarbon chain and the aggregation number are linearly related. On a mole solubilizate per mole surfactant basis, a detergent with 16 carbon atoms has twice the solubilizing capacity of a detergent with 32 carbon atoms. The solubilizing ability of nonionic detergents decreases as the saturated alkyl chain length is increased above 16 carbon atoms. This is in contrast to those found for the ionic surfactants, where increase in hydrocarbon chain length increases the amount solubilized (Arnarson and Elworthy 1981). In the study on Zwittergent series, Table 2, as the cmc of the detergent increases from the

least polar Zwittergent 316 to Zwittergent 310, a decreasing amount of enzyme activity was detected in the supernatant. This behavior is in contrast to the Tween series. For Zwittergent 316, cmc 0.012 mg/ml, when 12 mg/ml was used, 81% of enzyme activity was detected in the supernatant. For Zwittergent 312, cmc 1.2 mg/ml, when 1.2 mg/ml was used, only 35% of enzyme activity was detected in the supernatant and higher concentrations appeared to inactivate the enzyme. The results were in agreement with those of Mel'gunov and Akimova (1980) for the Ca^{2+} -ATPase enzyme.

The affinity of surfactants for the erythrocyte membrane appears to be a strictly linear function of the agent's alkyl chain length, as the hemolytic capacity and solubilizing capacity of the amphiphiles increase with increasing alkyl chain length. The surfactant concentration at which the complete breakdown of the cell membrane occurs decreases as the chain length of the agent increases. Both the methylene group chain and the head group of the amphiphile molecule partially embedded in the membrane bilayer reduce membrane stability (Zaslavsky et al. 1979). Thus, in the Zwittergent series, Table 2, detergents with longer alkyl chain length have greater solubilizing capacity.

The affinity for the liposomal membrane in a given homologous series of compounds parallels their alkyl chain length. It can be shown that the free binding energy is linearly dependent on the alkyl chain size for a given homologous series. The effect of the polar head group of the amphiphile molecule on the attached hydrophobic moiety seems to result in a gradient of hydrophilicity along the hydrocarbon chain. The specific feature of such a gradient depends on the nature of the head groups and its state in a given surrounding as well as on the structure of the hydrophobic fragment. The solubilizing capacity of the surfactants appears to depend on the hydrophobic tail length rather than on the nature of the agent's head group. This fact seems to indicate that the nature of the head group is im-

portant for the interactions of the surfactant with the organized structure of the lipid bilayer membrane (Zaslavsky et al. 1980).

In the Zwittergent series, the head group remains constant and the only structural difference between the various detergents is in the length of the hydrophobic alkyl chain, varying from 10 CH₂ for Zwittergent 310 to 16 CH₂ for Zwittergent 316. In Zwittergent series Z 312 to Z 316, a relatively constant amount of protein was solubilized from bovine erythrocytes (79 - 108% of total) at a constant ratio of Zwittergent concentration to cmc (300:1) (Table 5). Yet, the longer chain length compound, Zwittergent 316, was the detergent in the series that gave the highest degree of active enzyme activity in the supernatant (Table 2). The amount of enzyme activity solubilized by Z 316 increased with increasing concentration up to a 300:1 ratio of detergent concentration to cmc, whereas increasing the concentration of the shorter chain Zwittergents decreased the enzyme activity in the supernatant. In fact, with the shorter chain Zwittergents, maximum recovery of solubilized enzyme occurred at progressively decreasing ratios of detergent concentration to cmc (Table 2). A number of explanations can be advanced for this behavior. It is possible that Z 316 selectively solubilizes AChE above other proteins. However, this is unlikely, as the relative failure of the other Zwittergents was not due to retention of the AChE in the pellet at concentrations above the cmc. If all the Zwittergents solubilized AChE activity along with other proteins, then it appears that Z 316 maintains the enzyme active in the solubilized form, while the shorter chain Zwittergents are not as effective in supporting AChE activity. Detergents are known to solubilize membrane lipids as well as membrane proteins, and they may replace endogenous membrane lipids during solubilization. It may be that only Z 316 provides the amphiphilic properties necessary to maintain an active conformation, similar to that of amphiphilic phospholipids in the

membrane. Indeed, the most common fatty acid components in phospholipids contain a C₁₆ or greater chain length (Houslay and Stanley 1982), and the C₁₆ chain of Z 316 may simulate the interactions of AChE with these lipids. Specific dependence of enzyme activity on the length of the fatty acid chain rather than fluidity has been described for the (Ca²⁺+Mg²⁺)-ATPase of sarcoplasmic reticulum (East et al. 1984). Another alternative is that the short chain Zwittergents may interact with AChE in such a way that the chain intercalates with the protein in a way that causes nonspecific conformational perturbations leading to inactivation, while the longer chain Z 316 may be sterically excluded from such an interaction. It should be noted, however, that Z 316 produces overall activation of AChE activity, supporting a permissive interaction with the enzyme.

According to Madden and Cullis (1981), certain detergents, particularly Triton X-100, deoxycholate, octylglucoside, and lyso-phosphatidylcholine, can stabilize a bilayer organization for egg phosphatidylethanolamine at 37°C. A detergent-phospholipid molar ratio of R = 0.2 was required for the nonionic detergent Triton X-100 and octylglucoside. Residual detergent may serve to stabilize rather than destabilize bilayer organization. This may explain why certain detergents are inefficient in extracting membrane proteins, e.g. Tweens. In the present study, Z 316, Triton X-100 and Lubrol PX were found to increase enzyme activity after solubilization. Detergent enhancement of enzyme activity is frequently encountered in the literature. Ornithine decarboxylase from rat liver was activated slightly by 0.1% Tween 80 (Kitani and Fujisawa 1981). Similar results could be obtained by using Triton X-100 or bovine serum albumin instead of Tween 80. Triton X-100 enhanced the activity of lipoprotein lipase purified from bovine milk towards high density lipoprotein phosphatidylcholine (Eisenberg et al. 1981). Phosphatidylinositol kinase activity was markedly stimulated by detergents such as sodium deoxycholate, Triton X-100, Nonidet P40, Cutscum, and octylglucoside (Bostwick and Eichberg

1981). The optimum concentration of octylglucoside was just above its cmc of 25 mM. Among other detergents tested, 2% Miranol H2M and Lubrol PX enhanced enzyme activity up to 60% and 30%, respectively, of the maximum stimulation brought about by sodium deoxycholate. The Tween series, Lubrol WX and Brij 35 had a negligible effect. The maximum activation of enzyme activity by sodium deoxycholate occurred just below its cmc of 4 mM. The increase in enzyme activity could be due to binding of detergent monomers to the enzyme. Concentrations of Triton X-100 above 2% resulted in less enzyme activity; this may be due to the stripping away of active lipids from the enzyme and their replacement by detergent, with consequent denaturation of the enzyme (Bostwick and Eichberg 1981).

The relationship between chain length and solubilization of AChE in an active form could also be examined in the Lubrol class of detergents. Lubrol PX, has a shorter polyoxyethylene chain (9 - 10), attached to the cetyl-stearyl alcohol than Lubrol WX, which averages 17 polyoxyethylene chains (see Appendix 1). While both detergents solubilize similar amounts of protein from bovine erythrocytes, both at 100 mg/ml and 500 mg/ml (Table 5), Lubrol PX was more effective in maintaining the AChE active in the 200,000x g-soluble form, at either concentration examined (Table 4). Thus, at 100 mg/ml, Lubrol PX recovered 127% enzyme activity as compared to 38% with Lubrol WX. Higher concentrations of both Lubrol WX and Lubrol PX (500 mg/ml) decreased the overall enzyme recovery (Table 4). Detergents solubilize both phospholipids and proteins from membranes, and the ratio of solubilized phospholipids to solubilized proteins depends on the detergent concentration (Thang et al. 1980). The inhibition by higher Lubrol concentrations may be attributed to increased phospholipid solubilization, such that not only bulk lipids but also "boundary" (tightly bound) lipids are exchanged by the detergent and transferred into mixed micelles of lipid and detergent, resulting in an almost complete

separation of lipid from proteolipid (see Thang et al. 1980). This loss of key lipids may inactivate the AChE activity, particularly so in the presence of Lubrol WX. It should be noted that in Lubrol WX the polyoxyethylene chain length (17) is analogous to a side chain of 51 (17×3) atoms.

Similar results have been observed in other polyoxyethylene series. Lengthening the alkyl chain in polyoxyethylene monoalkyl ether surfactants causes a decrease in solubilizing capacity. It was found that the solubilizing capacity of $\text{CH}_3(\text{CH}_2)_{21}(\text{OCH}_2\text{CH}_2)_{21}\text{OH}$ (abbreviated BE_{21} where B = behenyl and E_n = ether) was lower than that of $\text{C}_{16}\text{E}_{20}$, and that of $\text{CH}_3(\text{CH}_2)_{31}-(\text{OCH}_2\text{CH}_2)_{41}\text{OH}$ ($\text{C}_{32}\text{E}_{41}$) was in turn lower than that of BE_{21} , although the micelle size increased with increasing hydrocarbon chain length (Arnason and Elworthy 1982).

Finally, Triton X-100, the most commonly used detergent for enzyme solubilization, including AChE from various sources, was found to be effective in solubilization of both erythrocyte membrane protein and enzyme activity (Table 5). Triton X-100 was found to be one of the most potent of the detergents examined, solubilizing more than 90% of protein and recovering 104% of AChE activity in the supernatant at 10 mg/ml. The high potency is consistent with its low cmc (0.16 mg/ml) (Houslay and Stanley 1982), its large micelle size and the favorable HLB. Surprisingly, however, Triton X-100 was not one of the most effective detergents for the purification of the enzyme, as considerably higher specific activities, following affinity chromatography, were obtained with Lubrol PX and Tween 20, while z 316 was the least effective of the compounds studied (Table 6).

The effect of Triton X-100 on human brain AChE has been studied with reference to its solubilization (Srinivasan et al. 1972). Triton X-100 levels of 0.2% to 2% have no effect on the total AChE activity in the residue, while inhibition is seen at 5% Triton X-100 level. In the supernatant, 0.2% to 2% Triton X-100 increased total enzyme activity from 3 to 7 fold. Inhibition is again seen at 5%

in the properties of the catalytic protein. The binding of enzyme to the membrane may be altered. This can lead either to the unmasking of hidden catalytic sites by release of an allosteric inhibitory mechanism or by conformational changes in the molecule due to the rupture of lipophilic bonds. Thus the increase in AChE activity after extraction with Triton X-100 may be a result of an alteration of the state of the protein (Srinivasan et al. 1972).

Nonionic detergents are invaluable in the isolation and characterization of integral membrane proteins. Almost all the commercially available nonionic detergents, being produced for industrial use, however, contain contaminants and additives which can lead to aberrant results in biochemical studies. For instance, the oxidizing impurities in Triton X-100 and Brij 35 reacted fairly rapidly with the SH group of the chromophore released in Ellman's procedure for the quantitative assay of thiol groups as well as with protein -SH molecules. Triton X-100 and Brij were found to contain the highest activity, whereas SDS and Tween 20 did not display measurable oxidizing properties. The impurities also increased upon standing, e.g. for Triton X-100, from less than 1 μM for a fresh solution to 136 μM after 8 weeks. The increase was probably due to catalysis by peroxide which is formed by oxygen or light oxidation. The addition of 1.3 mM EDTA reduced the rate of oxidation by 10 - 20%, a decrease found to be independent of EDTA concentration. The sulfhydryl oxidizing contaminants can be eliminated by reduction with sodium borohydride. The non-detergent impurities can be eliminated by silica gel column chromatography; and all metal ion contaminants can be eliminated by means of a mixed bed ion-exchange resin. The purified detergents were stable for at least two weeks when kept at room temperature in glass vials. The purification protocol did not affect the solubilizing properties of these detergents as far as AChE release from brain tissue is concerned. It is important to note that the Lowry method of protein assay, as performed in the presence of Triton X-100, was not found to be

affected by the contaminated detergents analyzed in the study (Ashani and Catravas 1980; Chang and Bock 1980).

Determination of the sedimentation coefficient of bovine erythrocyte AChE purified by affinity chromatography following solubilization by various detergents was used as an indication of the molecular structure of the enzyme solubilized by each detergent. Solubilization with Lubrol PX, Tween 20 and Triton X-100 yielded AChE showing relatively sharp peaks of activity corresponding to S values from 6.8 to 7.4 S. With Triton X-100 a broader peak was obtained, which suggested the presence of a 6.8 S component in addition to the 7.4 S component. These S values are not dissimilar to the value of 6.5 ± 0.2 S found by Rosenberry and Scoggin (1984) for the Triton X-100 purified enzyme from human erythrocytes, but somewhat higher than the value of 5.6 S determined for the globular AChE extracted from Torpedo species and 5.3 S to 6.3 S for two forms of human and rat erythrocyte AChE in the presence of Triton X-100 (Biagioni et al. 1982). Calculations performed, without correction for bound detergent, indicate that the enzyme purified by these three detergents in the present study is consistent with a dimeric structure, made up of monomers of 73,000 daltons. The molecular weight of the various forms calculated with reference to BSA, ranged from 128,000 to 151,000. When the amount of bound detergent was accounted for, human erythrocyte AChE was found to be a dimer of $160,000 \pm 8,000$ daltons (Rosenberry and Scoggin 1984). Whether this represents the native form of the enzyme in the erythrocyte, or rather the quaternary structure of the purified and solubilized enzyme, remains to be established.

AChE solubilized and purified following Zwittergent 316 behaved differently to the enzyme solubilized in the other detergents. In addition to the dimeric 7.1 S form, a large amount of enzyme activity appeared in an aggregated form. This

may be due to charge effects of enzyme bound to Z 316, or to aggregation of the detergent itself, but this was not investigated further.

Passage of detergent-purified forms of AChE through a polystyrene resin, Bio Beads SM-2, designed to deplete detergent from the preparation by adsorption of the alkyl chains to the hydrophobic surfaces, generally resulted in broadening of the peaks on sucrose gradients. In the case of the Z 316-solubilized enzyme, the large aggregated form was dispersed into smaller forms of 7.0 S and 4.4 S. The latter may represent a monomeric form of the enzyme (see Rosenberry and Scoggin 1984). This result tends to support the possibility that the aggregation of AChE by Z 316 was due to binding of the detergent to the enzyme. Despite the broadening of the peaks, the majority of AChE sedimented at between 7.0 S to 7.7 S (Table 7). These results contrast to those with human erythrocyte, where sucrose gradient sedimentation of both dimeric and monomeric forms of AChE in the absence of added Triton X-100 resulted in aggregation of the enzyme (Rosenberry and Scoggin 1984; Wiedmer et al. 1979). The latter authors found that aggregation occurred when the detergent concentration fell below the cmc, and was probably due to association of the enzyme through its hydrophobic domains (Rosenberry and Scoggin 1984). Failure of the bovine erythrocyte enzyme to aggregate following Bio Beads SM-2 treatment and sucrose gradient centrifugation in the present study may be due to tighter binding of detergent or to the absence of prominent aggregating hydrophobic domain in the bovine enzyme. Further work is required to investigate this aspect of the work.

An unexpected finding in this study was observed when the Lubrol PX-solubilized enzyme was passed through the Bio Beads SM-2 resin. Most of the enzyme activity was found to remain at the top of the sucrose gradient (Figure 4), suggesting that depletion of this detergent results in micelles or even perhaps mixed lipid-detergent liposomes which bind AChE. Taken together with the high

enzyme activity recovered with this detergent, this result could be of some practical usefulness in the future. Depletion of this detergent also yielded a larger 12 S species, which may be a tetrameric species.

Functional characteristics of the various detergent-purified AChE preparations were assessed by Arrhenius plots. In earlier work (Beauregard and Roufogalis 1977), a break in the Arrhenius plot of acetylcholine hydrolysis appeared to parallel the non-linear temperature dependence of $V_{\max}/K_{m(\text{app})}$, which reflects the conformational rearrangement of the enzyme-substrate complex, the rate limiting step at sub-saturating substrate concentrations (Roufogalis and Beauregard 1979). The break in the Arrhenius plot at around 16°C could be abolished by treatment of the enzyme with high salt (1.8 M NaCl) in the presence of phosphate buffer. At the same time, the phospholipid cardiolipin, found to be closely associated with AChE from bovine erythrocytes, could be removed by chloroform-methanol extraction, which was not possible before the high salt treatment. High salt treatment (1.8 M NaCl) causes functional but not physical dissociation of cardiolipin from the enzyme, as the resulting enzyme behaves as a monomer instead of a dimer by irradiation inactivation or target size analysis. A break in the Arrhenius plot at around 16°C was also observed in the present study with AChE purified with Tween 20 and Lubrol PX. Furthermore, the break was abolished after high salt treatment. Thus, the non-linear Arrhenius plot appears to be independent of the detergent used for extracting the enzyme species, and therefore represents an intrinsic property of the enzyme itself. It appears unlikely, therefore, that the nonionic detergents used in this study extracted lipid tightly bound to the AChE, which may be important for the regulation of its catalytic activity.

CONCLUSIONS

1. Various nonionic detergents were examined for their ability to solubilize bovine erythrocyte membrane proteins and to preserve enzyme activity in a soluble form.
2. β -D-Octylglucoside, one of the more hydrophilic detergents examined (cmc = 5.8 mg/ml), effectively solubilized both protein and AChE activity, but required large concentrations, with maximum solubilization occurring at more than ten times the cmc. Enzyme activity was rapidly lost when the enzyme was passed through a polystyrene Bio-Beads SM-2 resin.
3. A series of polyoxyethyleneglycol Tweens, from Tween 20 to Tween 80, showed poor efficiency in solubilization of both erythrocyte membrane protein and AChE activity. In the series with saturated alkyl fatty acid chains, the shorter C₁₂ chain in Tween 20 was found to be the most effective detergent. Despite its longer fatty acid chain (C₁₈), Tween 80 was the next most effective detergent, probably due to the unsaturation in the side chain. The poor efficiency of the Tweens is probably related to their high HLB values.
4. In a series of zwitterionic detergents, increasing the hydrocarbon chain length from C₁₀ (Z 310) to C₁₆ (Z 316) increased the activity of AChE in the soluble form, despite the similar efficiency of the detergents in solubilizing erythrocyte membrane protein. It is concluded that the longer chain length Zwittergents can support the enzyme activity in the solubilized state more effectively than the shorter chain length homologs.
5. A number of the detergents examined increased the total recovery of enzyme activity above that of the original activity measured in the membrane.

6. In the polyoxyethyleneglycol Lubrol detergents, the shorter polyoxyethylene chain (9 - 10) in Lubrol PX was more effective than the 17 chain Lubrol WX in maintaining soluble AChE activity, despite their similar efficiency in membrane protein solubilization. Higher concentrations of both detergents inactivated enzyme activity.
7. Triton X-100 was the most efficient of the detergents examined, consistent with its low cmc and HLB values.
8. Detergent-solubilized AChE could be purified by affinity chromatography on a phenyl-m-trimethylammonium side arm attached to Sepharose with a short spacer arm. Of the detergents examined, Lubrol PX and Tween 20 yielded enzyme with the highest specific activity, while Triton X-100 and Z 316 gave enzyme with considerably less specific activity.
9. Enzyme purified with Lubrol PX, Tween 20 and Triton X-100 yielded AChE which was dimeric, as determined from the sedimentation coefficient on a sucrose gradient. Z 316 yielded an aggregated form in addition to the dimer.
10. Passage of detergent-solubilized enzymes through a polystyrene Bio Beads SM-2 resin, to deplete them of detergents, resulted generally in broader peaks of enzyme activity on sucrose gradient sedimentation, but the S value of the majority of the enzyme was not altered dramatically. Z 316-solubilized enzyme was an exception, namely, Bio Beads SM-2 passage resulted in dispersion of the enzyme into smaller oligomers, including a form corresponding to a monomer. Depletion of Lubrol PX resulted in a micellar form of the enzyme.

11. Arrhenius plots of AChE purified with Lubrol PX and Tween 20 gave breaks at around 16°C , similar to the result reported previously from this laboratory with a variety of other enzyme forms. Furthermore, the break was lost following incubation of the enzyme with high salt (1.8 M NaCl) in phosphate buffer. These results indicate that this behavior is an inherent property of the bovine erythrocyte AChE, and is independent of the detergent or phospholipid environment.

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APPENDIX 1Lubrol Series

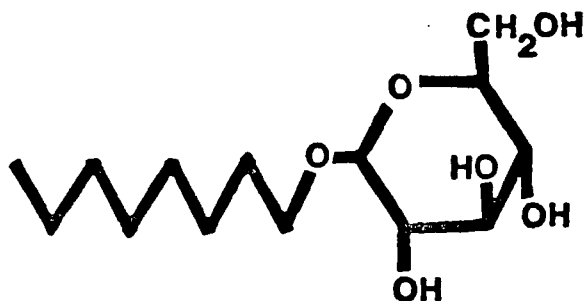
Lubrol PX: polyoxyethylene glycol (9-10) cetyl-stearyl alcohol

Lubrol WX: polyoxyethylene glycol (17) cetyl-stearyl alcohol

The general structural formula of the series is:

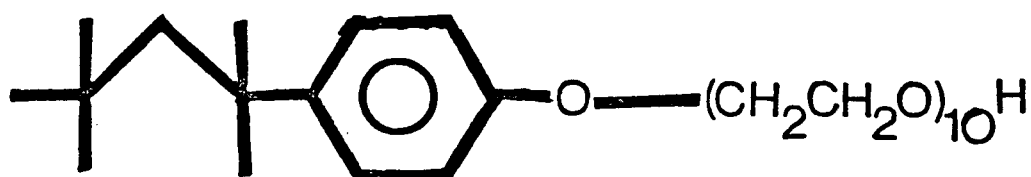
β-D-octylglucoside

It is also known as β-D-octylglucopyranoside. The structural formula is:



Triton X-100

Its chemical name is polyoxyethylene glycol p-isooctylphenyl ether. Its structural formula is:

Tween Series

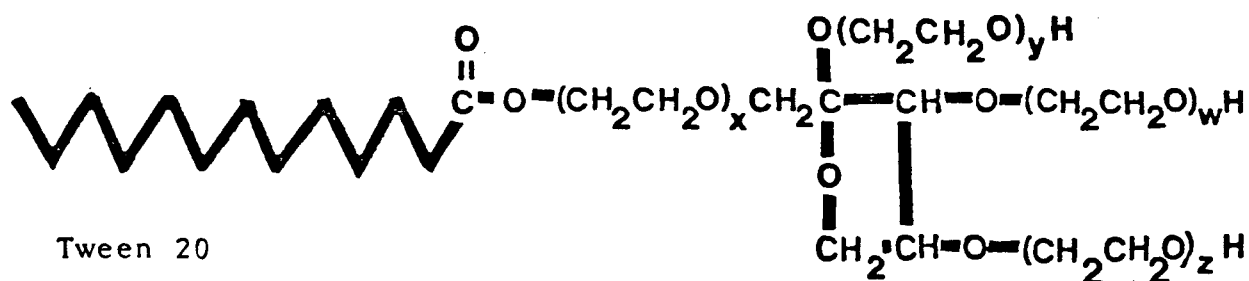
Tween 20: polyoxyethylene glycol (20) sorbitol monolaurate

Tween 40: polyoxyethylene glycol (20) sorbital monopalmitate

Tween 60: polyoxyethylene glycol (20) sorbital monostearate

Tween 80: polyoxyethylene glycol (20) sorbital monooleate

The general structural formula of the series is:



$$x + y + z + w = n$$

Zwittergent Series

Zwittergent 306: N-hexyl-N,N-dimethyl-3-ammonio-1-propanesulfonate

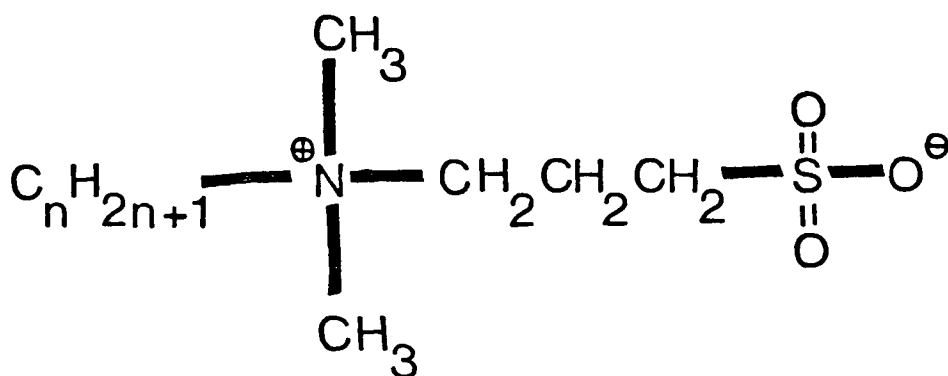
Zwittergent 308: N-octyl-N,N-dimethyl-3-ammonio-1-propanesulfonate

Zwittergent 310: N-decyl-N,N-dimethyl-3-ammonio-1-propanesulfonate

Zwittergent 312: N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate

Zwittergent 314: N-tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate

Zwittergent 316: N-hexadecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate



APPENDIX 2

Hydrophilic-lipophilic balance (HLB) value of some Tweens

Commerical Name	HLB	cmc (mg/ml)
Tween 60	14.9	0.027
Tween 80	15.0	0.013
Tween 40	15.6	0.029
Tween 20	16.7	0.060

APPENDIX 3

HLB value and its associated property

HLB range	Property
4 - 6	emulsifying agents (W/O emulsions)
7 - 9	wetting agents
8 - 18	emulsifying agents (O/W emulsions)
13 - 15	detergents
10 - 18	solubilizing agents

APPENDIX 4

Structure of the ligand coupled to Sepharose 2B:

N-((ξ-aminocaproyl)-m-aminophenyl) trimethylammonium bromide

