c) The membrane lipid fluidity has a direct influence on the catalytic activity of $(Na^+ + K^+)$-ATPase.

d) The ouabain interaction properties of beef brain $(Na^+ + K^+)$-ATPase were unchanged when the fluidity of the lipid matrix was varied. It is concluded that inhibition by ouabain of beef brain $(Na^+ + K^+)$-ATPase is not regulated by the physical state of the bulk lipid.

e) It is speculated that the increased chain length and the degree of unsaturation of the phospholipid acyl chains may be responsible for the decreased ouabain sensitivity observed with crab nerve $(Na^+ + K^+)$-ATPase.

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LIPID MODULATION OF THE OUABAIN SENSITIVITY OF $(Na^+ + K^+)$-ATPase

by

© MAHINDA YAPA ABEYWARDENA

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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TO MY PARENTS

ABSTRACT

The membrane fractions enriched in $(Na^+ + K^+)$-ATPase isolated from beef brain (homeotherm) and crab nerve (poikilotherm) showed markedly different sensitivities to inhibition by ouabain. The ID_{50} values to ouabain inhibition of the two enzyme preparations differed by more than two log units. Polyacrylamide gel electrophoresis revealed that polypeptide subunit pattern of the two enzymes were quite similar.

However, lipid analysis studies indicated that the lipid composition of the two enzyme preparations are quite different. The crab nerve enzyme preparation had an increased cholesterol/phospholipid ratio. In addition, the lipids of crab nerve enzyme were found to be more "fluid" and contained more long chain fatty acids compared to enzyme from beef brain.

The possible role of membrane lipid composition in regulating the ouabain interaction properties of $(Na^+ + K^+)$-ATPase was investigated. This was studied by reconstitution of both beef brain and crab nerve $(Na^+ + K^+)$-ATPase into liposomes of controlled lipid composition. The observations can be summarized as follows.

a) The reconstitution of $(Na^+ + K^+)$-ATPase into lipid bilayer structure was achieved by using a novel gel filtration procedure. This method resulted in the incorporation of over 80% of $(Na^+ + K^+)$-ATPase into liposomes.

b) The results strongly suggest that membrane lipids are capable of modulating the interaction of inhibitors such as ouabain, with the $(Na^+ + K^+)$-ATPase enzyme system.

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INTRODUCTION.

I. INTRODUCTION

A. General

Almost all animal cells maintain a low sodium (Na^+) and a high potassium (K^+) concentration inside the cell contrasting with the inverse situation in the extracellular fluid. In order to maintain this difference in ion distribution (which generates a transmembrane potential), the cell has evolved a so-called "pump" mechanism within the plasma membrane. Intracellular Na^+ ions are actively transported out of the cell whereas K^+ ions are transported into the cell by a tightly coupled, energy dependent process which is often referred to as the "Sodium Pump". While studying the Na^+ and K^+ fluxes in the red blood cell Schatzmann (1953) found that adenosine triphosphate (ATP) was required for active ion transport and that this process could be inhibited by very low concentrations of g-strophanthin, a representative member of the group of drugs known as cardiac glycosides. Some years later Skou (1957) discovered an adenosine triphosphatase (ATPase) enzyme in a fragmented membrane fraction of the leg nerve of the shore crab *Carcinus maenas*. This ATPase enzyme was unusual because in addition to Mg^{++} ions, it required the presence of both monovalent cations Na^+ and K^+ in a defined ratio for optimal activity. Although the activation of enzymes by certain alkali metal ions has been reported, many times before, all these other systems required only one ligand for full activation. The synergistic effects of Na^+ and K^+ discovered by Skou indicated that this enzyme was different from almost all other known enzyme systems. Of even greater interest to the present study, the Na^+ and K^+ stimulated component of the ATPase reaction was sensitive to inhibition by the same

cardiac glycoside, g-strophanthin (ouabain) used earlier by Schatzmann (1953) to inhibit active cation transport in the red cell. Skou (1957) suggested that this enzyme system which can be operationally defined as magnesium activated, sodium-plus-potassium-dependent, ouabain inhibited adenosine triphosphatase, which henceforth shall be referred to as $(Na^+ + K^+)$-ATPase (EC 3.6.1.3), might be involved in the transformation of chemical energy into a vectorial movement of Na^+ out of and K^+ into the cell. Since that time extensive investigation of this enzyme system has provided strong circumstantial evidence to support Skou's suggestion that the $(Na^+ + K^+)$-ATPase enzyme system is the energy transduction mechanism underlying the active transport of Na^+ and K^+ (Charnock and Opit, 1968; Whittam and Wheeler, 1970; Dahl and Hokin, 1974; Glynn and Karlish, 1975). The more recent enzyme reconstitution experiments which will be discussed below, have provided the proof that the $(Na^+ + K^+)$-ATPase is in fact the long sought sodium pump of mammalian cells.

The similarities between the ion transport process and the $(Na^+ + K^+)$-ATPase enzyme have been reviewed (Whittam and Wheeler, 1970) and discussed in detail (Glynn, 1968). Some of the similarities between the sodium pump and the enzyme activity include,

1) Tissues with high Na^+ and K^+ transport activity also possess high $(Na^+ + K^+)$-ATPase activity (Bonting and Caravaggio, 1963).
2) For optimal activity both active ion transport and enzyme activity require ATP, Mg^{++}, Na^+, K^+. In addition the optimal concentrations of these ligands required by both systems are identical (Post *et al.*, 1960).
3) Both monovalent cation transport and the $(Na^+ + K^+)$-ATPase are

inhibited by cardiac glycosides and their aglycones, and display the same structure-activity relationship towards these compounds (Abeles, 1969).

4) In whole cell preparations the $(Na^+ + K^+)$-ATPase displays the same orientational asymmetry as the sodium pump. Mg^{++}, ATP and Na^+ are required on the inside of the cell membrane, while K^+ and cardiotonic steroids affect both systems from the outside of the cell (Hoffman, 1970; Post *et al.*, 1960; Glynn, 1962; Hoffman, 1962; Laris and Letchworth, 1962; Whittam, 1962; Garrahan and Glynn, 1967).

In all some fourteen points of similarity exist between the coupled active transport process for Na^+ and K^+ and the $(Na^+ + K^+)$-ATPase enzyme system (Glynn, 1968). By 1970 most investigators agreed that this could not be coincidental and felt that the $(Na^+ + K^+)$-ATPase must be more than an energy source for ion transport, but that it could possibly be the "pump" mechanism itself. However, direct evidence for this idea was lacking until the reconstitution studies reported below were carried out.

B. Reconstitution Studies

Recently several investigators have demonstrated the ATP-dependent and ouabain inhibitable coupled transport of Na^+ and K^+ by purified $(Na^+ + K^+)$-ATPase enzyme preparations which have been reconstituted into phospholipid vesicles (liposomes) by a cholate dialysis method (Goldin and Tong, 1974; Hilden *et al.*, 1974; Goldin and Sweadner, 1975; Hilden and Hokin, 1975; Anner *et al.*, 1977; Goldin, 1977). Such reconstituted vesicles were able to exclude Na^+ from their interior while

they accumulated K^+ against a concentration gradient. These reconstitution experiments have provided the first direct evidence that the $(Na^+ + K^+)$-ATPase enzyme protein is indeed the molecular machine responsible for the vectorial movement of Na^+ and K^+ across the intact cell membrane. Many of the characteristics of reconstituted $(Na^+ + K^+)$-ATPase in liposomes were found to be identical to those observed in intact cell membrane systems. These similarities include,

1) Mg^{++} activated ATP, Na^+ and K^+ dependent coupled transport of Na^+ and K^+ (A transport property).
2) The transport process is sensitive to inhibition by ouabain (A transport property).
3) The stoichiometry of Na^+/K^+ pumped per ATP hydrolyzed is 1.43 : 1, a ratio which is very close to that observed in red blood cell and nerve membranes (1.5 : 1). However, a ratio of 1.5 : 1 for ATPase-liposomes has been reported recently (Goldin, 1977). (A transport property).
4) The substrate specificity for nucleoside triphosphatases is similar for both processes. (Both a transport and an enzymatic property).
5) Absolute specificity for Na^+ at the 'sodium site' is still maintained but Rb^+ will substitute for K^+. (Both a transport and an enzymatic property).
6) Under suitable conditions either Na^+-Na^+ or K^+-K^+ exchange occurs. (A transport property).
7) The ouabain binding site is on the opposite surface to the Na^+ and ATP activation sites. (A property of both systems).

However, even in the best oriented liposome systems, about half of the reconstituted ATPase molecules have lost their orientational asymmetry with respect to the *in vivo* situation.

C. Structural Aspects

The $(Na^+ + K^+)$-ATPase enzyme system has been isolated and purified from a variety of sources including the outer medulla of guinea-pig, rabbit, sheep, dog and pig kidneys (Charnock and Post, 1963a; Kyte, 1971a; Lane *et al.*, 1973; Jørgensen, 1974a; Lane *et al.*, 1978), the salt-gland of ducks (Hopkins, 1974) and the rectal-gland of the dogfish *Squalus acanthias* (Hokin *et al.*, 1973), as well as the electric-organ of the electric eel *Electrophorus electricus* (Perrone *et al.*, 1975). Polyacrylamide gel electrophoresis of purified $(Na^+ + K^+)$-ATPase in the presence of SDS has yielded only two major protein components despite the variation in tissues, species and isolation procedures (Kyte, 1971a and 1971b; Kyte 1972a and 1972b; Wallick *et al.*, 1979). The molecular weight of the larger polypeptide components appears to be about 90,000-100,000 daltons, whereas the smaller polypeptide which has been shown to be a sialoglycoprotein has an apparent molecular weight of about 45,000 daltons (Lane *et al.*, 1973; Jørgensen, 1974b; Kyte, 1975; Perrone *et al.*, 1975). The mass ratio of large to small polypeptide has been reported to vary from 1:7 (Kyte, 1972b) to 1:9 (Lane *et al.*, 1973), 2:3 (Hokin *et al.*, 1973), 2:8 (Jørgensen, 1974b) and 2:0 (Perrone *et al.*, 1975). The functional unit of the enzyme appears to be a dimer with each monomer consisting of one large polypeptide chain and one glycoprotein subunit. The evidence for this suggestion arises from cross linking studies (Kyte,

1972b; Kyte, 1975; Giota, 1976); the molecular weight of the $(Na^+ + K^+)$-ATPase dimer is calculated to be around 250-280,000 daltons. This figure appears to be in close agreement with the values obtained by other methods such as radiation inactivation (Kepner and Macey, 1968), steady-state phosphorylation and ouabain binding studies (Hokin *et al.*, 1973; Lane *et al.*, 1973; Jørgensen, 1974b).

Labelling of the enzyme protein with ^{32}P from $[\gamma^{32}P]$-ATP in the presence of Mg^{++} and Na^+, followed by polyacrylamide gel electrophoresis in SDS have revealed that the ^{32}P migrates exclusively with the larger polypeptide fragment (Alexander and Rodnight, 1970; Kyte, 1971b; Uesugi *et al.*, 1971).

The concentration of the large polypeptide fragment increases in proportion to the specific activity of $(Na^+ + K^+)$-ATPase. This polypeptide unit appears to contain the Na^+ dependent phosphorylation site in addition to possessing sulphydryl (-SH) groups with specific affinity to ATP, Na^+, K^+ and ouabain (Jørgensen, 1975a). These latter characteristics have been used to identify this protein as the catalytic subunit of the $(Na^+ + K^+)$-ATPase.

In studying the site of Na^+ dependent phosphorylation of the catalytic subunit, Kahlenberg, Galsworthy and Hokin (1967 and 1968) have reported a glutamyl phosphate at the phosphorylation site, whereas Post and Kume (1973) suggested that the phosphorylation from ATP occurs at an aspartic acid residue. Nishigaki *et al.* (1974) have provided further evidence which indicated that the Na^+ and Mg^{++} dependent phosphorylation of catalytic subunit by $[\gamma^{32}P]$-ATP occurs at the β-carboxyl group of an aspartic acid residue. While the identity of the phosphorylation site

may not be certain, all investigators agree that an acylphosphate is formed in this reaction.

The smaller polypeptide present in the purified $(Na^+ + K^+)$-ATPase does not appear to bear any catalytic functions. At the present time, there is no direct evidence to assign a functional role to this glycoprotein subunit although several suggestions have been made concerning this (Kyte, 1972b). The glycoprotein can be covalently cross-linked to the catalytic subunit suggesting that they are in close proximity to one another (Kyte, 1972b). Moreover, antibodies raised against the glycoprotein appear to inhibit the $(Na^+ + K^+)$-ATPase in a concentration dependent manner, indicating that the glycoprotein is an integral part of the $(Na^+ + K^+)$-ATPase (Jean *et al.*, 1975; Rhee and Hokin, 1975; Jean and Albers, 1977). Conversely, the immunochemical studies of Jean *et al.* (1975) and the photoaffinity labelling studies of Ruoho and Kyte (1974), Hegyvary (1975), Forbush *et al.* (1978) and Forbush and Hoffman (1979a,b) all indicate that the binding site for cardiac glycoside is located on the catalytic subunit.

It is known that in the intact cell the hydrolysis of ATP occurs at the interior surface of plasma membrane, whereas cardiotonic steroids interact only from the outer surface of the cell membrane. Since it has been shown that both the phosphorylation site and the ouabain binding site reside on the catalytic subunit, it is clear that the catalytic subunit of the $(Na^+ + K^+)$-ATPase must completely span the plasma membrane. This is quite feasible for a protein of this molecular weight and an average membrane thickness between 70-100 Å.

D. Catalytic Aspects

The reaction sequence of ATP hydrolysis is thought to occur in a step-wise manner. Although the detailed reaction mechanism of $(Na^+ + K^+)$-ATPase has not yet been fully elucidated, several models have been proposed (Charnock and Post, 1963b; Opit and Charnock, 1965; Siegel and Goodwin, 1972; Froehlich *et al.*, 1976). The following sequences of reactions taken from Schwartz (1976) illustrates the complexity of the mechanism by which the simultaneous transport of Na^+ and K^+ is thought to occur.

$$E_1 + Mg{\cdot}ATP \xrightleftharpoons{Na_1} (E_1 \sim P)\text{-}Na + ADP \qquad 1$$

$$(E_1 \sim P)\text{-}Na \xrightleftharpoons{(Mg^{++})?} (E_2 \sim P) + Na_o \qquad 2$$

$$H_2O + (E_2 \sim P) \xrightleftharpoons{K_o} E_2\text{-}K + P_1 \qquad -3$$

$$E_2\text{-}K \rightleftharpoons E_2 + K_1 \qquad 4$$

$$E_2 \xrightleftharpoons{ATP} E_1 \qquad 5$$

In the above scheme, E_1 and E_2 represent different conformational states of the non-phosphorylated and phosphorylated ($E_1 \sim P$ and $E_2 \sim P$) enzyme. P_i is inorganic phosphate and Na_1, K_1, Na_o, K_o denote intracellular and extracellular sodium and potassium respectively. Glynn and his coworkers (Glynn *et al.*, 1971) have referred to each of these steps as the

partial reactions of the system. Earlier reports as well as recent studies indicate that the catalytic subunit undergoes conformational changes during the reaction cycle which include Na^+ dependent phosphorylation of the enzyme (Step 1) and K^+ dependent hydrolysis of the phosphoenzyme (Step 3); (Opit and Charnock, 1965; Titus and Hart, 1974; Giotta, 1975; Jørgensen, 1975b; Jørgensen, 1977; Jørgensen and Kloods, 1978). However, recent studies by Kudoh *et al.* (1979) indicate that the reaction sequence of $(Na^+ + K^+)$-ATPase is more complex than that illustrated above, since the functional unit of $(Na^+ + K^+)$-ATPase appears to operate as a dimer. Thus the terminology $\begin{matrix} E\sim P \\ | \\ E\sim P \end{matrix}$ has now been introduced into the reaction mechanism.

The phosphorylation of the enzyme can be achieved not only by ATP, but other nucleotides have been shown to be effective as well. However ATP appears to be the most effective substrate (Charnock *et al.*, 1963), while CTP and ITP have moderate effects. GTP serves as a poor substrate and no ouabain sensitive hydrolysis of UTP was observed (Hokin and Yoda, 1964; Matsui and Schwartz, 1966; Towle and Copenhaver, 1970; Hegyvary and Post, 1971).

The cation requirement of the $(Na^+ + K^+)$-ATPase has been investigated extensively by several workers. The requirement for Na^+ at the 'sodium site' is absolute. Several other mono-valent cations can replace K^+ at the 'potassium site' with an effectiveness of $K^+ > Rb^+ > NH_4^+ > Cs^+ > Li^+$ (Skou, 1965). Tl^+ has also been shown to be effective with ten times the affinity of K^+ (Britten and Blank, 1968; Skul'skii *et al.*, 1973). The presence of Mg^{++} is essential for the ATPase reaction. Other divalent cations such as Mn^{++}, Co^{++} can substitute for Mg^{++} but with decreased

efficiency (Rendi and Uhr, 1964; Atkinson *et al.*, 1968; Atkinson and Lowe, 1972). Fe^{++} and Ca^{++}-dependent hydrolysis of ATP which is not stimulated by Na^+ and K^+ has been reported (Rendi and Uhr, 1964; Charnock and Opit, 1968; Atkinson and Lowe, 1972). Thus, Ca^{++}, Fe^{++} and several other divalent cations including Zn^{++}, Cu^{++}, Ba^{++} and Sr^{++} were found to inhibit the Mg^{++}-dependent reaction, hence the inhibition of $(Na^+ + K^+)$-ATPase activity (Rendi and Uhr, 1964; Charnock and Opit, 1968; Toda, 1968; Bowler and Duncan, 1970; Donaldson *et al.*, 1971; Robinson, 1973; Ting-Beall *et al.*, 1973). Quite recently vanadate has been shown to inhibit the enzyme at very low concentrations. There is considerable interest and speculation in the possible biological role of this "trace element" as potentially inhibitory concentrations of this metal have been reported in skeletal and heart muscles as well as in kidney (Cantley *et al.*, 1977; Beauge and Glynn, 1978; Cantley *et al.*, 1978). It seems certain that, much attention will be paid to the inhibitory action of vanadate on $(Na^+ + K^+)$-ATPase in the future. The role of Ca^{++} in inhibiting the enzyme activity is also of some interest, since it may serve as a possible physiological modulator of the 'Sodium-Pump' activity (Baker, 1972; Tobin *et al.*, 1973).

It has been shown that the operation of the Sodium-Pump is not limited to Na^+-K^+ exchange but other modes of operation also exist. As Glynn and Karlish (1975) pointed out these alternative modes include,

a) Na^+-K^+ exchange (the normal mode)

b) Reversed Na^+-K^+ exchange

c) Na^+-Na^+ exchange

d) K^+-K^+ exchange

e) Uncoupled Na^+ efflux.

Many of these processes are thought to be associated with individual partial reactions of the $(Na^+ + K^+)$-ATPase (Glynn, 1971).

E. Phosphatase Activity

It has been well established that $(Na^+ + K^+)$-ATPase preparations exhibit a K^+-dependent ouabain inhibitable phosphatase activity (EC 3.6.1.7) which may represent the terminal step in the sequence of reactions catalyzed by $(Na^+ + K^+)$-ATPase (Judah *et al.*, 1962; Albers *et al.*, 1965; Albers and Koval, 1966; Glynn *et al.*, 1971; Koyal *et al.*, 1971; Uesugi *et al.*, 1971; Dahl and Hokin, 1974). The purification of $(Na^+ + K^+)$-ATPase leads to a parallel increase in the phosphatase activity (Ahmed and Judah, 1964; Nagai *et al.*, 1966; Jørgensen *et al.*, 1971; Uesugi *et al.*, 1971). Further evidence in support of the concept that the phosphatase reaction is an integral part of the $(Na^+ + K^+)$-ATPase comes from the observations that both activities display similar responses to various ligands and the "sideness" of the action of these ligands were similar (Garrahan *et al.*, 1969; Rega *et al.*, 1970; Garrahan and Rega, 1972).

Compounds with phosphate groups with a higher free energy of hydrolysis than a typical phosphate ester such as p-nitrophenylphosphate, acetylphosphate, carbamylphosphate and umbelliferonephosphate will serve as artificial substrates for the K^+-dependent hydrolytic activity of the $(Na^+ + K^+)$-ATPase (Fujita *et al.*, 1966; Yoshida *et al.*, 1966; Dahl and Hokin, 1974). The natural substrate for this reaction is thought to be provided from the Na^+-dependent phosphorylation of the $(Na^+ + K^+)$-ATPase. Thus, *in vivo* the phosphorylated intermediate of the enzyme, $E_2 \sim P$ which as indicated before is an acylphosphate, may serve as the substrate for

the K^+-dependent partial reaction (Judah and Ahmed, 1962; Sach *et al.*, 1967; Yoshida *et al.*, 1969; Dahl and Hokin, 1974). The phosphatase reaction has been investigated by many workers and the similarities and differences in the properties of the K^+-dependent phosphatase and the $(Na^+ + K^+)$-ATPase have been reported in detail (Emmelot and Bos, 1966; Fujita *et al.*, 1966; Nagai and Yoshida, 1966; Israel and Titus, 1967; Yoshida *et al.*, 1969; Askari and Koyal, 1971; Glynn *et al.*, 1971). It therefore seems perfectly reasonable to accept that this 'partial reaction' is indeed the site of ouabain inhibition of the stepwise reactions of $(Na^+ + K^+)$-ATPase.

F. Receptor for Digitalis?

For the pharmacologist the inhibition of $(Na^+ + K^+)$-ATPase by cardiotonic steroids is perhaps the most interesting aspect of the system. Repke (1963) was the first to suggest that $(Na^+ + K^+)$-ATPase may be the pharmacologic receptor for digitalis. Since that time numerous studies have provided evidence for and against this hypothesis (Akera, 1969; Lee and Klaus, 1971; Hokin and Dahl, 1972; Schwartz *et al.*, 1974; Schwartz *et al.*, 1975; Schwartz, 1976; Akera, 1977). However, now it is generally agreed that the pharmacological response of cardiac glycoside on the heart is a consequence of interaction of these drugs with myocardial $(Na^+ + K^+)$-ATPase (Wallick *et al.*, 1979). Several laboratories were able to show good correlation between the occupation of receptors (i.e. $(Na^+ + K^+)$-ATPase) and myocardial inotropy (Ku *et al.*, 1974; Gelbart and Goldman, 1977; Wallick *et al.*, 1979; Charnock *et al.*, 1980c). It is known that there is a wide variation in the sensitivity of $(Na^+ + K^+)$-ATPase

for inhibition by ouabain and other cardiac glycosides, among different tissues and species. *In vitro*, the inhibitory potencies of cardiac glycosides appear to depend on the temperature and on the concentration of ligands present in the incubation media (Albers *et al.*, 1968; Allen and Schwartz, 1969; Sen *et al.*, 1969; Allen and Schwartz, 1970; Allen *et al.*, 1970; Tobin and Sen, 1970). Conditions which favour the formation of the phosphorylated enzyme complex ($E_1 \sim P$), also favour the inhibition of the system by ouabain and other cardiac glycosides. Thus Mg^{++}, Na^+, ATP and other nucleoside triphosphatases that phosphorylate the enzyme system favour, whereas K^+ reduce, the binding of cardiac glycosides to $(Na^+ + K^+)$-ATPase (Tobin *et al.*, 1972; Akera *et al.*, 1978). The role of ligands in influencing the formation of enzyme-drug complex may perhaps explain the considerable variation found in the literature in the inhibitory potency of a given cardiotonic steroid. Although the conditions favouring the formation of phosphorylated intermediate would lead to an increased inhibition of the $(Na^+ + K^+)$-ATPase by cardiac glycosides, phosphorylation of the enzyme is not a prerequisite for ouabain binding. Indeed, it has been shown that the enzyme-ouabain complex can exist even in the absence of ligands and/or the substrate, ATP (Mandel *et al.*, 1977).

The existence of two populations of ouabain binding sites on the $(Na^+ + K^+)$-ATPase has been suggested (Inagaki *et al.*, 1974; Hansen, 1976; Charnock *et al.*, 1977; Frickle and Klaus, 1977; Schoner *et al.*, 1977; Van-Alstyre, 1978). More recently, Rhee and Hokin (1979) have reported that they observed only 50% inhibition of ouabain binding by antibody raised against the purified $(Na^+ + K^+)$-ATPase or its catalytic subunit.

This observation may substantiate the claim that the ouabain binding sites can exist in at least two environments.

G. Purification

In the intact membrane, the $(Na^+ + K^+)$-ATPase enzyme system is an intrinsic membrane protein which is surrounded by lipids. The fact that it is an integral component of the cell membrane has produced considerable difficulty in the purification of this enzyme system. All purification procedures involve the use of detergents and/or chaotropic agents such as NaI. Mainly two approaches have been made to this problem. In one of these, the enzyme is isolated in a membrane bound form. The other method involves the removal of enzyme protein from its lipid matrix (i.e. solubilization) followed by the purification of the solubilized enzyme.

The isolation of the $(Na^+ + K^+)$-ATPase in a membrane bound form is usually achieved by mild treatment with relatively low concentrations of ionic detergents, such as deoxycholate (DOC) which primarily extract the extrinsic proteins from the membrane (Jørgensen *et al.*, 1971; Kyte, 1971a). By this procedure the $(Na^+ + K^+)$-ATPase enzyme remains membrane bound while less tightly bound proteins are removed. Higher DOC concentrations are known to solubilize the enzyme. The enzyme preparation of Kyte (1971a) was prepared by solubilizing the $(Na^+ + K^+)$-ATPase from the membrane by DOC at a high salt concentration. Centrifugation over glycerol followed by agarose gel filtration of the solubilized enzyme has produced over 90% pure $(Na^+ + K^+)$-ATPase from the outer medulla of the dog kidney (Kyte, 1971a; Goldin and Tong, 1974).

The solubilization of $(Na^+ + K^+)$-ATPase may also be achieved by non-

ionic polyoxyethylene ethers such as lubrol, which are known to be less prone to inactivate the enzyme than ionic detergents (Swanson *et al.*, 1964; Banerjee *et al.*, 1970). In these latter procedures the solubilization process now depends on the length of the polyoxyethylene chain and also on the weight ratio of detergent to protein (Swanson *et al.*, 1964). After solubilization of the protein the free detergent is then removed usually by prolonged zonal centrifugation. Subsequently the fractionation of the solubilized protein is achieved by precipitating out all of the proteins with 1 M ammonium sulphate, followed by a decrease in the ionic strength in the medium by dilution to 0.4 M ammonium sulphate. This step appears to resolubilize "impurities" from the precipitated proteins leaving the $(Na^+ + K^+)$-ATPase in a membranous form (Uesugi *et al.*, 1971; Hokin *et al.*, 1973). This latter technique has resulted in enzyme protein over 90-95% pure from both *Squalus acanthias* rectal gland and eel electric organ (Perrone *et al.*, 1975). These preparations have specific activities of about 900-1500 µmol Pi released/mg protein per hour. Recent work reported from Skou's laboratory indicates that the $(Na^+ + K^+)$-ATPase from *Squalus acanthias* can also be solubilized in an active form using the non-ionic detergent octaethyleneglycoldodecyl ether yielding a preparation with a specific activity of about 2300 µmol Pi released/mg protein per hour. Gel filtration of the solubilized enzyme in Sepharose 4B-CL has revealed two peaks both with enzyme activity. It is of great interest that even at this level of purification both peaks contained phospholipid in addition to the enzyme protein (Esmann *et al.*, 1979; Skou and Esmann, 1979).

The criteria that have been employed in establishing the "purity"

of $(Na^+ + K^+)$-ATPase include,

a) specific activity (highly purified preparations have specific activities of about 1000-2300 μmol Pi released/mg protein/hr).

b) SDS-polyacrylamide gel patterns.

It should be born in mind that both of these criteria have some drawbacks. For example the specific activity of the preparation can be misleading if the enzyme has been activated or inactivated by detergents. In other words, the highest enzyme activity may be observed at a particular lipid : protein ratio, whereas the "naked" but highly purified enzyme protein has little or no activity! It is of particular interest to this study to note that all so-called "highly purified" enzyme preparations reported to date retained at least some of their endogenous phospholipids irrespective of the method of preparation (Kyte, 1971a; Hokin *et al.*, 1973; Perrone *et al.*, 1975; Esmann *et al.*, 1979).

It is also known that different SDS-polyacrylamide gel systems differ in their resolving power and the sensitivity of staining of protein. In addition, the permissability for complete penetration of all the protein into the gel varies with each gel system (Dahl and Hokin, 1974).

H. Lipid Requirement

Biological membranes can be viewed as fluid bilayers of lipid with globular proteins embedded within them. The membranes are not static structures but are in a dynamic state (Singer and Nicolson, 1972). It has also been recognized that in this "sea of lipid" there appear to be areas of restricted mobility caused by certain protein-protein, lipid-

protein and/or lipid-lipid interactions (Singer and Nicolson, 1972; Edidin, 1974; Singer, 1974; Nicolson, 1976; DePierre and Ernster, 1977). The phenomenon of lipid-protein interaction has been well documented for the $(Na^+ + K^+)$-ATPase enzyme system and it is now known that the $(Na^+ + K^+)$-ATPase reaction required the presence of phospholipids for optimal activity.

The complete delipidation of the enzyme by detergents, phospholipases or organic solvents results in an irreversible inactivation of the $(Na^+ + K^+)$-ATPase (Hegyvary and Post, 1969; Roelofson *et al.*, 1971; Goldman and Albers, 1973). In contrast, partial delipidation of the system usually by detergent extraction yields an inactive enzyme which can be reactivated by adding certain phospholipids. The non-ionic detergent lubrol-wx and the anionic detergent deoxycholate (DOC) are most commonly employed for this purpose. Delipidation by phospholipases is mainly achieved by treating the enzyme preparations with either phospholipase A (PPL-A) or phospholipase-C (PPL-C). However because of the possible impurities of these phospholipases, there is some doubt about the specificity of action of lipolysis (Roelofsen and Van Deenen, 1973). The delipidation by organic solvents is rarely used (Roelofsen *et al.*, 1971). The lipid depletion/reactivation of the $(Na^+ + K^+)$-ATPase by these methods will be discussed in more detail below (Subsections (1) - (3) inclusive).

While it is agreed that the $(Na^+ + K^+)$-ATPase requires phospholipid for optimal activity there has been some disagreement about the specificity of the phospholipid effective in reactivating the lipid depleted enzyme preparations. Some investigators have found a specific requirement for negatively charged phospholipids such as phosphatidylserine (PS)

(Ohnishi and Kawamura, 1964; Fenster and Copenhaver, 1967; Wheeler and Whittam, 1970; Kimelberg and Papahadjopoulos, 1972) and phosphatidylglycerol (PG) (Kimelberg and Papahadjopoulos, 1972). However, there are other reports which indicate that a variety of acidic phospholipids (Taniguchi and Tonomura, 1971; Tanaka *et al.*, 1971; Hokin and Hexum, 1972), acidic or neutral phospholipids (Tanaka and Strickland, 1965; Tanaka, 1969; Palatini *et al.*, 1972) or even cholesterol may serve as the essential "lipid" (Noguchi and Freed, 1971; Jarnefelt, 1972). As Kimelberg and Papahadjopoulos have pointed out, these discrepancies may be due to,

a) The manner in which the lipid is removed.

b) The purity of the phospholipid used for reactivation.

c) The amount of lipid removed.

d) The presence of residual activity which is partly a function of the amount of lipid removed.

(1) Delipidation by Detergents

The observation by Ohnishi and Kawamura (1964) that the $(Na^+ + K^+)$-ATPase required a specific phospholipid (PS) for its activity, led to the beginning of a new chapter in the concept of lipid-protein interactions of this enzyme system. In the same year Tanaka and Abood (1964) reported that the phospholipid depletion by ammonium sulphate $[(NH_4)_2SO_4]$ fractionation of the DOC treated rat brain $(Na^+ + K^+)$-ATPase, paralleled the loss of enzyme activity. The activity of the lipid depleted preparations could be restored by the addition of various phospholipids, of which dipalmitoyl-L-lecithin was the most effective. Further evidence in

support of these findings was reported by Tanaka and Strickland (1965) who found animal lecithin to be most effective in reactivating the delipidated $(Na^+ + K^+)$-ATPase. However, the purity of the commercial lecithin employed in these studies has been questioned, since Fenster and Copenhaver (1967) using essentially the Tanaka and Strickland preparation observed no activation by phosphatidylcholine (PC). Fenster and Copenhaver (1967) have provided evidence that contaminating PS in the commercial lecithin was responsible for the activation of the $(Na^+ + K^+)$-ATPase. The activating effect of PS was subsequently confirmed by several investigators (Tanaka, 1969; Tanaka and Sakamoto, 1969; Towle and Copenhaver, 1970). Some years later Wheeler and Whittam (1970a, 1970b) reported reactivation by PS of the solubilized $(Na^+ + K^+)$-ATPase prepared according to the method of Tanaka and Strickland (1965). In addition, Wheeler and Whittam also reported some activation of the enzyme with phosphatidylinositol (PI) as well as with phosphatidic acid (PA). Although they have explained these results on the basis of impurities in the lipids they used for reactivation, paper chromatography of PA did not reveal the presence of phosphatidylserine. More recently, Palatini *et al.* (1972) have used the non-ionic detergent lubrol to solubilize the enzyme from bovine heart, after a preliminary extraction with NaI. Subsequent fractionation of the solubilized enzyme with $(NH_4)_2SO_4$ was followed by washing in salt solutions. This method of preparation resulted in $(Na^+ + K^+)$-ATPase, depleted of phospholipids and cholesterol with a substantial loss of enzyme activity. Reconstitution of this particular enzyme preparation was most effective with either PS or diphosphatidylglycerol (DPG), but PC was also capable of restoring the enzyme activity to some extent. On the other

hand, phosphatidylethanolamine (PE) which is weakly acidic at neutral pH, caused a complete activation of the delipidated enzyme which was comparable to that induced by a total phospholipid extract from bovine brain. However, both PC and PE exerted their activating effects only at high phospholipid concentrations in comparison to the highly acidic phospholipids, PS and DPG which were effective at relatively low concentrations. In contrast, cholesterol by itself, added to the delipidated $(Na^+ + K^+)$-ATPase as an aqueous suspension had no effect. Kimelberg and Papahadjopoulos (1972) have reported similar observations (PS and PG were most effective), when they used Tanaka and Strickland's enzyme preparation in which lipid depletion was achieved by the deoxycholate/$(NH_4)_2SO_4$ fractionation procedure.

After an exhaustive series of experiments Wheeler *et al.* (1975) reported that reactivation of the lubrol extracted $(Na^+ + K^+)$-ATPase could be achieved by adding exogenous phospholipids bearing a net negative charge. In these experiments PS and PG were most effective while PI, PE, PC and DPG produced either very little or no effect. These workers also found a significant correlation between the extent of reactivation and the amount of residual enzyme activity which remained after the lipid depletion step. These observations are in close agreement with those reported by Kimelberg and Papahadjopoulos (1972) for DOC extracted $(Na^+ + K^+)$-ATPase from rabbit kidney. Palatini *et al.* (1977) have introduced the concept of lipid "specificity" by fatty acid acyl chain characteristics rather than by phospholipid polar head groups, by suggesting that for the reactivation of lubrol solubilized $(Na^+ + K^+)$-ATPase, a lamellar arrangement of phospholipids having fluid acyl chains which bear a

sufficient density of negative charges is necessary. This conclusion was based on the observation that incorporation of enzyme protein into phospholipid liposomes made from either natural or synthetic acidic phospholipids resulted in restoration of the enzyme activity. By contrast, incorporation of enzyme protein into uncharged liposomes of the neutral lipid phosphatidylcholine failed to reactivate the lipid depleted enzyme, unless negative charges were introduced into the liposome bilayer. PC liposomes exhibited an activating effect after acquiring an appropriate density of negative charges with either dicetylphosphate or oleate which alone did not reactivate. These claims by Palatini's group are in agreement with the very recent findings of De Caldenty and Wheeler (1979). In confirming the observations of Palatini *et al.* (1977) De Caldenty and Wheeler also found that reactivation of the lubrol extracted rabbit kidney $(Na^+ + K^+)$-ATPase could be achieved with phospholipid dispersions carrying a net negative charge. The reactivation required a lamellar configuration of the lipid molecules. The positively charged compound cetyltrimethylammonium was completely ineffective; another observation which is in accordance with Palatini's claim that PC liposomes made positively charged by introducing ethylhexadecyldimethylammonium did not reactivate the delipidated $(Na^+ + K^+)$-ATPase. Moreover, De Caldenty and Wheeler (1979) also concluded that in addition to the net negative charge present, the nature of the acyl chains of the phospholipids is also a key factor in controlling the "correct" interaction between the protein moiety of $(Na^+ + K^+)$-ATPase and the lipid. The effect of acyl chain fluidity on the ATPase activity has been investigated by several groups and their observations will be discussed below.

(2) Extraction with Organic Solvents

Organic solvent extraction of the $(Na^+ + K^+)$-ATPase usually leads to a complete inhibition of activity which cannot be reconstituted by added phospholipids. However, it was shown that if the extraction was performed at -75°C, then reactivation of enzyme activity was possible, but cholesterol was now the most effective "lipid", whereas phospholipids produced only marginal activation (Nogouchi and Freed, 1971; Jarnefelt, 1972). No follow-up studies have been performed to explain this unusual finding.

(3) Treatment with Phospholipases

Diverse effects have been observed when enzyme preparations were depleted of endogenous phospholipids by treatment with phospholipases. Taniguchi and Tonomura (1971) reported the treatment of ox-brain microsomes with Naja naja venom, which contains PPL-A_2 and with PPL-C from *Clostridium welchii*. The $(Na^+ + K^+)$-ATPase activity was reduced to 5-25% and 40% of the control value by the treatment with venom or PPL-C respectively. The residual enzyme activities indicate that lipid depletion by either phospholipase was only a partial process. When added to the venom treated enzyme, PI caused 50% reactivation, but either PS or PE had no effect. However, mixtures of either PI + PS or PI + PS + PE, caused complete reactivation of $(Na^+ + K^+)$-ATPase activity to the control level. Phosphatidylserine but not PI, reactivated the PPL-C treated preparations. In the following year Hokin and Hexum (1972) confirmed these findings and extended the list to include PA and didodecylphosphate as the phospholipids effective in reactivating the PPL-A treated $(Na^+ + K^+)$-ATPase. In

addition, Hokin and Hexum reported that the inactivation of $(Na^+ + K^+)$-ATPase upon treatment with PPL-A is not due to the liberated lysophosphatides. Goldman and Albers (1973) employed PPL-A and PPL-C to alter the phospholipids associated with the $(Na^+ + K^+)$-ATPase of electric eel electroplax. Like Taniguchi and Tonomura (1971) they also observed that with PPL-C only partial inhibition of ATPase activity occurred, yet the treatment resulted in the removal of 95% of the total PC and 60-70% of the PE. On the other hand, PS was not affected by PPL-C and remained unchanged. Conversely, treatment with PPL-A caused a complete inhibition of the enzyme activity. The loss of activity paralleled the loss of PS and PE, but did not correlate well with the removal of intact PC or the appearance of lysophosphatides. In a well controlled series of experiments Roelofsen and Van Deenen (1973) showed that the $(Na^+ + K^+)$-ATPase of erythrocyte ghosts is completely inhibited by treatment with PPL-A_2 and PPL-C. The observation with PPL-C is interesting since as stated above earlier investigators achieved only 40-50% decrease in the $(Na^+ + K^+)$-ATPase activity (Taniguchi and Tonomura, 1971; Goldman and Albers, 1973). Roelofsen and Van Deenen have also demonstrated that the conversion of anionic PS into neutral PE by the enzyme phosphatidylserine decarboxylase inhibited $(Na^+ + K^+)$-ATPase activity, but that this effect was apparent only after 87% of the total PS had been decarboxylated. Decarboxylation of the last 13% of the PS molecules (after unmasking the "latent sites") caused a complete inhibition of the ATPase activity. Treatment with phospholipase-D, which converts phospholipids into phosphatidic acid, caused about 30-40% increase in the $(Na^+ + K^+)$-ATPase activity. It is of interest to note that this latter treatment caused a

drastic reduction in the associated (Mg^{++})-ATPase activity of the enzyme preparation. In contrast to the findings of Roelofsen and Van Deenen (1973), De Pont *et al.* (1973) reported an absence of inhibition of ($Na^+ + K^+$)-ATPase activity when the PS of the brain microsomal enzyme preparations was treated with phosphatidylserine decarboxylase.

The products of lipolysis of phospholipids by PPL-C are mainly diglycerides. In contrast, treatment with PPL-A leads to the production of free fatty acids and lysophosphatides. In addition to the detergent like effect of lysophosphatides, the fatty acids themselves are inhibitory to enzyme activity and it has been shown that under these conditions the addition of serum albumin to the incubation mixture leads to an increase in the ($Na^+ + K^+$)-ATPase activity (Taniguchi and Tonomura, 1971). Presumably this occurs by the binding of free fatty acids to the albumin. However, lysophosphatides appear to cause little or no inhibitory effect at the concentrations generated by PPL-A action on the membrane matrix (Goldman and Albers, 1973). While Roelofsen and Van Deenen's experiments are free of the technical objections that may be raised when impure PPL-A is used, the results of Goldman and Albers (1973) with PPL-A have been critisized by De Pont *et al.* (1973) on the grounds that the effects observed could well be due to the production of inhibitory fatty acids.

More recently, by treating a highly purified ($Na^+ + K^+$)-ATPase with PS-decarboxylase and phosphatidylinositol specific PPL-C, De Pont *et al.* (1978) have concluded that neither PS nor PI is <u>essential</u> for the ATPase activity. Only the combination of the phospholipid converting enzymes resulted in a loss of 44% of the ($Na^+ + K^+$)-ATPase activity. The authors

have also reported that the enzyme does not require more than 90 phospholipid molecules per mole $(Na^+ + K^+)$-ATPase for proper functioning. In another recent communication which co-appeared with that of De Pont *et al.* (1978), Mandersloot, Roelofsen and De Gier (1978) have presented evidence that PI is the endogenous activator of the $(Na^+ + K^+)$-ATPase. The reactivation of the PPL-A_2 treated microsomes was achieved by adding lipid vesicles of negatively charged glycerophospholipids such as PS, PG and PI. The neutral phospholipids phosphatidylcholine and PE had no effect unless cholate was present. Cholate by itself did not restore the enzyme activity. This observation is in agreement with the data of Hilden and Hokin (1976) that in a reconstituted liposome system PC can replace the endogenous lipids of a highly purified $(Na^+ + K^+)$-ATPase maintaining the coupled transport of Na^+ and K^+. Based on the claim by Walker and Wheeler (1975) that the negative charge on the lipid structure is essential for the enzyme activity of the lipid-protein complex, or the charge is only required to bring about the proper interaction between the exogenous lipid and enzyme protein, Mandersloot *et al.* (1978) speculated that the presence of cholate induces the necessary negative charge on the lipid structure thus making PC liposomes effective in restoring the $(Na^+ + K^+)$-ATPase activity. These results, which indicate that PI is the "essential lipid" of the $(Na^+ + K^+)$-ATPase, are in direct conflict with the observations by De Pont *et al.* (1978), in spite of the fact that both groups obtained their enzyme preparations from the same source, the rabbit kidney. However, at least one important difference is apparent. Mandersloot *et al.* have used a rabbit kidney microsomal fraction enriched with $(Na^+ + K^+)$-ATPase which was prepared according to

the method of Tashima and Hasegawa (1975). Only 70% of the total enzyme activity was sensitive to inhibition by ouabain and their preparations had a $(Na^+ + K^+)$-ATPase activity of about 20 µmol Pi released/mg protein per hour. In contrast, the highly purified $(Na^+ + K^+)$-ATPase employed by De Pont's group was prepared according to the method of Jørgensen (1974a) and had a final specific activity of 1300 µmol Pi released/mg protein per hour. This latter enzyme preparation was free of ouabain insensitive (Mg^{++})-ATPase activity, and was shown to be at least 95% pure on a protein basis as determined by SDS-gel electrophoresis. This difference in the purity of the enzyme preparations probably accounts for the reported discrepancy over the role of PI in the function of $(Na^+ + K^+)$-ATPase.

From the results discussed so far, it is not possible to conclude which if any "specific phospholipd" is essential for the proper function of the $(Na^+ + K^+)$-ATPase. As Roelofsen and Van Deenen (1973) have pointed out, much of the discrepancies in the literature may be due to the presence of one or more of the following factors,

a) The phospholipases used for lipid depletion may have been impure.
b) Similar uncertainty about the purity of the phospholipids used for reactivation.
c) The presence of residual lipid in the delipidated preparations.
d) Some, so-called "delipidized" preparations retained some activity.
e) Both inactivation and reactivation are often incomplete.
f) Full reactivation is only achieved with preparations still showing considerable residual activity.

I. Effect of Lipid Fluidity

From the aforementioned information it is clear that the molecular structure of phospholipid is a key factor involved in reactivating the lipid depleted $(Na^+ + K^+)$-ATPase. However, the physical properties of phospholipids may also be of importance for the reactivation process. Since the physical state of lipids is a function of temperature, the activating effects of lipids can be expected to vary with the temperature. Thus, one would expect to detect changes in the activity-temperature relationship of $(Na^+ + K^+)$-ATPase depending on the nature of the lipid activating the enzyme.

The non-linearity of the activity-temperature relationship has been reported by several workers (Gruener and Avi-Dor, 1966; Swanson, 1966; Neufeld, 1970). Charnock *et al.* (1971) have reported that the Arrhenius plots of enzyme activity at various temperatures (i.e. log V_m vs 1/T) of DOC treated $(Na^+ + K^+)$-ATPase prepared from rabbit kidney, display a distinct break at about 20°C. The authors have also noted that the value for the activation energy above the inflection point (T_c), is significantly less than the corresponding value below the T_c. Such abrupt changes in the activation energies of the enzyme reaction could be a result of a change in the state of some component essential to the reaction, and are thought to be related to the changes in the physical state of the lipid molecules associated with the enzyme in the membrane (Linden and Fox, 1973). In both biomembranes and in synthetic phospholipid systems these temperature dependent transitions mainly involve the fatty acyl chains of the phospholipids which exist in an ordered quasi-crystalline state below some critical temperature and a disordered

liquid-crystalline state above the T_c. Kimelberg and Papahadjopoulos (1972) studied the influence of both polar head group and fatty acyl chain fluidity on reactivation of the delipidated rabbit kidney $(Na^+ + K^+)$-ATPase. In addition to finding that both PS and PG were equally effective in reconstituting the ATPase activity of their system, they also observed that maximal reactivation was obtained when the fatty acyl chains were fluid. The activating effect of dipalmitoyl-phosphatidylglycerol (DPPG), was inhibited below its transition temperature or in the presence of cholesterol. Arrhenius plots of the DPPG reconstituted enzyme were found to be non-linear. By employing the electron paramagnetic resonance (EPR) technique, Grisham and Barnett (1973) have shown that in purified $(Na^+ + K^+)$-ATPase preparations such discontinuities in the temperature-activity relationship reflect the solid-fluid transitions of the fatty acyl chains of the phospholipids required for enzyme function. The observed inhibitory effect of cholesterol on reconstituted $(Na^+ + K^+)$-ATPase (Kimelberg and Papahadjopoulos, 1972; Kimelberg and Papahadjopoulos, 1974; Kimelberg, 1975) may be due to the effect of cholesterol immobilizing the fatty acyl chains of phospholipids (Kimelberg, 1976). From these reports, it is tempting to suggest that the immediate lipid environment of the $(Na^+ + K^+)$-ATPase contains little or no cholesterol. Indeed, the existence of such a cholesterol poor microenvironment around a transmembrane enzyme protein has been reported for the Ca^{++} transporting ATPase of the sarcoplasmic reticulum (Warren *et al.*, 1975). These workers have shown that in the presence of phospholipid, cholesterol is excluded from the immediate lipid environment of the (Ca^{++})-ATPase macromolecular protein structure. Moreover, the

"fluidity" of the amphiphilic environment appears to regulate the (Ca^{++})-ATPase activity (Lee *et al.*, 1974; Warren *et al.*, 1974).

Taniguchi and Iida (1972) have reported that the treatment of ($Na^+ + K^+$)-ATPase preparations with PPL-A resulted in a temperature-activity relationship which yielded a single straight line Arrhenius plot, in contrast to the untreated enzyme preparations which yielded a non-linear Arrhenius plot. The reconstitution of the PPL-A treated enzyme with both PS and PI restored the discontinuity. Charnock *et al.* (1973) have validated these findings and also reported that neither mild detergent treatment nor PPL-C, altered the activation energies or the critical temperature at which the discontinuity in the Arrhenius plot occurred. According to Tanaka and Teruya (1973) the activity temperature curve of the ($Na^+ + K^+$)-ATPase is determined not by the "source" of the enzyme, but appears to depend on the lipid moiety activating the enzyme. By employing a fluorescent probe technique Charnock and Bashford (1975) have provided supporting evidence to the concept of modulation of enzyme activity by the fluidity of the lipid associated with the protein macromolecule, and claimed that the thermal dependent enzyme activity of their ($Na^+ + K^+$)-ATPase preparation was regulated by the physical state of the lipid adjacent to the enzyme protein.

When studied by differential scanning calorimetry, Kimelberg and Papahadjopoulos (1974) demonstrated that the DOC/$(NH_4)_2SO_4$ fractionated enzyme reconstituted with dimyristoyl (DM)-, dipalmitoyl (DP)-, distearoyl (DS)- and dioleoyl (DO)-phosphatidylclycerol, displayed activity-temperature curves which reflected the phase behaviour of these substituted PG's. For the saturated PG's (DMPG, DPPG, DSPG) the discontinuity

in the Arrhenius plots occurred 1-8°C lower than the initial rise of the endothermic transition in the pure lipid. On the other hand, the Arrhenius plot of ATPase reconstituted with unsaturated DOPG was found to be linear and correlated with the fact that this lipid does not undergo a phase transition within the temperature range studied. In contrast, bovine PS which has a heterogeneous fatty acyl chain composition, showed a broad transition at 13°C and the reconstituted enzyme displayed a discontinuity in the slope of the Arrhenius plot at approximately 15°C. The absence of a sharp discontinuity on the Arrhenius plot of lubrol solubilized ($Na^+ + K^+$)-ATPase after reconstitution with DOPG has also been reported more recently by Palatini *et al.* (1977). In contrast to the unsaturated DOPG, the preparations reconstituted with DMPG displayed a "break" at about 20°C. These results are in agreement with the values reported by Kimelberg and Papahadjopoulos (1974) for their DOC extracted enzyme. These observations as well as those of Grisham and Barnett (1973) and Almeida and Charnock (1977) imply that the enzyme protein has an ordering or restricting effect on the adjacent membrane lipids without necessarily affecting the temperature of the phase transition in the bulk membrane lipids. In a very recent study Charnock *et al.* (1980a) have estimated that less than 2% of the lipids may be involved in certain phase-transitions associated with thermally dependent enzyme behavior.

J. Lipid Analysis

A number of workers have reported that the purification of ($Na^+ + K^+$)-ATPase from a microsomal fraction results in an increase in

the phospholipid : protein ratio when expressed on a µg lipid phosphorus per mg protein basis (Jørgensen, 1974a; Wheeler *et al.*, 1975; De Pont *et al.*, 1978). However the reported values for this ratio vary considerably; 18 from the $(Na^+ + K^+)$-ATPase from beef brain (Uesugi *et al.*, 1971), 24 (Jørgensen, 1974a) and 33 for preparations from the outer medulla of rabbit kidney (De Pont *et al.*, 1978), 209 for the enzyme from *Electrophorus* electroplax and 389 when the rectal gland of *Squalus acanthias* was the source (Perrone *et al.*, 1975). Interestingly, this increase in lipid : protein ratios is also associated with an increase in the specific activity of these preparations. Since the purification results in a concentration of the enzyme protein any phospholipid associated with the purified protein moiety should be necessary for the proper function of the $(Na^+ + K^+)$-ATPase. Based on this assumption, several groups have carried out detailed lipid analysis of the purified $(Na^+ + K^+)$-ATPase without much success. Hokin and Hexum (1972) reported that their purification procedure leads to an increase in the PS content of the enzyme. This claim was supported by Kawai *et al.* (1973), who also found an enrichment of PS during purification, but the percent relative increase was less than that reported by Hokin and Hexum (1972), despite the much higher specific activity which was achieved. More recently, De Pont *et al.* (1978) found an increase in PS content from 7.9% in the microsomal fraction to 13.1% in the purified $(Na^+ + K^+)$-ATPase prepared according to the method of Jørgensen (1974a). In contrast, Wheeler *et al.* (1975) failed to observe an increase in the content of a specific phospholipid during purification despite the fact that the purification led to a 50-fold increase in the specific activity. In

addition, their detailed lipid analysis indicated no selective removal of any particular type of lipid during solubilization and inactivation with either DOC or lubrol. According to Roelofsen and Van Deenen (1973) only 13% of the total PS molecules are involved in the $(Na^+ + K^+)$-ATPase activity. If only such a small fraction of a specific phospholipid is involved in maintaining the enzyme activity, the changes that might occur upon purification would be relatively small, thus making the detection of these alterations extremely difficult.

K. Involvement of Lipid in Ouabain Binding

From the information discussed above it is clear that phospholipids are essential for $(Na^+ + K^+)$-ATPase activity. It is less clear whether lipids are either necessary for, or are capable of modulating the interaction of specific inhibitors (such as ouabain) with the enzyme system. The reports in the literature provide contradictory findings. For example, Tanaka and Strickland (1965) reported that in the absence of lecithin, even 1 mM ouabain was without effect in suppressing the residual $(Na^+ + K^+)$-ATPase activity present in the lipid depleted enzyme. Yet Taniguchi and Iida (1971) by using ox-brain microsomes treated with either PPL-A or PPL-C have concluded that binding of ouabain to $(Na^+ + K^+)$-ATPase does not require phospholipid directly. The validity of this latter claim seems questionable since after either phospholipase treatment their preparations retained significant amounts of phospholipids. Harris *et al.* (1973) found no significant change in the amount of $[^3H]$-ouabain bound to $(Na^+ + K^+)$-ATPase preparations from ox brain, after PPL-C treatment, although some 75% of the ouabain sensitive ATPase

activity was abolished after the lipolysis. From their observations Harris, Swanson and Stahl (1973) concluded that the phospholipids removed by the PPL-C treatment did not play a major role in the binding of ouabain to their brain microsome preparations. In agreement with Taniguchi and Iida (1971) and Harris *et al.* (1973), Goldman and Albers (1973) also observed no alteration in the binding of ouabain after treatment of an enzyme preparation from the electric organ of electric eel with PPL-C. However, in contrast to Harris *et al.* (1973), Goldman and Albers (1973) observed no significant change in the ouabain-sensitive ATPase activity of their enzyme preparation after treatment with PPL-C. In addition, Goldman and Albers (1973) also found that PPL-A digestion of $(Na^+ + K^+)$-ATPase eliminated both ATPase activity and ATP dependent ouabain binding. In the same year Taniguchi and Iida (1973) reported that $(Na^+ + K^+)$-ATPase can exist in at least two different ouabain binding conformations. Phospholipid appeared to play a role in one conformational state which was induced by either $(Mg^{++} + K^+ + ATP)$, $(Mg + P_i)$ or Mg^{++} alone. The other conformer of the enzyme seems to be directly related to the phosphorylation of the enzyme as it was found in the presence of $(Mg^{++} + ATP)$ or $(Mg^{++} + Na^+ + ATP)$, and here these investigators reported that phospholipids did not affect the binding of ouabain. The binding of ouabain to $(Na^+ + K^+)$-ATPase that had been partially delipidated with DOC has been observed by Chipperfield and Whittam (1973). However, in direct conflict with the findings of Taniguchi and Iida (1973), Chipperfield and Whittam observed a three-fold increase in the amount of ouabain bound in the presence of $(Mg^{++} + Na^+ + ATP)$ after the addition of exogenous phospholipids to their delipid-

ated ($Na^+ + K^+$)-ATPase preparation. In other words, this increase in ouabain binding to the reactivated preparation correlates with the stimulation of Na^+-dependent ATPase activity. The findings of Chipperfield and Whittam (1973) suggest that membrane phospholipids may play a major role in the binding of ouabain to the ($Na^+ + K^+$)-ATPase. It should be noted that the preparations used by Taniguchi and Iida (1973) retained high residual ouabain sensitive ATPase activity (about 30% of the initial control value), after treatment with PPL-A. The lipid depleted preparations used by Chipperfield and Whittam (1973) were low in residual ouabain sensitive ATPase activity (only about 3% of phospholipid dependent enzyme activity), and the specific activity of their enzyme preparation from rabbit kidney, was an order of magnitude lower than that used by Taniguchi and Iida (1973).

More recently, Charnock, Almeida and To (1975) investigating the temperature-activity relationship of the ($Na^+ + K^+$)-ATPase have observed that the ouabain dependent but not the cation dependent response of the enzyme to temperature had altered markedly after mild lipolysis with PPL-A. On these grounds, Charnock *et al*. have suggested that only some portion of the membrane lipids may play a role in the binding of cardiac glycosides to the ($Na^+ + K^+$)-ATPase. The fluidity of the lipid matrix has been reported <u>not</u> to influence the rate of ouabain binding (Wallick and Schwartz, 1974). However, the accuracy of the interpretation of the data of Wallick and Schwartz has been questioned by Charnock *et al*. (1977). In contrast to the findings of Wallick and Schwartz (1974), Charnock *et al*. found a non-linear effect of temperature upon the rate of [^{3}H]-ouabain binding. Treatment with detergents resulted in the

abolition of the discontinuity in the Arrhenius plots of ouabain binding, but did not change the non-linear Arrhenius plots for cation activation of the enzyme. These workers have therefore suggested that the lipids which influence the binding of ouabain may not be as closely associated with the "active centre" of the ATPase protein, as those lipid moieties which are involved in the cation dependent response. That is, Charnock and his colleagues are suggesting an asymmetric distribution of lipids around the $(Na^+ + K^+)$-ATPase macromolecule. In agreement with both Taniguchi and Iida (1973) and Hansen (1976), Charnock, Simonson and Almeida (1977) also observed two different types of ouabain binding sites and speculated that only one site is in close contact with the phospholipids of the $(Na^+ + K^+)$-ATPase "active centre".

Information discussed so far indicates the contradictory nature of the results reported by various workers, on the possible involvement of lipid in ouabain binding. Quite recently several investigators have tried to resolve this problem by studying the kinetics of ouabain binding to enzyme preparations depleted of endogenous phospholipids. However, the information available appears to be no clearer than that reported several years earlier. By using a highly purified enzyme preparation Lane *et al.* (1978) reported that the total number of ouabain binding sites on the enzyme was not altered by PPL-A treatment. Moreover, the rate of $[^3H]$-ouabain binding in the presence of $(Mg^{++} + ATP)$ was unchanged after lipolysis. With $(Mg^{++} + ATP + Na^+)$ the rate of ouabain binding to the lipid depleted enzyme was slightly reduced after treatment, with PPL-A whereas in the presence of either $(Mg^{++} + P_i)$ or $(Mg^{++} + ATP + Na^+ + K^+)$ the initial rates of ouabain binding were reduced to

approximately 50% of that of the untreated enzyme. These observations by Lane *et al.* (1978) appear to substantiate the earlier report by Taniguchi and Iida (1973) who also observed about 40% decrease in the initial rate of ouabain binding to PPL-A treated brain microsomes, in the presence of either (Mg^{++} + P_i) or (Mg^{++} + ATP + Na^+ + K^+). Recently, Goodman and Wheeler (1978) using a lubrol solubilized and reconstituted (Na^+ + K^+)-ATPase prepared from rabbit kidney have reported that in the presence of (Mg^{++} + ATP + Na^+) the amount of ouabain bound was the same in both lipid depleted and reconstituted enzymes. This claim by Goodman and Wheeler is in close agreement with the observations of both Taniguchi and Iida (1973) and Lane *et al.* (1978) when using PPL-A treated preparations. However, in contrast to these two groups of investigators [i.e. Taniguchi and Iida (1973) and Lane *et al.* (1978)], who found no difference in the amount of ouabain bound to either control or PPL-A treated preparations, Goodman and Wheeler (1978) observed that in the presence of (Mg^{++} + ATP) no ouabain binding to the delipidated preparation had occurred. Similarly, (Mg^{++} + P_i) promoted binding only to the reconstituted enzyme. From these observations Goodman and Wheeler (1978) concluded that ouabain binding induced by (Mg^{++} + ATP) and (Mg^{++} + P_i) was completely dependent on the phospholipid essential for enzyme activity. The finding of Goodman and Wheeler that phospholipid was an absolute requirement for ouabain binding differs from that of Taniguchi and Iida (1973) in that these latter workers observed only a slight increase in the amount of ouabain bound at equilibrium, when their PPL-A treated brain microsome preparation was reactivated with exogenous phospholipids. However, it should be recalled that the preparations of

Taniguchi and Iida had retained significant amounts of residual phospholipids and ATPase activity after delipidation.

A possible explanation for at least some of the apparent discrepancy in the literature over the role of lipid in ouabain binding has been offered by Whittam and his colleagues. It has been shown recently by Hallam and Whittam (1975) and Whittam *et al.* (1976) that the Mg^{++} and (Mg^{++} + P_i) dependent ouabain binding varies considerably with the methods used to prepare the (Na^+ + K^+)-ATPase enzyme. They attributed these variations to the presence or absence of vesicles in the enzyme preparation. Another possible explanation has been offered by Goodman and Wheeler who pointed out that in the presence of (Mg^{++} + P_i), the enzyme undergoes a conformational change necessary for ouabain binding, and that this change is phospholipid dependent. Alternatively, the phospholipids may be essential for the initial interaction of Mg^{++} and P_i with the (Na^+ + K^+)-ATPase before undergoing any conformational change. In addition, it should be born in mind that observations discussed above were obtained by using (Na^+ + K^+)-ATPase preparations isolated from different species and tissues. Thus, it is possible that the lipid composition of the membrane preparations may be a decisive factor, involved in modulating the interaction of ouabain with the (Na^+ + K^+)-ATPase. Therefore, the apparent conflicting information found in the literature may at least in part be due to the differences in the lipid composition of the membrane among different species and tissues. The effect of changes in the lipid composition of membrane on the interaction of ouabain with (Na^+ + K^+)-ATPase enzyme system has been studied by several workers. These observations which assign a regulatory role for

membrane lipids in ouabain binding are discussed below.

Additional information on the possible lipid modulation of ouabain sensitivity of the $(Na^+ + K^+)$-ATPase has been reported by Charnock and Simonson (1977). The $(Na^+ + K^+)$-ATPase isolated from the axons of the walking legs of the cold water crab, *Cancer magister*, displayed a marked decrease in the sensitivity to inhibition by ouabain when compared to the enzyme isolated from a mammalian source such as beef brain. The crab nerve $(Na^+ + K^+)$-ATPase preparations showed a decreased rate of ouabain binding as well as a decreased amount of ouabain bound under equilibrium conditions. It is well known that the lipid composition of membranes of poikilotherms differs from that of homeotherms. The crustacea and other marine animals are known to contain more unsaturated fatty acids with longer chain lengths. This results in the membranes of marine animals demonstrating phase transitions at lower temperatures than those seen in the membranes of homeotherms. In other words increased fluidity of the membrane results from changes in unsaturation and chain length of the membrane fatty acids. The increased mobility of lipids associated with the crab nerve $(Na^+ + K^+)$-ATPase as compared to the lipids of beef brain enzyme has been observed by the fluorescence polarization technique (Charnock and Simonson, 1977).

It is now well documented that there is a seasonal variation in the membrane lipid composition of hibernating animals (Aloia *et al.*, 1974; Goldman, 1975; Blaker and Moscatelli, 1978). In addition to the changes in the lipid composition, seasonal variations in the $(Na^+ + K^+)$-ATPase activity as well as its cardiac glycoside receptor properties have been reported (Goldman and Willis, 1973a, 1973b; Charnock and Simonson, 1978;

Charnock *et al.*, 1980b, 1980c). As suggested by Charnock and Simonson (1978), the changes in the properties of the $(Na^+ + K^+)$-ATPase that have been observed during hibernation are more likely to be due to alterations in the lipid components of the membrane rather than any structural or molecular changes in the $(Na^+ + K^+)$-ATPase protein.

Thus the role of phospholipids in the function of $(Na^+ + K^+)$-ATPase can be summarized as follows,

a) The $(Na^+ + K^+)$-ATPase requires phospholipids for optimal activity.

b) Reactivation requires negatively charged phospholipids arranged in a lamellar configuration.

c) In addition to the polar head group of the phospholipids, the fluidity and length of the fatty acid acyl chains play a major role in the regulation of enzyme activity.

d) The "breaks" in the Arrhenius plots of the temperature-activity relationship of $(Na^+ + K^+)$-ATPase are possibly due to the phase transition of membrane lipids adjacent to the ATPase protein.

e) Under certain experimental conditions, phospholipids can modulate the binding of ouabain to the $(Na^+ + K^+)$-ATPase.

L. Rationale

From the information discussed in the introduction it is apparent that the lipids of the membrane matrix are able to influence the activity of $(Na^+ + K^+)$-ATPase. However, the observations on the role of lipids in modulating the ouabain sensitivity of $(Na^+ + K^+)$-ATPase are less clear. The present study is concerned with this latter aspect of the $(Na^+ + K^+)$-ATPase.

The working hypothesis for this study is based on the observation that:

a) The crab nerve $(Na^+ + K^+)$-ATPase reflects far less sensitivity to inhibition by ouabain when compared with the same membrane-associated enzyme from beef brain.

b) The membrane lipid composition of pokilotherms differs from that of the homeotherms.

This information strongly suggests that the sensitivity of $(Na^+ + K^+)$-ATPase to ouabain inhibition resides mainly if not completely in the lipid component of the enzyme. In order to test this hypothesis it is necessary to,

a) Purify and delipidate the $(Na^+ + K^+)$-ATPase from both crab axon and beef brain.

b) The purified, delipidated enzyme from both beef brain and crab leg nerve should be incorporated into liposomes prepared from lipids extracted from microsomal fractions enriched with beef

brain and crab nerve ($Na^+ + K^+$)-ATPase respectively, in an attempt to reconstitute the enzyme activity and hence the ouabain sensitivity.

c) Using the reconstituted preparations ID_{50} values for inhibition by ouabain should be obtained.

d) The cross-over study.

The reconstitution of beef brain enzyme in a lipid bilayer composed of lipids extracted from crab nerve ($Na^+ + K^+$)-ATPase and *vice versa*, followed by the construction of dose-response curves for inhibition by ouabain in each case.

Thus, if successful, these experiments should enable us to achieve some insight into the concept of lipid modulation of the ouabain sensitivity of ($Na^+ + K^+$)-ATPase.

MATERIALS AND METHODS

II. MATERIALS AND METHODS

A. General

Distilled water which was passed through a series of deionizing columns was used to prepare all the solutions. Prior to use all solutions were filtered through a millipore filtration system of 0.8 μ pore size.

B. Chemicals

Albumin (bovine serum), ATP (disodium salt), deoxycholic acid (sodium salt), glycylglycine, L-histidine, lactic dehydrogenase (type II), mercaptoethanol, β-nicotinamide adenine dinucleotide - reduced form (cyclohexylamine salt), ouabain octahydrate, phospho-enol-pyruvate (tri-monocyclohexylammonium salt), L-α-phosphatidylcholine (type III from egg yolk), pyruvate kinase (type II), sodium dodecyl sulphate, Sepharose 4B-CL and DNP-L-serine were all purchased from the Sigma Chemical Co., Missouri, U.S.A.

The synthetic lecithins dimyristoyl, dipalmitoyl, distearoyl, distearoyl and dioleoyl L-α-phosphatidylcholines were purchased from Supelco, Inc. (Bellefonte, Pa.). 1-palmito, 2-oleoyl-L-α-phosphatidylcholine and cholesterol were products of Serdary Research Laboratories (London, Ont.). Tris-base (ultra pure) was obtained from Schwarz/Mann (Orangeburg, N.Y.) and Sephadex G-50 (coarse) was supplied by Pharmacia (Canada) Ltd; 2,6-di-tert-butyl-p-cresol (BHT), H_4-EDTA and sucrose (Analar®) were from BDH Chemicals Ltd. Blue dextran, chloroform, 1,4-dioxane (scintanalyzed®), glacial acetic acid, methanol, naphthalene, PPO (2,5-diphenyloxazole - scintanalyzed), sodium azide and toluene (scintanalyzed) were purchased from the Fisher Scientific Co. Ltd.,

New Jersey, U.S.A. The Eastman Kodak Co. Ltd. (Rochester, N.Y.) provided the following: acrylamide, ammonium peroxy disulphate, bromophenol blue (sodium salt) and N',N'-methylene bisacrylamide. [Carboxyl-^{14}C] deoxycholic acid-sodium salt (specific activity 52 mCi/mmol) was from Amersham/Searle and [G-^{3}H] ouabain (specific activity 12 Ci/mmol) was obtained from the New England Nuclear (Canada) Ltd.

Tris-ATP was prepared by passing Na_2-ATP (Sigma) through a column of Dowex-50W (H^+ form, 200-400 mesh), purchased from Bio Rad Laboratories (U.S.A.), followed by adjustment of the pH to 7.5-7.7 with Tris (base).

All other chemicals were analytical grade and purchased from either British Drug House (Chemicals) Ltd., or Fisher Scientific Co. Ltd.

C. Tissues

(1) Beef Brain

Fresh beef brains were obtained from the Gainers Ltd. (Edmonton) and transported to the laboratory on crushed ice. After removing large blood vessels, the cerebral cortex was cut into approximately 100 g portions and immediately frozen in liquid N_2. The frozen tissue was stored at -20°C until required.

(2) Crab Nerve

Axons were isolated from the walking legs of the cold water crab *Cancer magister*, collected from a depth of 14-17 fathoms (ambient temperature 4-8°C), off Barkley Sound, Vancouver Island. The animals were kept alive in sea water (ambient temperature 6-8°C) in an aquarium at the Bamfield Marine Biology Station for 8-10 days prior to dissection. The legs were cut off, while the animal was alive and the nerves were

quickly removed and immersed in an ice cold solution of 0.9% NaCl - 10 mM Tris - 2 mM EDTA, pH 7.6 (storage medium). The pooled axons (approximately 10 g) from several animals were transferred (along with a small volume of storage medium) into small plastic vials and rapidly frozen in an acetone/dry ice mixture. The aliquots of frozen axons were stored at -20°C.

D. Enzyme Preparations

(1) Beef Brain $(Na^+ + K^+)$-ATPase

Untreated preparations of $(Na^+ + K^+)$-ATPase from the cerebral cortex of beef (*Bos taurus*) were obtained by a procedure similar to that first described by Charnock and Post (1963a) but recently modified by Charnock *et al.* (1977). The method was based on the differential centrifugation technique.

Frozen brain tissue (approximately 100 g) was weighed and allowed to thaw in 250 mM sucrose - 30 mM L-histidine - 20 mM Tris (base) - 1 mM H_4-EDTA, pH adjusted to 6.8 with 1 N HCl (homogenizing buffer). The thawed tissue was cut into small pieces (approximately 0.5 g) and homogenized in a ratio of 1 g tissue to 10 ml buffer. The homogenization was carried out at 4°C by using a Polytron Homogenizer (model PT 10-20-350D; Brinkmann Instruments Ltd.) fitted with a generator (type PT 20) and operated at setting 8 for 10 seconds. All of the following differential centrifugation steps were performed at 4°C in a refrigerated Sorvall RC-2B centrifuge fitted with a SS 34 rotor.

The homogenate was centrifuged at 1000 x g_{max} for 15 min to remove large cellular debris. The lysosomes and the mitochondrial particles

were removed by centrifuging at 9000 x g_{max} for 20 min. The resultant supernatant was then centrifuged at 46,000 x g_{max} for 30 min. to yield a "heavy microsomal" pellet. The soluble fraction was discarded while the pellet was washed twice by resuspension and sedimentation in 250 mM sucrose - 20 mM Tris (base) - 1 mM H_4-EDTA, pH 7.6 (resuspending buffer). The washed pellets were resuspended in this buffer and stored at -20°C as 1 ml aliquots in 1.5 ml Brinkmann-Eppendorf micro test tubes, after freezing rapidly in liquid N_2.

(2) Crab Nerve ($Na^+ + K^+$)-ATPase

A microsomal fraction enriched in ($Na^+ + K^+$)-ATPase from crab axons was prepared according to the general procedure of Charnock and Post (1963a) as later modified in this laboratory (Charnock and Simonson, unpublished data).

The axons (approximately 10 g) which were stored in a salt solution, were thawed in 250 mM sucrose - 30 mM L-histidine - 20 mM Tris (base) - 2 mM H_4-EDTA, pH 7.6 (homogenizing buffer). The nerves were washed twice using this medium by resuspending and centrifuging at 5000 x g_{max} for 15 min, in a Sorvall RC-2B refrigerated centrifuge fitted with a SS 34 rotor. This step was done to remove the NaCl which was present in the storage medium. The wet weight of the pellet was noted, and the axons were homogenized in a ratio of 1 g tissue to 20 ml homogenizing buffer. The homogenization was carried out at 4°C, by using the Polytron Homogenizer described above, which was operated at setting 8. Separate bursts of 15 sec, 10 sec, 10 sec (for a total of 35 seconds) were employed with short periods of cooling in ice between bursts to

homogenize the axons. After removing the cellular debris by a low speed spin (2000 x g_{max} for 15 min in a Sorvall RC-2B centrifuge) the supernatants were pooled and centrifuged at 123,000 x g_{max} for 60 min to obtain a microsomal pellet. This latter centrifugation step was performed in a Beckman refrigerated ultracentrifuge equipped with a 60 Ti fixed angle rotor. The microsomes were washed twice by resuspension and centrifugation at 123,000 x g_{max} in sucrose buffer (250 mM sucrose - 20 mM Tris (base) - 2 mM H_4-EDTA, pH 7.6). The washed pellets were resuspended in sucrose buffer and stored as 1 ml aliquots, after freezing in liquid N_2.

(3) Deoxycholate Treatment of $(Na^+ + K^+)$-ATPase

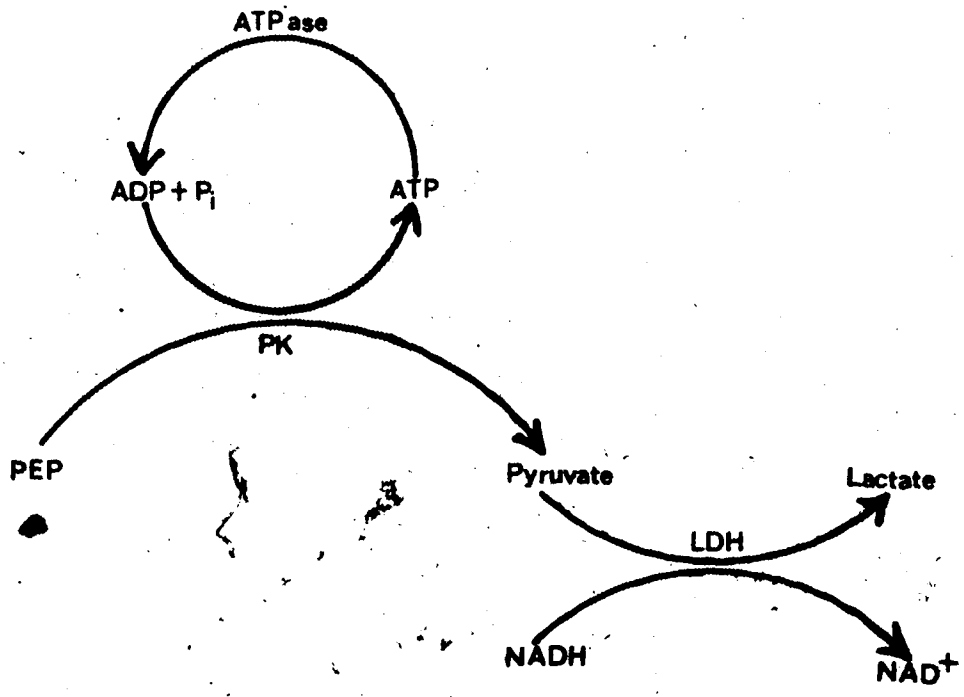
After determining the initial protein concentration, the untreated preparation of $(Na^+ + K^+)$-ATPase (either beef brain enzyme or crab nerve ATPase) was diluted with Tris-EDTA buffer (20 mM Tris - 1 mM EDTA, pH 7.6) to a final protein concentration of 1 mg/ml in the presence of 5 mM Na_2-ATP. An equal volume of 0.2% sodium deoxycholate solution (in Tris-EDTA buffer, pH 7.6) was added to the enzyme suspension, to achieve a final detergent/protein ratio of 2/1 (w/w). The detergent/enzyme mixture was then incubated at 30°C with gentle agitation in a Dubnoff shaking water bath. After 30 min. the extraction was stopped by the addition of an equal volume of ice cold water. The detergent extracted membranes were recovered by centrifugation at either 46,000 x g_{max} for 30 min (beef brain enzyme) or 123,000 x g_{max} for 60 min (crab nerve enzyme). The resultant pellet was washed, resuspended and stored in the appropriate buffer (depending on the source of the enzyme) in a manner identical

to that described for the untreated membrane fraction for either beef brain or crab nerve respectively.

(4) Assay of Enzyme Activity

All enzyme preparations were assayed for both ouabain sensitive and ouabain insensitive, as well as sodium sensitive and sodium insensitive ATPase activity. In all instances the temperature of the assay medium was maintained at 37 ± 0.2°C.

Enzyme activity was determined by a coupled optical assay system, similar to that described previously (Schoner *et al.*, 1967; Schwartz *et al.*, 1969; Charnock *et al.*, 1977; Charnock and Simonson, 1977). This method involves the cyclic regeneration of ATP via pyruvate kinase (PK), in the presence of phospho-enol-pyruvate (PEP). The spectrophotometric assay is linked to the oxidation of NADH via lactic dehydrogenase (LDH) and can be illustrated (Mahler and Cordes, 1966) as follows.



The end result is the stoichiometric conversion of ATP by ATPase to NAD, which was continuously monitored at 340 nm, using a Gilford-2400 recording spectrophotometer, connected to a jacketed ethylene/glycol constant temperature bath (Haake NK 22). The assay medium contained, in a final volume of 3 ml, 3.14 mM phospho-enol-pyruvate (trimonocyclohexylammonium salt), 22 U/ml pyruvate kinase, 13.5 U/ml lactic dehydrogenase, 0.28 mM NADH (cyclohexylamine salt) and 80 mM NaCl in 100 mM glycylglycine buffer (pH 7.6), which contained 250 mM sucrose, 2 mM $MgSO_4 \cdot 7H_2O$, 0.2 mM H_4-EDTA and 20 mM KCl. After temperature equilibration, either 10 or 20 µl aliquot of enzyme suspension was added to the cuvette. This was immediately followed by the addition of Tris-ATP (to a final concentration of 1.5 mM) which initiated the reaction. Enzyme activity was calculated by using the linear portion of the slope of the tracing and the specific activity expressed as µmol ATP hydrolysed/mg protein per hour at 37°C.

Mg^{++}-ATPase (ouabain insensitive ATPase) activity was followed in the presence of 2 mM ouabain. Ouabain sensitive ATPase activity was obtained by subtracting the Mg^{++}-ATPase activity from the total ATPase activity, which was measured in the presence of Mg^{++}, K^+ and Na^+. Sodium insensitive ATPase activity was measured in an identical assay medium to that used for the determination of total ATPase activity except that 80 mM NaCl was omitted from the reaction. Thus, sodium stimulation of the enzyme (i.e. sodium sensitive ATPase activity) was obtained from the difference in enzyme activity in the presence and absence of 80 mM Na^+.

(5) Determination of Enzyme Protein Concentration

The protein concentration of an enzyme preparation was estimated by a modification of the method of Lowry *et al.* (1951), as described by Peterson (1977). The main difference in this modified Lowry procedure is that it includes 1% SDS (final) in the alkali reagent for rapid denaturation and solubilization of membrane protein. This is of key importance since lipids in membrane proteins are known to interfere with the Lowry assay procedure and the inclusion of SDS, may alleviate possible interference from detergent and/or membrane lipid (Peterson, 1977).

E. SDS-Polyacrylamide Gel Electrophoresis

The subunit pattern of untreated ($Na^+ + K^+$)-ATPase preparations isolated from beef brain and crab nerve was studied by the SDS-polyacrylamide gel electrophoresis technique according to the method of Fairbanks *et al.* (1971).

5.6% polyacrylamide gels containing 1% SDS were prepared in 10 cm long glass tubes with an internal diameter of 6 mm. The enzyme preparation (2-4 mg/ml) was solubilized at 37°C for 1 hr, in a solution containing 1% SDS-5% sucrose-10 mM Tris-1 mM EDTA-40 mM mercaptoethanol (pH 8.0). After adding the tracking dye (0.05% bromophenol blue), 50-100 µl aliquots (approximately 50-100 µg protein) of the solubilized enzyme preparation were applied to the tops of the gels. Electrophoresis was performed in electrophoresis buffer (200 mM sodium acetate-100 mM Tris-20 mM EDTA, pH 7.4), containing 1% SDS, at a current of 8 mA per gel, in an electrophoresis apparatus connected to an Ortec-4100 pulsed constant electrophoresis power supply. Electrophoresis was carried out

until the tracking dye had moved to the end of the gel (approximately 4-5 hours). After electrophoresis, the gels were stained with coomassie brilliant blue and destained. The destaining procedure was carried out according to the method of Webber and Osborn (1969). After destaining the gels were stored in 7.5% acetic acid. The gels were scanned at 550 nm using a Gilford 2400 spectrophotometer coupled with a linear transport accessory (Gilford 2410-S).

F. Lipid Extraction

(1) Preparation of Total Lipid Extract

Total lipids from untreated ($Na^+ + K^+$)-ATPase preparations were extracted with chloroform:methanol according to the method of Bligh and Dyer (1959).

An untreated membrane fraction enriched in ($Na^+ + K^+$)-ATPase (either from beef brain or crab nerve) was prepared according to the method described above, except that the final pellet was taken up in distilled water (pH 7.6 with 1 M Tris), to yield a protein concentration of about 5-10 mg/ml. The lipids from this aqueous membrane suspension were extracted by the addition of 10 volumes of chloroform:methanol (2:1). The lipid containing lower organic solvent phase was collected and the residue was re-extracted with chloroform:methanol (2:1) saturated with water. The pooled solvent phases were transferred to a preweighed round bottom flask and rotary evaporated *in vacuo* to dryness. The lipid film was further dried under vacuum over Sicapent® (BDH Chemicals) for 24-48 hours, weighed and then taken up in a desired volume of chloroform. The lipid extract was stored under nitrogen, in the presence of 0.001%

2,6-di-tert-butyl-p-cresol (BHT) at -20°C and used within two weeks of preparation.

(2) Determination of Phospholipid and Cholesterol Content

Phosphate assays were conducted according to the method of Fiske and Subbarow (1925) to determine the phospholipid content of the total lipid extracts of $(Na^+ + K^+)$-ATPase. The cholesterol content of lipid extracts was measured by a colorimetric method based on the Libermann-Burchard reaction as described by Huang *et al.* (1961). The absorbance of the samples was measured using either a Beckman DB-G Spectrophotometer or a Gilford 300-N micro-sample spectrophotometer.

G. Incorporation of $(Na^+ + K^+)$-ATPase into Liposomes

Incorporation of $(Na^+ + K^+)$-ATPase into liposomes was achieved by column chromatography technique. The method employed was similar to that described for the incorporation of aminopeptidase (Wacker *et al.*, 1976), human acetylcholinesterase (Hall and Brodbeck, 1978) and $T_{(is)}$ hydrophobic peptide from MN glycoprotein (Allen *et al.*, 1980) into lecithin liposomes.

(1) Preparation of Sephadex G-50 Columns

Dry Sephadex G-50 (coarse) beads were swollen overnight at room temperature in 100 mM KCl - 20 mM Tris (base) - 1 mM EDTA, pH 7.6, which contained 0.02% sodium azide as a preservative, henceforth referred to as KTEA or eluting buffer. After swelling, the fine gel particles were aspirated out, and the gel slurry was washed several times with the eluting buffer. Before packing the swollen gel was deaerated (by using

a built in laboratory vacuum system) to remove the air bubbles trapped in the gel slurry. The fairly thick slurry of deaerated gel was transferred into the column, by pouring carefully down the glass wall. An eluent reservoir was connected to the column and filled with the eluting buffer. In order to achieve even sedimentation of the beads, the flow (under gravity) was started immediately after filling the column.

The columns used for gel filtration in Sephadex G-50, were obtained from Bio Rad Laboratories and were of two sizes. Small column (1.5 cm x 50 cm) was packed to a height of about 40 cm and samples up to 4 ml were eluted. The large column (2.0 cm x 60 cm) was packed to a bed volume of about 150 cm^3 and used for samples up to 8 ml. The void volume and the column volume of each gel column was determined by using approximately 1-2 ml of a mixture of blue dextran and DNP-L-serine. The larger molecular weight substance, blue dextran was excluded in the void volume (blue colour), while the DNP-L-serine having a smaller molecular weight, ran in the column volume as a sharp yellow band.

(2) Preparation of Sepharose 4B-CL Column.

Sepharose 4B-CL which was supplied as a pre-swollen thick suspension in distilled water containing 0.02% sodium azide was diluted with KTEA buffer. The fine bead particles were aspirated out, and the gel was equilibrated with several changes of buffer at room temperature for 4-5 hours. The gel was deaerated before packing. The thick slurry of gel was poured into the column (1.5 cm x 50 cm) and eluted with KTEA buffer. The beads were packed to a height of 40 cm, under an operating pressure of 10-15 cm H_2O. Samples up to 3 ml were eluted in this column.

The void volume and column volume of the Sepharose 4B-CL column was determined by running a sample of a mixture of blue dextran and DNP-L-serine.

(3) Preparation of Liposomes Containing $(Na^+ + K^+)$-ATPase

For the preparation of $(Na^+ + K^+)$-ATPase containing lipid vesicles a phospholipid/protein molar ratio of 750:1 was used, assuming an average molecular weight of 250,000 g/mole and 750 g/mole for $(Na^+ + K^+)$-ATPase enzyme protein and phospholipid respectively.

An aliquot of lipid (usually 10-15 mg in $CHCl_3$) was evaporated to dryness under a stream of N_2 and further dried at 20°C for 45-60 min *in vacuo* by rotary evaporation in a Buchler flash evaporator. The dried lipid film was flushed with nitrogen and sealed with parafilm. To the dried lipid, 1% solution of deoxycholate (sodium salt) in 20 mM Tris - 1 mM EDTA (pH 7.6) was added to yield a detergent/phospholipid molar ratio of about 3:1. The detergent-lipid mixture was vortexed thoroughly until clear.

A sample of partially delipidated (with DOC) $(Na^+ + K^+)$-ATPase preparation (2-5 mg/ml) was thawed and diluted (if necessary) with 20 mM Tris-1 mM EDTA, pH 7.6, so that when added to the detergent-lipid mixture the final protein concentration would range from 1.0 - 1.5 mg/ml. The enzyme preparation was then added to the DOC-lipid mixture, vortexed and incubated at room temperature for 5 min. The enzyme/lipid/detergent mixture was then applied, (by using a Pasteur pipet) to a column of Sephadex G-50, which had been pre-equilibrated with eluting buffer. The enzyme/lipid/detergent mixture was eluted at a flow rate of 1 ml/min,

with KTEA buffer. The flow rate was controlled by means of a constant hydrostatic pressure and fractions of either 2 ml or 4 ml (depending on the size of the column) were collected by using an LKB (Ultro-Rac 7000) or an ISCO (Golden Retriever-370) fraction collector.

The turbid fractions (i.e. void volume) containing liposomes and enzyme protein were pooled and centrifuged at 161,000 x g_{max} for 60 min, in a Beckman L3-40 refrigerated ultracentrifuge fitted with a titanium (60 Ti) rotor. The resultant pellet was taken up in 2-3 ml of KTEA buffer and applied to a column of Sepharose 4B-CL which had been pre-equilibrated with the same buffer. The sample was eluted at a flow rate of 9 ml/hr, by means of a constant hydrostatic pressure and 2 ml fractions were collected. The most turbid fractions of the void volume were pooled (4-6 ml) and concentrated by centrifugation at 161,000 x g_{max} for 60 min. The resultant pellet was taken up in about 1.0 - 1.5 ml of 20 mM Tris - 1 mM EDTA, pH 7.6 and assayed for $(Na^+ + K^+)$-ATPase activity as described above (Section D. 4.). The $(Na^+ + K^+)$-ATPase preparation reincorporated into a liposome bilayer was stored at 4°C and used within 24-48 hours.

A small aliquot of reconstituted ATPase preparation was viewed under an electron microscope (JEM 7A) after negative staining with a 1% solution of phosphotungstate.*

Where the endogenous lipids of an enzyme preparation were serially substituted with added lipid, the procedure employed was similar to that described above for liposome preparation from mixed or "total" lipids.

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* The electron microscopy was performed by Mr. G. Duchon of the Department of Pharmacology at the University of Alberta.

The only difference was that the enzyme preparation was exposed to a mixture of lipid-detergent three times, instead of a single exposure as mentioned before. All three mixtures of lipid plus detergent contained identical quantities of lipid and detergent. The incorporation of enzyme protein into liposomes was achieved by the gel-filtration technique as described before. In brief, a suspension of $(Na^+ + K^+)$-ATPase was added to lipid-detergent mixture and ATPase-liposomes were formed by detergent depletion on a Sephadex G-50 column. The void volume was concentrated by centrifugation and reintroduced to a mixture of lipid and detergent for the second time. The enzyme/lipid/detergent mixture was then gel filtered on a column of Sephadex G-50 which had been pre-equilibrated with eluting buffer. The void volume was pooled, concentrated and exposed to a lipid-detergent mixture for the third time. Deoxycholate was removed from this enzyme/lipid/detergent mixture by gel filtration on Sephadex G-50. ATPase-liposomes eluted from the Sephadex column were concentrated by sedimentation at 161,000 x g_{max} for 60 min. The resultant pellet was taken up in KTEA buffer and gel filtered on a Sepharose 4B-CL column as described before. The term, "serially substituted enzyme" will be used to describe an enzyme preparation, incorporated into liposomes by this method.

In order to avoid possible thermal-denaturation of the enzyme, the initial reconstitution experiments were carried out at 4°C in a cold room. However, it was found that the reincorporation of $(Na^+ + K^+)$-ATPase into liposomes could be accomplished successfully at room temperature, without significantly affecting the enzyme activity. Therefore, unless otherwise indicated, all of the reconstitution experiments

reported in this presentation were performed at room temperature.

In a preliminary series of experiments the separation of DOC by Sephadex G-50 filtration and the elution profile of the enzyme protein was followed by using radiolabelled compounds of [^{14}C]-deoxycholate and [^{3}H]-ouabain respectively. A small aliquot (10-20 µl) of [^{14}C]-deoxycholate (1.1 x 10^{6} dpm/ml) was added to the detergent-lipid mixture and vortexed. The enzyme protein was labelled with tritiated ouabain according to the method of Charnock *et al*. (1977). 2 ml of enzyme (2.5 mg/ml) was incubated in the binding solution which consisted of 100 mM glycylglycine - 2 mM $MgSO_4$ - 0.2 mM H_4-EDTA - 80 mM NaCl - 2.5 mM Na_2-ATP, pH 7.6 with 1 M Tris-base. The incubation medium also contained 5 x 10^{-7}M (final) unlabelled ouabain plus 10 µl of [^{3}H]-ouabain (1.0 x 10^{9} dpm/ml). This mixture was incubated at 37°C for 30 min. in a Dubnoff metabolic shaking incubator. At the end of the incubation the enzyme-ouabain mixture was added to the [^{14}C]-DOC/lipid mixture, vortexed and incubated for 5 min., at room temperature. This mixture, which contained (Na^{+} + K^{+})-ATPase labelled with [^{3}H]-ouabain, phospholipid and [^{14}C] labelled deoxycholate was then applied on to a column of Sephadex G-50 and eluted with KTEA buffer. Fractions of 2 ml were collected by using an LKB (Ultro-Rac 7000) fraction collector. A 0.5 ml aliquot was withdrawn from each fraction and counted for both [^{14}C] and [^{3}H] in 10 ml of 13.5% (v/v) toluene-dioxane fluor containing both 0.5% (w/v) PPO and 10% (w/v) napthalene. The samples were counted to 2% error in a Beckman LS-100 liquid scintillator.

In addition, a small aliquot from each fraction was analyzed for the presence of protein and for inorganic phosphate (P_i) content which

was obtained according to the method of Fiske and Subbarow (1925).

The liposomes and the [^{3}H]-labelled ATPase protein which was eluted in the void volume of G-50 column, were concentrated by centrifugation (161,000 x g_{max} for 1 hr) and the resuspended pellet was gel filtered in Sepharose 4B-CL. Fractions of 2 ml were collected and a 0.5 ml sample from each fraction was counted for both [^{14}C] and [^{3}H]. In addition each fraction eluted from the Sepharose column was analyzed for the presence of enzyme protein and also for the P_i content. The ATPase liposome eluted in the Sepharose 4B-CL void volume were used for density gradient experiments which were carried out as follows.

(4) Sucrose Density Gradient Centrifugation

Linear density gradients of sucrose (10 ml) consisting of 15-40% sucrose (w/w) in 100 mM KCl-20 mM Tris-1 mM EDTA, pH 7.6, were prepared using a Chrismac-(model DGM-15) gradient forming device. The gradients were chilled to 4°C overnight before use. A sample (200-500 µl) of the Sepharose 4B-CL void volume was layered on each gradient. The tubes were then centrifuged at 4°C in a Beckman swing-out rotor (SW 41) using a Beckman L3-40 ultracentrifuge at 208,000 x g_{max} for 6 hr. After centrifugation a long needle was introduced through each gradient. With the aid of a 1 ml syringe and a three-way stopcock, twenty 0.5 ml fractions were withdrawn from each gradient. The detection of liposomes was carried out using two different methods. In one of these, the absorbance at 330 nm of each fraction (0.5 ml), was measured by using a quartz micro cuvette on a Gilford 2400 spectrophotometer, and served as a qualitative measurement. For a quantitative measurement of liposomes, a

small aliquot from each fraction was analyzed for the P_i content. In order to detect the presence of enzyme protein, a 200 µl sample from each fraction was counted for the presence of [^{3}H]. In addition, each fraction was assayed for the presence of protein. In order to follow the linearity of the gradient, the refraction of sucrose of each fraction was measured by using a refractometer (Fisher Scientific Co. Ltd.).

H. Ouabain Dose-Response Curves

The inhibition of ($Na^+ + K^+$)-ATPase activity at not less than twenty different concentrations of ouabain was followed by using the coupled assay method described above. All assays were carried out at 37 ± 0.2°C. The enzyme activity was measured in the presence of 2 mM Mg^{++}, 20 mM K^+ and 80 mM Na^+ plus varying concentrations of ouabain. The total enzyme activity was measured using an identical assay medium but contained no ouabain. The basal enzyme activity (i.e. in the absence of 80 mM Na^+) was also determined and used to calculate the minimum ouabain concentration which produced a maximum inhibition of the ($Na^+ + K^+$)-ATPase activity. The maximum inhibition by ouabain occurred when the ouabain inhibited enzyme activity was similar to the basal activity which was observed in the absence of 80 mM Na^+.

I. Analysis of Results

When possible the results are expressed as mean ± SEM. The ouabain concentration which caused a 50% inhibition (i.e. ID_{50} value) of ($Na^+ + K^+$)-ATPase activity was calculated by using the linear portion of the log dose response curve (usually between 20-80% inhibition). The ID_{50}

value was obtained by computer regression analysis and the mean ID_{50} value for each enzyme preparation was calculated by using results from no less than three separate experiments. The difference in ID_{50} values between enzyme preparations were analyzed by means of student t-test (unpaired), at the 1% significance level.

RESULTS

III. RESULTS

A. Control Preparations

Microsomal membrane fractions prepared from either beef brain or crab nerve were assayed for $(Na^+ + K^+)$-ATPase activity as described in Materials and Methods. The $(Na^+ + K^+)$-ATPase activity was always measured by both sodium stimulation and inhibition by ouabain. This was necessary since $(Na^+ + K^+)$-ATPase from crab nerve has been reported to be less sensitive to inhibition by ouabain than the $(Na^+ + K^+)$-ATPase prepared from beef brain (Charnock and Simonson, 1977).

It has also been reported (Charnock *et al.*, 1977) that partial delipidation of membrane preparations enriched in $(Na^+ + K^+)$-ATPase with deoxycholate, would cause a considerable increase in the $(Na^+ + K^+)$-ATPase activity. Thus, the untreated membrane fractions from both beef brain and crab nerve were extracted with deoxycholate at a detergent/protein ratio of 2/1 (w/w) as described in Materials and Methods. The results are summarized in Table 1.

The results shown in Table 1 indicate that deoxycholate treatment of enzyme preparations from beef brain had resulted in a considerable activation of $(Na^+ + K^+)$-ATPase activity. As a result of the detergent extraction, the specific activity of ouabain-sensitive beef brain $(Na^+ + K^+)$-ATPase increased from 41.2 to 113.8 μmol ATP hydrolyzed/mg protein/hr. In contrast, the specific activity of crab nerve $(Na^+ + K^+)$-ATPase was not altered by the detergent treatment. This observation suggested that enzyme preparations isolated from crab axons have a different profile to activation by deoxycholate, compared to the

TABLE 1

COMPARISON OF THE EFFECT OF DOC ON THE SPECIFIC ACTIVITIES OF BEEF BRAIN AND CRAB NERVE ENZYME PREPARATIONS

Enzyme preparation	n	Specific activity*			% ATPase sensitive to sodium	% ATPase sensitive to ouabain
		Total ATPase	S.S. ATPase†	O.I. ATPase#		
Beef brain						
untreated	9	49.3 ± 2.9	41.4 ± 2.6	41.2 ± 2.5	84 ± 1.2	84 ± 1.0
DOC-treated**	11	131.3 ± 7.1	110.1 ± 6.6	113.8 ± 6.8	84 ± 0.8	86 ± 0.7
Crab nerve						
untreated	9	14.8 ± 2.8	13.9 ± 2.5	12.1 ± 2.2	94 ± 0.8	82 ± 1.1
DOC-treated**	11	14.9 ± 1.6	14.0 ± 1.5	13.0 ± 1.4	92 ± 1.5	86 ± 1.7

* µmol ATP hydrolyzed/mg protein/hr. at 37°C; Values given are Mean ± SEM.

† sodium stimulated ATPase.

ouabain inhibitable ATPase.

** Treatment with deoxycholate was carried out as described in Materials and Methods.

$(Na^+ + K^+)$-ATPase prepared from beef brain. Therefore, in a separate experiment, the effect of varying concentrations of deoxycholate (i.e. different ratios of detergent/protein) on the specific activity of both beef brain and crab nerve was investigated. The treatment with deoxycholate was carried out as described in Materials and Methods except that different concentrations of DOC solutions were used to achieve the desired detergent/protein ratios. Fig. 1 illustrates the results of this experiment where the data are presented as % $(Na^+ + K^+)$-ATPase activity remaining after treatment with deoxycholate. From these results it is clear that beef brain $(Na^+ + K^+)$-ATPase can be activated considerably by detergent extraction. Optimum activation occurred after extraction with DOC at a detergent/protein ratio of 3/1. In contrast, DOC treatment did not result in a significant increase in the specific activity of $(Na^+ + K^+)$-ATPase prepared from crab nerve. Both enzymes were inactivated at higher detergent/protein ratios. Although DOC treatment did not cause a significant enhancement in the specific activity of crab nerve ATPase, neither was it significantly decreased by this level of DOC. Therefore, all enzyme preparations (both beef brain and crab nerve) used in subsequent experiments were treated with deoxycholate at a detergent/protein ratio of 2/1 (w/w).

In an earlier study Charnock and Simonson (1977) had provided evidence that untreated preparations of $(Na^+ + K^+)$-ATPase obtained from beef brain and crab nerve have markedly different responses to inhibition by ouabain. This claim was re-examined by using detergent extracted enzyme preparations, isolated from beef brain and axons of the walking legs of the cold water crab *Canoer magister*. For both enzyme

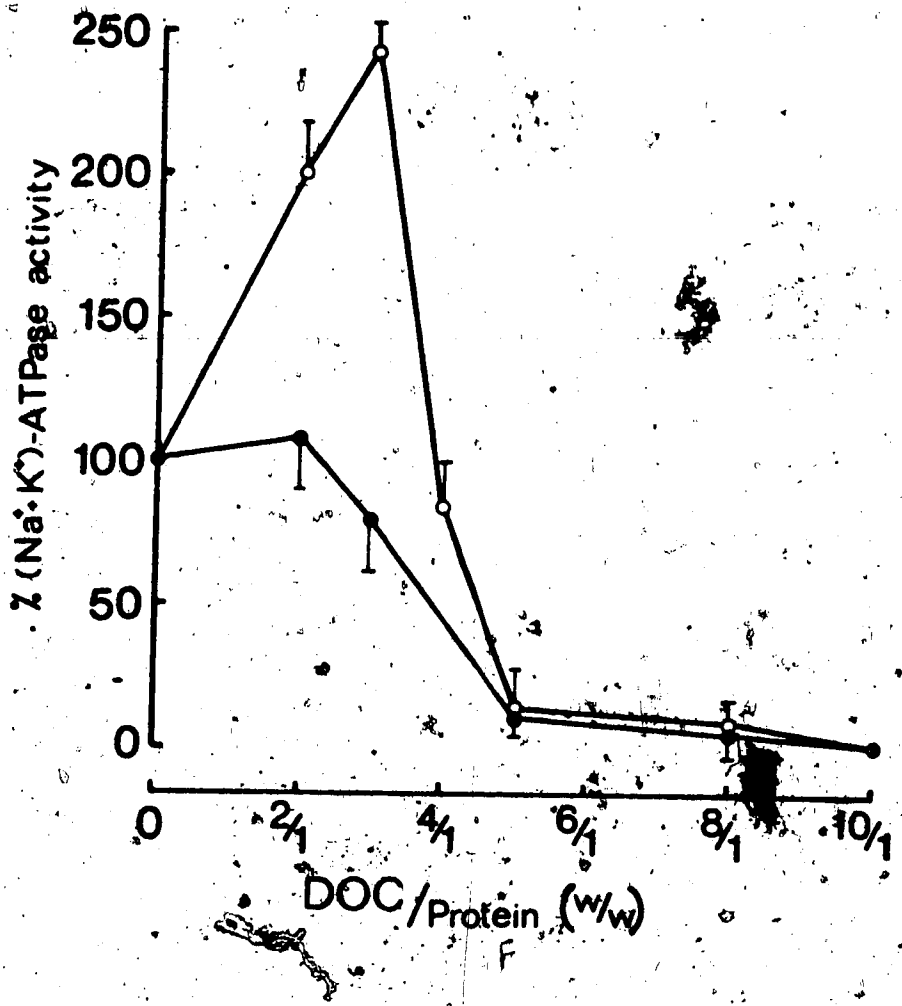


Fig. 1. Comparison of the effect of deoxycholate on $(Na^+ + K^+)$-ATPase from beef brain and crab nerve. Membrane fractions enriched in $(Na^+ + K^+)$-ATPase were treated with varying concentrations of deoxycholate at 30°C for 30 min. The treated membranes were recovered by centrifugation, washed and assayed for activity as described in Materials and Methods. (○) beef brain; (●) crab nerve. The points represent Mean ± SEM of 3 determinations.

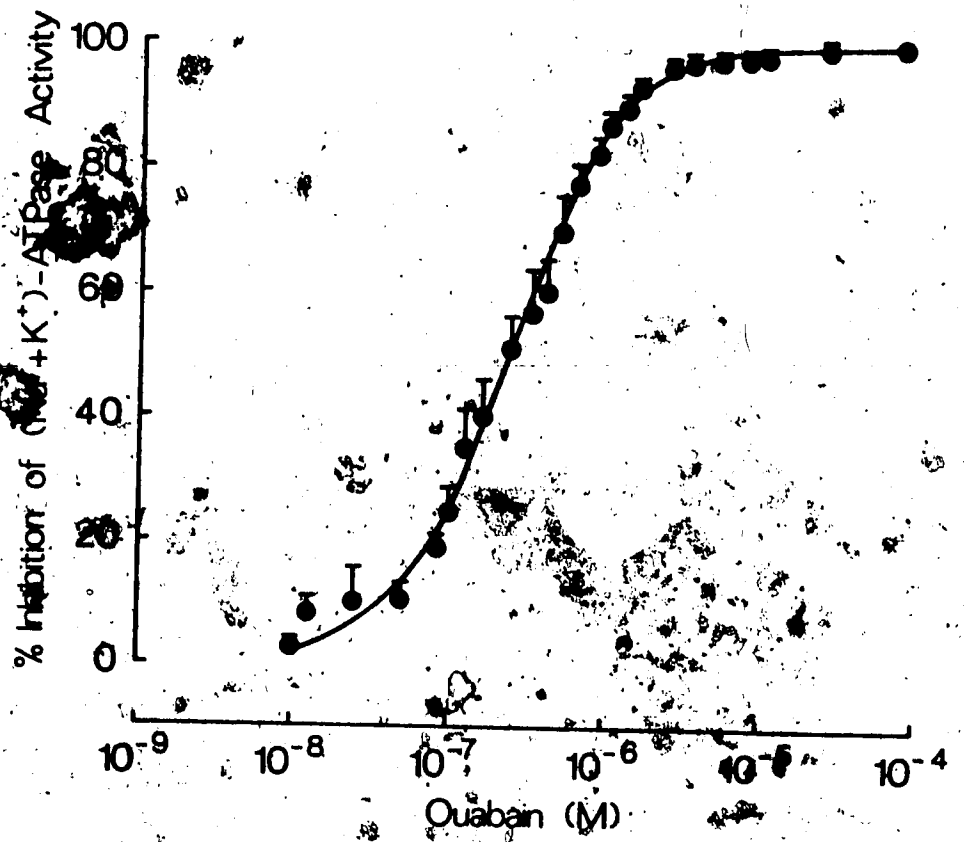


Fig. 2. Dose-response curve of ouabain inhibition of DOC extracted $(Na^+ + K^+)$-ATPase from beef brain. Enzyme preparations were treated with 0.1% deoxycholate for 30 min. as described in Materials and Methods. Enzyme activity was determined at 37°C in the presence of 2 mM Mg^{++}, 20 mM K^+, 80 mM Na^+ plus variable concentrations of ouabain as indicated. 100% inhibition of $(Na^+ + K^+)$-ATPase occurred when the ouabain inhibited enzyme activity was similar to that of basal activity which was observed in the absence of 80 mM Na^+. The points represent Mean ± SEM of 4 determinations. The value for one-half maximal inhibition (ID_{50}) was calculated by computer regression analysis and is $3.06 \pm 0.44 \times 10^{-7}$ M.

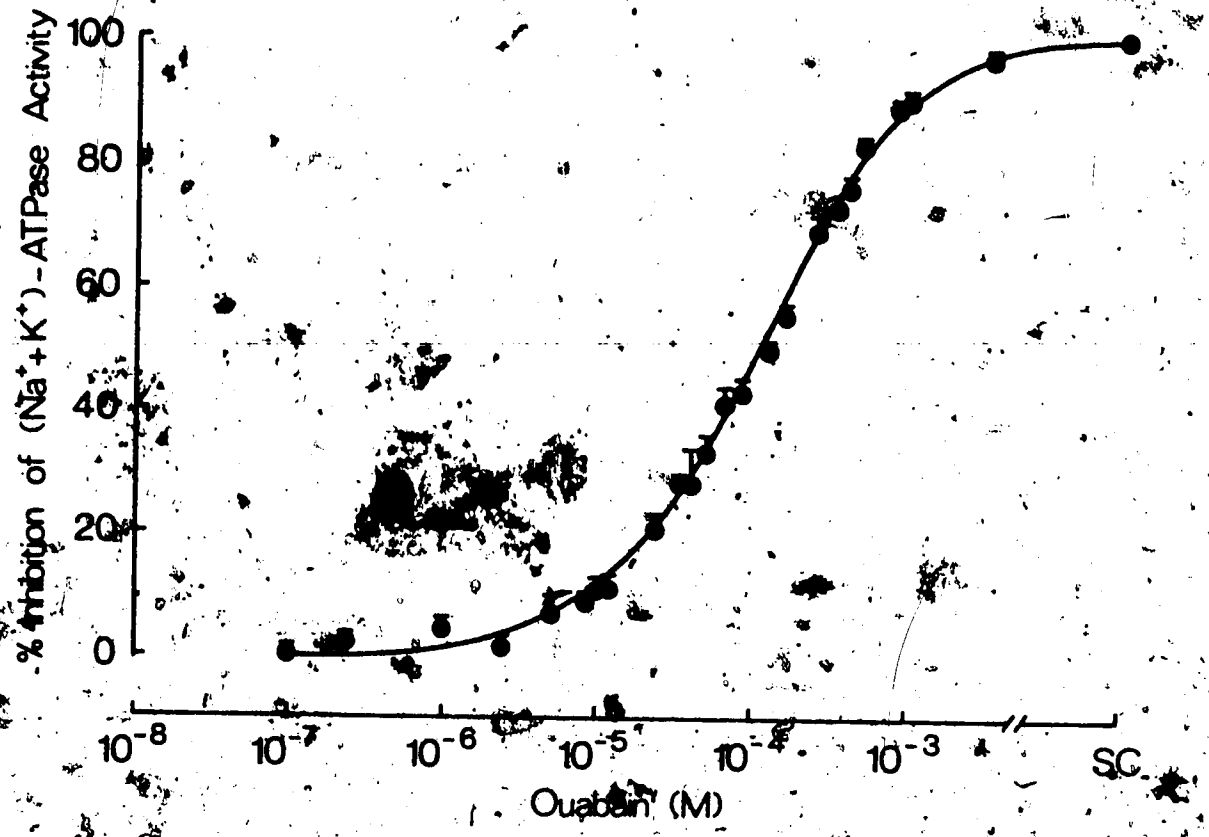


Fig. 3. Dose-response curve of ouabain inhibition of DOC extracted $(Na^+ + K^+)$-ATPase from crab nerve. Enzyme preparations were treated with 0.1% deoxycholate as described in Materials and Methods. Enzyme activity was determined at 37°C in the presence of 2 mM Mg^{++}, 20 mM K^+, 80 mM Na^+ plus variable concentrations of ouabain as indicated. 100% inhibition of $(Na^+ + K^+)$-ATPase occurred when the ouabain inhibited enzyme activity was similar to that of basal activity which was observed in the absence of Na^+. SC indicates saturating concentration of ouabain. The points represent Mean ± SEM of 4 determinations. The value for one-half maximal inhibition (ID_{50}) was calculated by computer regression analysis and is $1.51 \pm 0.09 \times 10^{-4}$ M.

preparations dose-response curves to inhibition by ouabain were constructed as described in Materials and Methods. The results are shown in Figures 2 and 3 respectively. It can be seen from these results that $(Na^+ + K^+)$-ATPase from crab nerve had a markedly reduced sensitivity to inhibition by ouabain, in that the two ID_{50} values differed by more than 2 log units. The mean value for one-half maximal inhibition of beef brain $(Na^+ + K^+)$-ATPase was $3.06 \pm 0.44 \times 10^{-7}$M. In contrast, the mean ID_{50} value for $(Na^+ + K^+)$-ATPase from crab nerve was significantly increased ($P < 0.01$) at $1.51 \pm 0.09 \times 10^{-4}$M. These observations are in agreement with those reported earlier by Charnock and Simonson (1977).

B. Polyacrylamide Gel Electrophoresis

The observations in Figures 2 and 3 naturally raise the question, why there is such a marked difference in the mean ID_{50} values of the two microsomal preparations of $(Na^+ + K^+)$-ATPase to inhibition by ouabain. One simple explanation would be that a difference in the structure of $(Na^+ + K^+)$-ATPase from crab nerve is responsible for this decrease in sensitivity to inhibition by ouabain (Fig. 3). Therefore, the extent of structural similarity between $(Na^+ + K^+)$-ATPase isolated from beef brain and crab nerve was studied by comparing their polypeptide patterns as shown by SDS-polyacrylamide gel electrophoresis. From the results presented in Fig. 4 and Fig. 5 it is clear that the two enzyme preparations had identical polypeptide patterns. Both enzyme preparations contained three major and two minor bands. In addition, the relative positions of these bands in the gels were very similar.

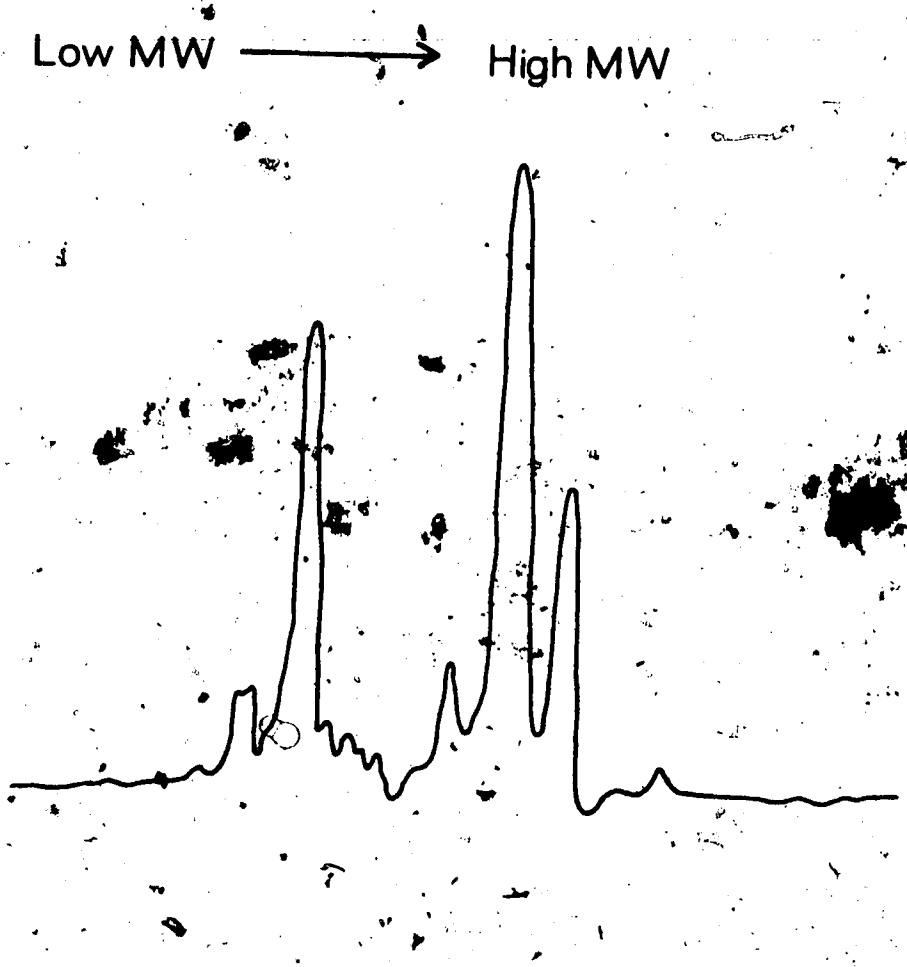


Fig. 4. Polyacrylamide gel electrophoretic pattern of $(Na^+ + K^+)$-ATPase isolated from beef brain.

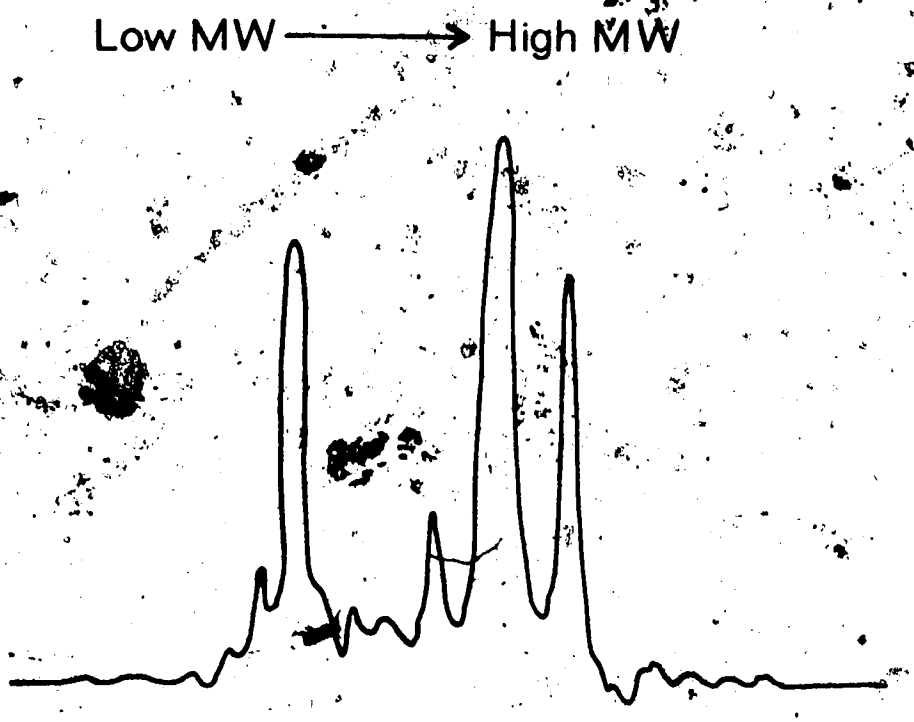


Fig. 5. Polyacrylamide gel-electrophoretic pattern of $(Na^+ + K^+)$-ATPase isolated from crab nerve.

Also, these electrophoretic patterns are similar to those reported by Uesugi *et al.* (1971) for bovine brain $(Na^+ + K^+)$-ATPase. From these results it seems likely that the difference in ID_{50} values to inhibition by ouabain cannot be readily explained by a major structural dissimilarity between the two enzyme proteins.

C. Membrane Lipid Composition

Information discussed in the introduction to this thesis suggests that the lipids of biological membranes exert a modulating effect upon the behaviour of $(Na^+ + K^+)$-ATPase. In addition, phopholipids appear to play a role in the binding of ouabain to this enzyme system. Thus, it is conceivable that a difference in lipid composition of the membrane is responsible for the decreased sensitivity of crab nerve $(Na^+ + K^+)$-ATPase to inhibition by ouabain. In order to investigate this possibility, it was necessary to analyze the lipid composition of membrane fractions prepared from both beef brain and crab axons. The extraction of lipids and determination of phospholipid and cholesterol contents were carried out as described in Materials and Methods. Table 2a summarizes the results of this experiment. These data indicate that lipid extracted from crab nerve $(Na^+ + K^+)$-ATPase had a higher content of cholesterol compared to the lipid extract prepared from $(Na^+ + K^+)$-ATPase isolated from beef brain. There was almost a two-fold increase in the molar ratio of cholesterol/phospholipid of crab nerve $(Na^+ + K^+)$-ATPase compared to enzyme preparations from beef brain. Tabe 2b shows the fatty acid composition of total lipid extracts prepared from membrane fractions enriched in beef brain and crab nerve

TABLE 2a

VARIATION IN THE LIPID COMPOSITION OF $(Na^+ + K^+)$-ATPase FROM BEEF BRAIN AND CRAB NERVE*

	mole %		molar ratio
	cholesterol	phospholipid	$\frac{\text{cholesterol}}{\text{phospholipid}}$
Beef brain $(Na^+ + K^+)$-ATPase	42.7	57.2	0.75
Crab nerve $(Na^+ + K^+)$-ATPase	57.0	43.1	1.32

* Total lipid extracts from untreated membrane fractions enriched in $(Na^+ + K^+)$-ATPase were prepared as described in Materials and Methods.

TABLE 2b

VARIATION IN THE FATTY ACID COMPOSITION OF $(Na^+ + K^+)$-ATPase FROM BEEF BRAIN AND CRAB NERVE*

	% Total lipids	
	Beef brain enzyme	Crab nerve enzyme
Saturated fatty acids		
14:0	1.8	trace
15:0	trace	trace
16:0	21.7	10.4
17:0	0.3	0.4
18:0	19.6	11.0
20:0	0.2	3.3
22:0	0.2	6.0
24:0	1.1	1.5
Total saturates	44.9	32.6
Unsaturated fatty acids		
14:1	0.2	trace
15:1	trace	trace
16:1	1.8	1.5
17:1	0.1	0.2
18:1	19.6	15.8
18:2 (ω-6)	0.8	0.9
18:3	0.1	0.9
20:1	trace	5.2
20:2 (ω-6)	0.4	1.4
20:3 (ω-3)	0.5	0.9
20:4 (ω-4)	6.2	3.0
20:5 (ω-3)	trace	21.2
21:1	1.1	trace
22:1	1.2	trace
22:4 (ω-6)	3.1	1.6
22:5 (ω-3)	1.1	0.2
22:6 (ω-3)	12.7	13.4
24:1	2.2	trace
Total unsaturates	51.1	66.2

* This study was carried out by Dr. R. Gibson of the Department of Pediatrics at the Flinders University, Bedford Park, South Australia.

$(Na^+ + K^+)$-ATPase. It can be seen from these results that total lipid extract of $(Na^+ + K^+)$-ATPase from crab nerve contained more unsaturated fatty acids than the lipid extract of beef brain $(Na^+ + K^+)$-ATPase. The total lipid extract of beef brain enzyme contained about 51.1% unsaturates whereas in crab nerve enzyme this value had increased by 15% to 66.2%. In addition these results show that crab nerve $(Na^+ + K^+)$-ATPase contained fatty acids with longer chain lengths, compared to the lipids from beef brain enzyme. For example, it can be seen that saturated, 22:0 contributed only 0.2% to the total lipids of beef brain $(Na^+ + K^+)$-ATPase. In contrast, 6% of the total lipids of $(Na^+ + K^+)$-ATPase from crab nerve was 22:0. Among the unsaturates a dramatic difference was seen in the content of polyunsaturate, 20:5 (ω-3), between the two enzymes. The beef brain enzyme contained only trace amounts of 20:5 (ω-3) while 21.2% of total lipids of crab nerve $(Na^+ + K^+)$-ATPase were enriched in this polyunsaturated fatty acid.

D. Incorporation of $(Na^+ + K^+)$-ATPase into Liposomes

From the results given in Tables 2a and 2b it can be seen that there is a significant difference in the lipid composition between the two enzyme preparations isolated from beef brain and crab nerve respectively. Thus, it seems possible that this difference in lipid composition of the membrane may account for the decreased sensitivity of crab nerve $(Na^+ + K^+)$-ATPase to inhibition by ouabain. However, in order to correlate this finding with the decreased ouabain sensitivity of crab nerve enzyme preparation, the $(Na^+ + K^+)$-ATPase of the crab has to be introduced into a bilayer composed of lipid extracted from beef brain.

enzyme, followed by the construction of a dose-response curve to ouabain inhibition of the reconstituted enzyme. The reconstitution of a beef enzyme system in lipids extracted from the crab constitutes a "cross-over" study. Therefore, enzyme protein was introduced into a "foreign" lipid environment by reconstitution of $(Na^+ + K^+)$-ATPase protein into liposomes of controlled lipid composition.

(1) Effect of Sonication

Currently, there are a number of different experimental procedures available by which proteins can be incorporated into lipid vesicles (liposomes). Preferably, the ideal method of reconstitution should allow the incorporation of protein into unilamellar vesicles without considerable loss of enzyme activity or other damage to the molecular structure of the enzyme. It has been reported that the reconstitution of certain membrane proteins into lipid vesicles can be achieved by the so-called "sonication method" (Racker *et al.*, 1975; Racker, 1977; Hall and Brodbeck, 1978). Therefore, in a preliminary experiment, the feasibility of this method for the reconstitution of $(Na^+ + K^+)$-ATPase into liposomes was investigated. The results are summarized in Fig. 6, which shows the % $(Na^+ + K^+)$-ATPase activity which remained in the preparations after sonication for varying periods of time. It is clear that sonication of $(Na^+ + K^+)$-ATPase resulted in almost complete loss of ATPase activity. After 10 min. of sonication only 27% of the initial ATPase activity was present. After 30 min. the activity was further decreased by 10% and at 60 min. only 10% of the initial $(Na^+ + K^+)$-ATPase activity was present. The protection of the active site of

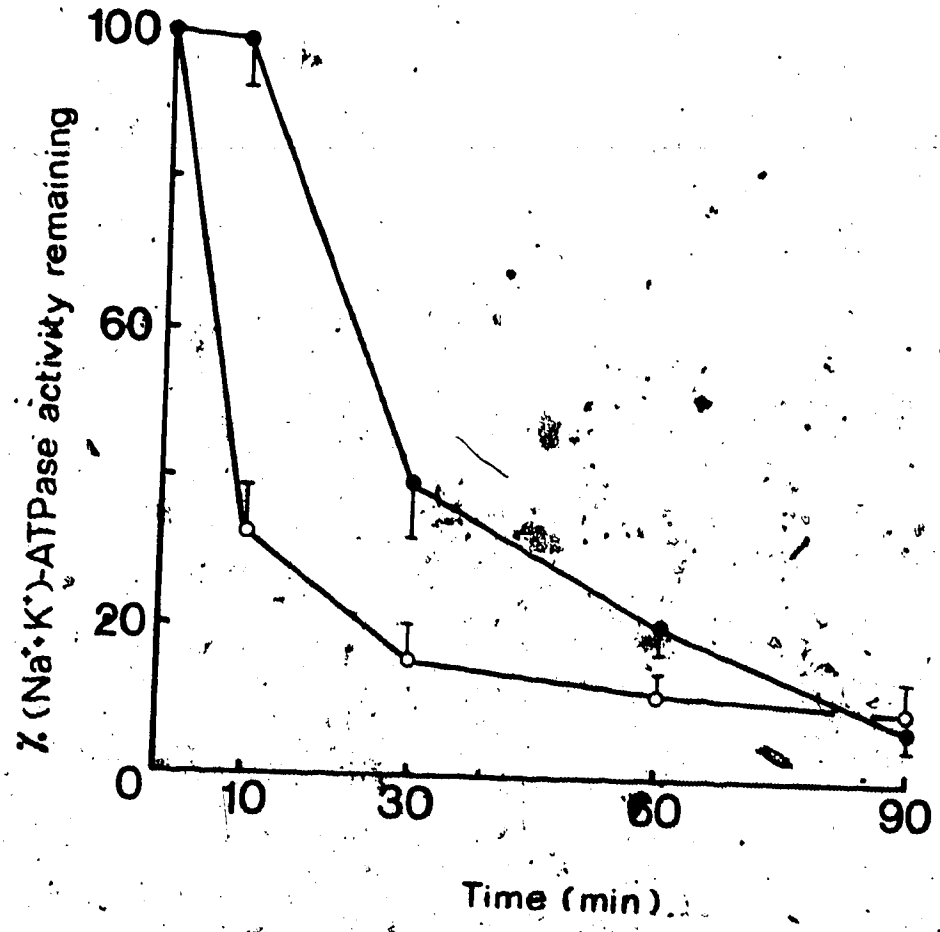


Fig. 6. Effect of sonication on $(Na^+ + K^+)$-ATPase activity. An enzyme preparation from beef brain was diluted to a final concentration of 1.5 mg/ml with 20 mM Tris - 1 mM EDTA, pH 7.6 and sonicated in a bath type sonicator. (○) enzyme; (●) enzyme + 5 mM ATP. The points represent Mean ± SEM of 3 determinations.

ATPase protein with its substrate (ATP) appeared to produce complete protection from loss of activity for 10-15 min. However, as the sonication time increased, there was a gradual loss of enzyme activity. A 60% loss of activity was seen after sonication for 30 min. At 60 min. only 22% of the initial $(Na^+ + K^+)$-ATPase activity was observed. However, incorporation of enzyme proteins into liposomes by the "sonication method" ususally requires more than 60 min. of sonication time. Thus, it is clear that this particular method cannot be employed to accomplish the reconstitution of $(Na^+ + K^+)$-ATPase into liposomes without disrupting its molecular structure. Therefore, some other method of reconstitution was required for the incorporation of $(Na^+ + K^+)$-ATPase, in an active form, into the lipid bilayer structure of liposomes. With this goal in mind, the incorporation of $(Na^+ + K^+)$-ATPase into liposomes by a "detergent depletion method" was investigated. Because of the lengthy time required for published dialysis procedures, a much faster gel filtration procedure was examined.

(2) Incorporation by Detergent Depletion

The experiments were carried out as described in Materials and Methods. In brief, egg phosphatidylcholine was dissolved in 1% solution of deoxycholate. In order to follow the separation of deoxycholate by gel filtration, a small aliquot of [^{14}C]-labelled deoxycholate was added to this lipid/detergent mixture. A sample of beef brain $(Na^+ + K^+)$-ATPase was labelled with [^{3}H]-ouabain and added to the [^{14}C]-DOC/lipid mixture and vortexed thoroughly. This mixture, which contained egg phosphatidylcholine, [^{14}C]-DOC and $(Na^+ + K^+)$-ATPase

labelled with [^{3}H]-ouabain was then passed through a column of Sephadex G-50. The results are shown in Fig. 7 where it can be seen that both liposomes (indicated by the presence of inorganic phosphate) and enzyme protein showed similar elution profiles in that both were co-eluted at the void volume (fractions 14-19) of the Sephadex G-50 column. In contrast, [^{3}H]-ouabain showed two separate peaks, one of which overlapped with enzyme protein and liposomes (fractions 14-19). The second peak was much larger in size and was found between fractions 26-52. On the other hand, [^{14}C]-deoxycholate showed only one peak which was eluted at a later stage (fractions 31-52) from the column. It is clear that the void volume (fractions 14-19) did not contain any [^{14}C]-deoxycholate. The material eluted at the void volume (fractions 14-19) of the Sephadex G-50 column was concentrated by centrifugation and further fractionated on a Sepharose 4B-Cl column in order to separate the liposome incorporated (free) enzyme protein. This procedure resulted in the elution profiles shown in Fig. 8. It is clear from these results that the liposomes showed a somewhat broad distribution in that all fractions 12-24 showed the presence of P_i. However, it can be seen that these fractions consisted of two peaks. A larger sharp peak can be seen between fractions 13-17 and this was immediately followed by a smaller peak which was eluted between fractions 18-24. It is also clear that both [^{3}H]-ouabain and enzyme protein had quite similar elution profiles. However, in contrast to liposomes they both showed two completely separated peaks. The first peak appeared to overlap with the elution pattern of liposomes and indicated a broad distribution (fractions 12-24). The second peak was eluted at a later stage from the column (fractions 32-40) and was completely separated from the

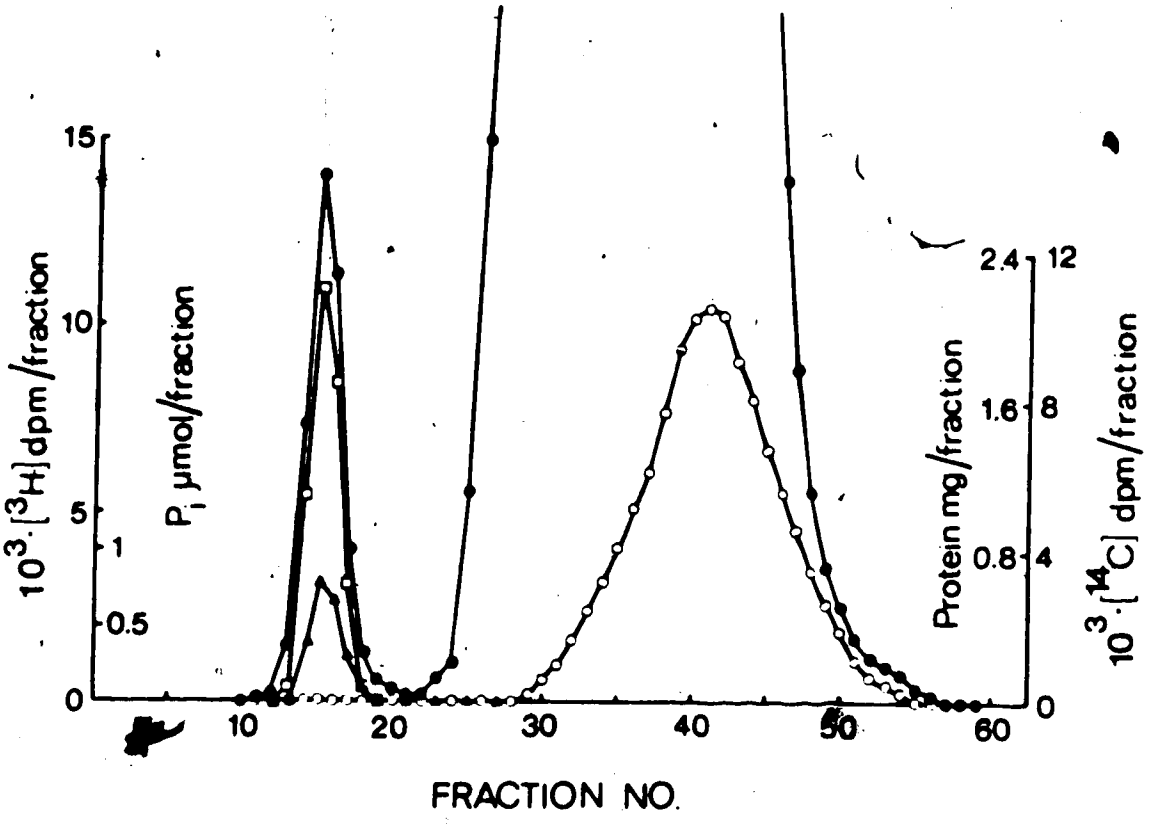


Fig. 10. Incorporation of $(Na^+ + K^+)$-ATPase into liposomes by detergent depletion using gel filtration (control experiment). Removal of deoxycholate by Sephadex G-50 chromatography. The experiment was carried out as described in Materials and Methods. Tritiated ouabain labelled beef brain $(Na^+ + K^+)$-ATPase was added to a solution of 1% deoxycholate. The detergent/enzyme mixture was then gel filtered on a column of Sephadex G-50. (○) deoxycholate; (●) ouabain; (▲) inorganic phosphate; (□) protein.

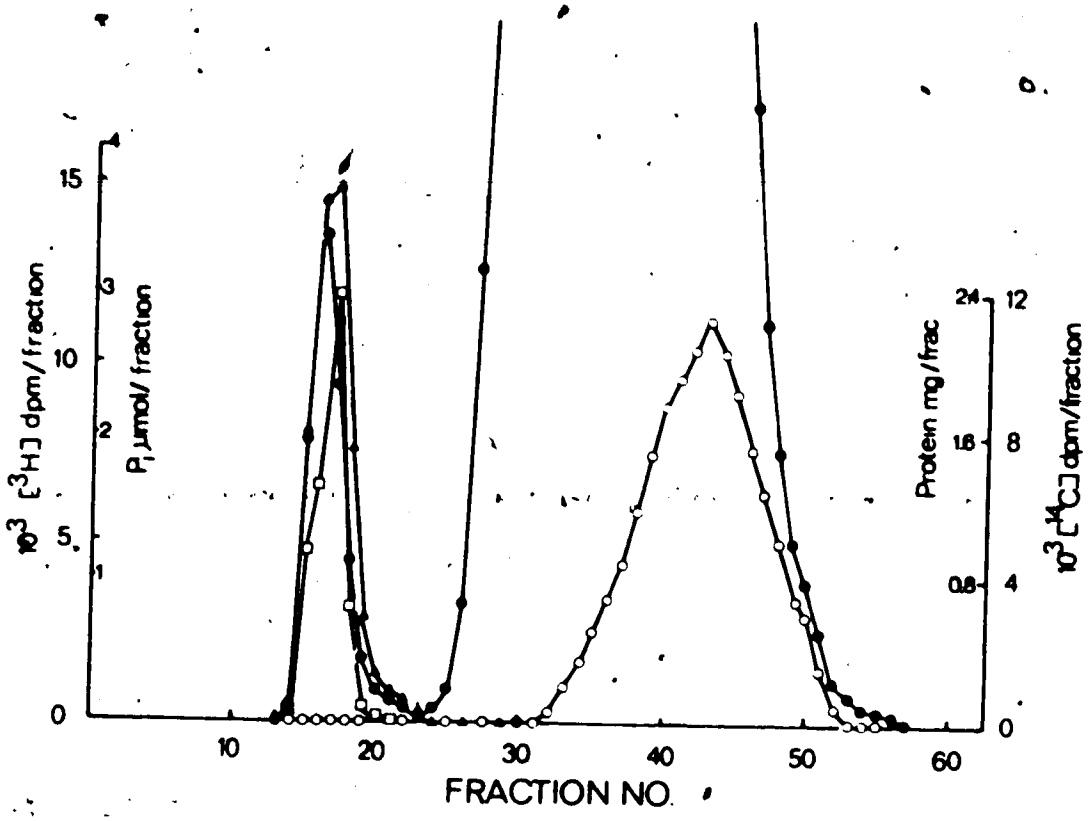


Fig. 7. Incorporation of $(Na^+ + K^+)$-ATPase into liposomes by detergent depletion using gel filtration. Removal of deoxycholate by Sephadex G-50 chromatography. The liposomes containing $(Na^+ + K^+)$-ATPase were prepared as described in Materials and Methods. Tritiated ouabain labelled beef brain $(Na^+ + K^+)$-ATPase was added to egg phosphatidylcholine which was dissolved in 1% deoxycholate. The detergent/lipid/enzyme mixture was applied onto a column of Sephadex G-50 and eluted with buffer which contained 100 mM KCl - 20 mM Tris - 1 mM EDTA - 0.02% NaN_3, pH 7.6. (○) deoxycholate; (●) ouabain; (▲) inorganic phosphate; (□) protein.

Fig. 8. Incorporation of $(Na^+ + K^+)$-ATPase into liposomes by detergent depletion using gel filtration. Rechromatography of the void volume of the Sephadex G-50 column (fractions 14-19, Fig. 7) on a column of Sepharose 4B-CL. The experiment was performed as described in Materials and Methods. The material eluted in fractions 14-19 (Fig. 7) from Sephadex G-50 column was pooled and concentrated by centrifugation at 161,000 x g_{max} for 60 min. The pellet was taken up in 20 mM Tris - 1 mM EDTA, pH 7.6 and passed through a column of Sepharose 4B-CL which had been pre-equilibrated with 100 mM KCl - 20 mM Tris - 1 mM EDTA - 0.02% NaN_3, pH 7.6. (●) ouabain; (▲) inorganic phosphate (P_i); (□) protein. None of the fractions contained deoxycholate. Thus, for clarity, the symbol representing deoxycholate has been omitted.

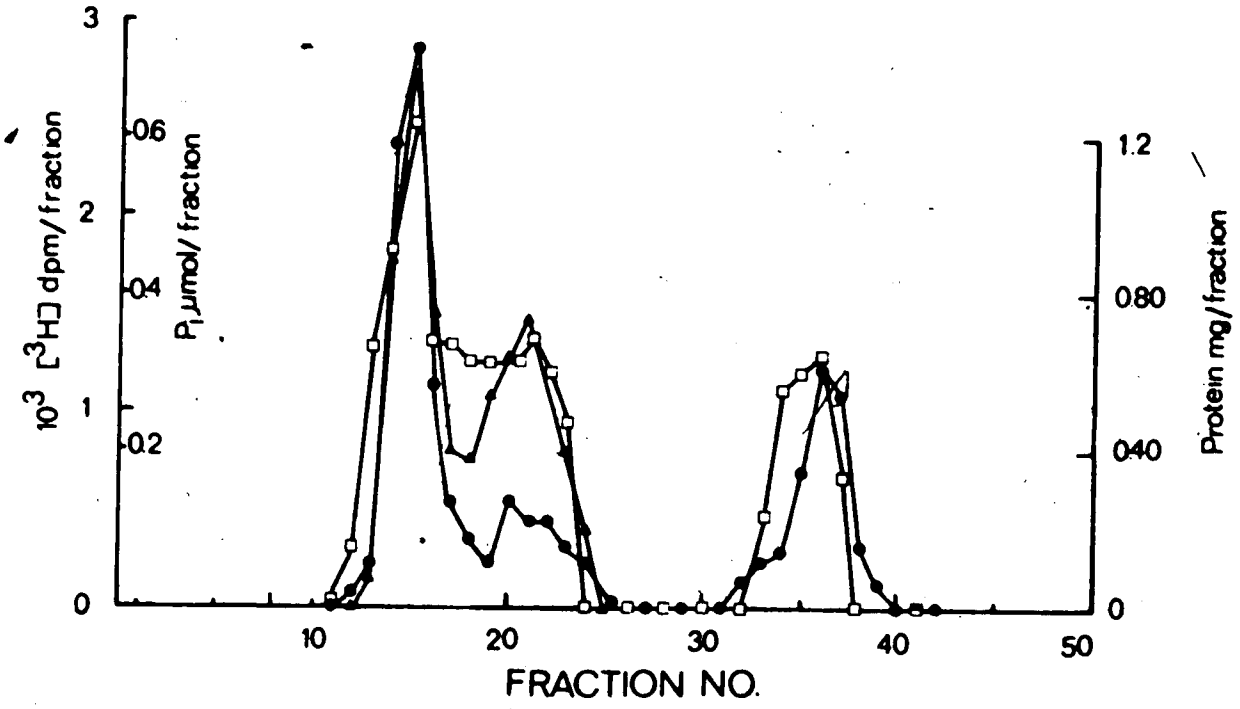
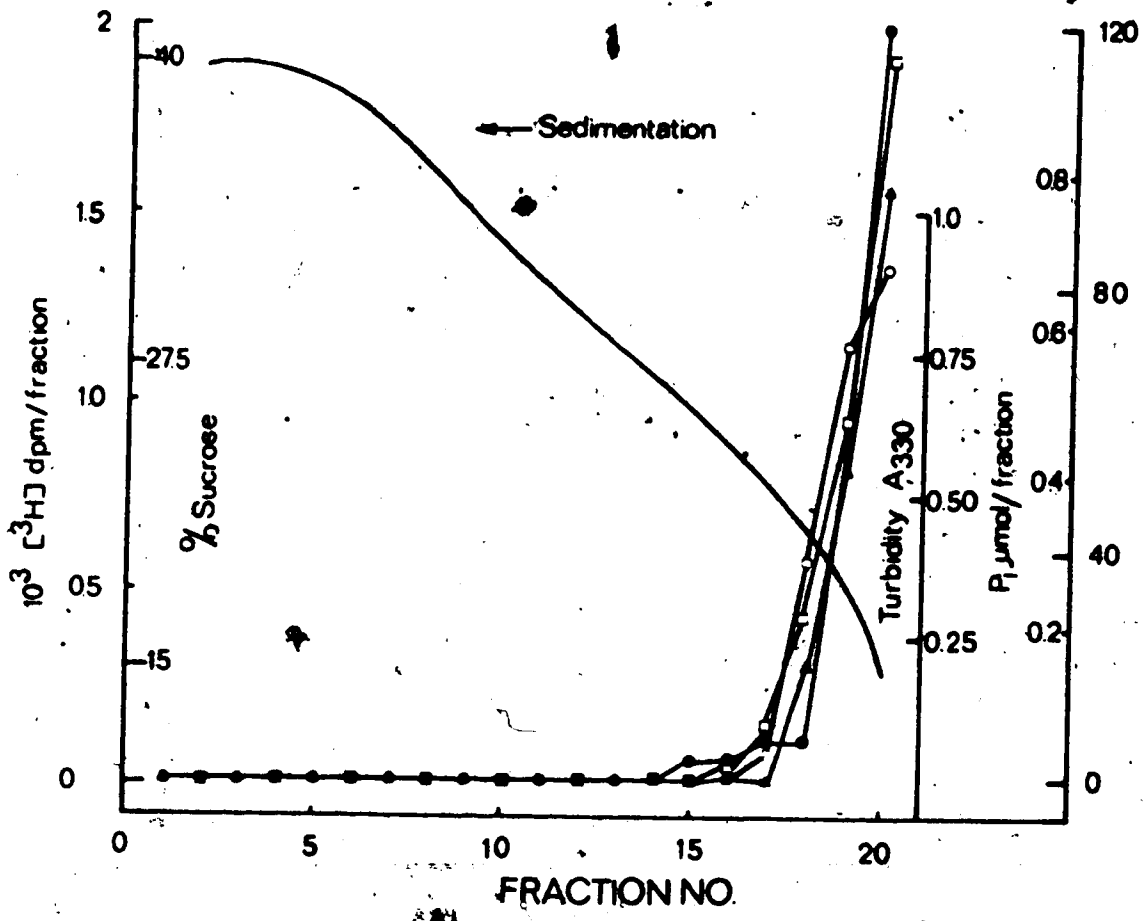

3
2
1
0
10^3 [^{3}H] dpm/fraction
0.6
0.4
0.2
P_i µmol/ fraction
1.2
0.80
0.40
0
Protein mg/fraction
10
20
30
40
50
FRACTION NO.

Fig. 9. Incorporation of $(Na^+ + K^+)$-ATPase into liposomes by detergent depletion using gel filtration. Sucrose density gradient analysis of ATPase-liposomes eluted at the void volume of Sepharose 4B-CL column (fractions 13-17, Fig. 8). A 1.0 ml sample of linear sucrose gradient (15-40%) was made by using a Chrismac® gradient forming device. The sample (200-500 µl) was layered on top of the gradient and centrifuged at 208,000 x g_{max} for 6 hrs in a swing-out rotor (Beckman SW 41) using a Beckman L3-40 ultracentrifuge. After centrifugation twenty, 0.5 ml fractions were withdrawn from the gradient. (—) sucrose; (○) turbidity - A_{330}; (●) ouabain; (▲) inorganic phosphate (P_i); (□) protein.


Sedimentation
10^3 [^{3}H] dpm/fraction
% Sucrose
Turbidity A330
P_i µmol/ fraction
Protein µg/fraction
FRACTION NO.
2
1.5
1.0
0.5
0
40
27.5
15
1.0
0.75
0.50
0.25
0.8
0.6
0.4
0.2
120
80
40
0
0
5
10
15
20

first peak. As mentioned in the legend to Fig. 8, none of these protein fractions contained [^{14}C]-deoxycholate. It should be noted that even co-elution of lipid and protein could not by itself be taken as evidence for protein-lipid interaction. Therefore, in order to establish that the incorporation of ATPase protein into liposomes had occurred, rather than a simple collection of aggregated free enzyme and lipids, the ATPase-liposomes eluted from the Sepharose 4B-CL column, at the void volume (fractions 13-17, Fig. 8) were further fractionated by centrifugation on a linear density gradient of sucrose. The results are summarized in Fig. 9. From this experiment it is clear that inorganic phosphate (P_i), the increase in turbidity at 330 nm, [^{3}H]-ouabain and enzyme protein were all present only on the top of the gradient and that none of these markers were located below fraction #16 with the majority located between fractions 18-20.

Figures 10, 11 and 12 summarize the results of the essential control experiments which were performed in the absence of exogenous lipid. Firstly, in these experiments a mixture of enzyme and detergent was gel-filtered on Sephadex G-50 and the eluted fractions examined. The results are shown in Fig. 10, where it can be seen that elution profiles of enzyme protein, [^{3}H]-ouabain, inorganic phosphate and [^{14}C]-deoxycholate were quite similar to those observed in the "test" experiment described in Fig. 7. The ($Na^+ + K^+$)-ATPase protein was eluted at the void volume (fractions 13-18). In addition, P_i was also present in the void volume. However, it should be noted that since the ($Na^+ + K^+$)-ATPase was eluted on Sephadex G-50 in the absence of exogenous lipid, the total amount of P_i eluted in the void volume (fractions 13-18, Fig. 10) was much less

than that observed in the "test" experiment (compare fractions 14-19, Fig. 7). The elution patterns of [^{3}H]-ouabain and [^{14}C]-deoxycholate were almost identical to those seen in Fig. 7. [^{3}H]-Ouabain showed an elution profile which consisted of two separate peaks. A relatively small peak was observed between fractions 13-18 which was co-eluted with ATPase protein and P_i. The much larger second peak was observed between fractions 23-54. In contrast, deoxycholate was eluted as a single peak between fractions 29-54. It is also clear that the void volume (fractions 13-18) contained no [^{14}C]-deoxycholate. The material eluted at the void volume was concentrated by centrifugation and further fractionated on a column of Sepharose 4B-CL (Fig. 11). From these experiments it can be seen that both ($Na^+ + K^+$)-ATPase protein and [^{3}H]-ouabain had quite similar elution patterns. Both were co-eluted at the void volume (fractions 12-17) and also showed retarded peaks which overlapped (fractions 31-37). On the other hand, P_i was present only in fractions 12-17. The ATPase protein eluted from Sepharose 4B-Cl column at the void volume (fractions 12-17, Fig. 11) was then subjected to density gradient centrifugation in sucrose (Fig. 12). It is clear that both ATPase protein and [^{3}H]-ouabain had travelled deeper into the gradient compared to the "test" experiment described in Fig. 9. Thus, in contrast to the results given in Fig. 9, fractions 9-20 indicated the presence of both enzyme protein and ouabain. In addition, a relatively small amount of P_i and an increase in turbidity were both found on the top of the gradient (fractions 19-20). Therefore, it is evident that these results are quite different to those observed in the "test" experiment (Fig. 9) where enzyme-protein, ouabain, P_i and an increase in turbidity all

Fig. 11. Incorporation of $(Na^+ + K^+)$-ATPase into liposomes by detergent depletion using gel filtration (control experiment). Rechromatography of the void volume of the Sephadex G-50 column (fractions 13-18, Fig. 10) on a column of Sepharose 4B-CL. The experiment was performed as described in Materials and Methods. The material eluted in fractions 13-18 (Fig. 10) from Sephadex G-50 column was pooled and concentrated by centrifugation at 161,000 x g_{max} for 60 min. The pellet was taken up in 20 mM Tris - 1 mM EDTA, pH 7.6 and passed through a column of Sepharose 4B-CL which had been pre-equilibrated with 100 mM KCl - 20 mM Tris - 1 mM EDTA - 0.02% NaN_3, pH 7.6. (●) ouabain; (▲) inorganic phosphate (P_i); (□) protein. None of the fractions contained deoxycholate. Thus, for clarity, the symbol representing deoxycholate has been omitted.

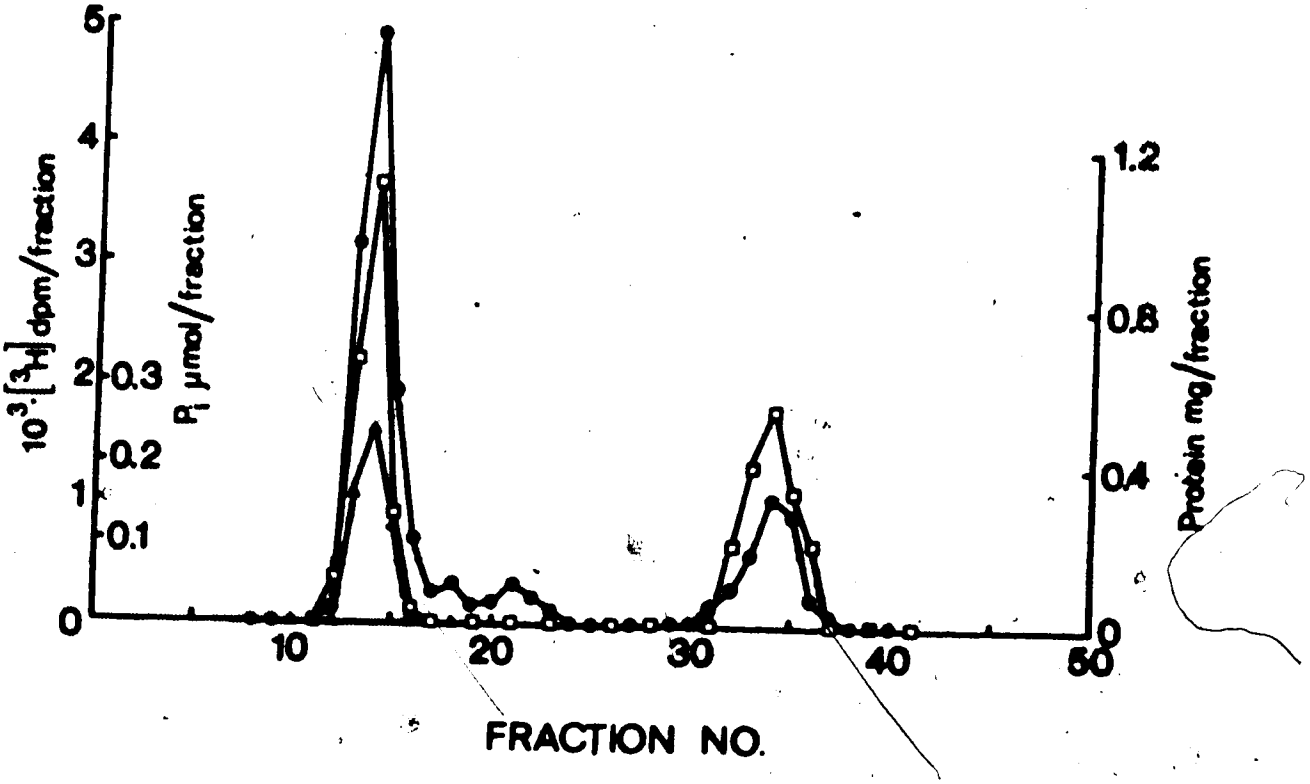
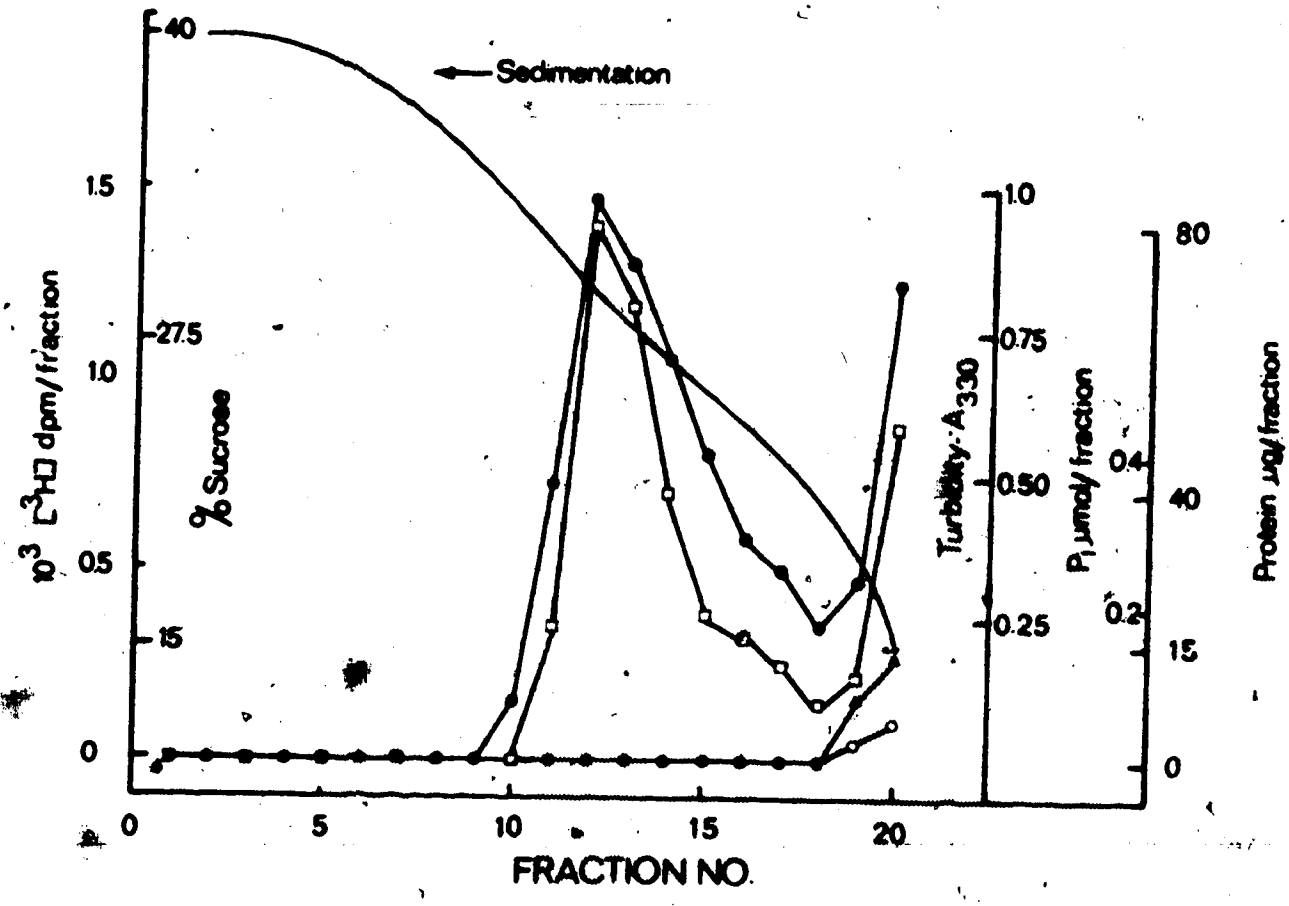

5
4
3
2
1
0
$10^3 \cdot [^3H]$ dpm/fraction
0.3
0.2
0.1
P_i µmol/fraction
1.2
0.8
0.4
0
Protein mg/fraction
10
20
30
40
50
FRACTION NO.

Fig. 12. Incorporation of ($Na^+ + K^+$)-ATPase into liposomes by detergent depletion using gel filtration (control experiment). Sucrose density gradient analysis of the material eluted at the void volume of Sepharose 4B-CL column (fractions 12-17, Fig. 11). A 10 ml sample of linear sucrose gradient (15-40%) was made by using a Chrismac® gradient forming device. The sample (200-500 µl) was layered on top of the gradient and centrifuged at 208,000 x g_{max} for 6 hrs in a swing-out rotor (Beckman SW 41) using a Beckman L3-40 ultracentrifuge. After centrifugation twenty 0.5 ml fractions were withdrawn from the gradient. (—) sucrose; (○) turbidity A_{330}; (●) ouabain; (▲) inorganic phosphate; (□) protein.


40
Sedimentation
1.5
1.0
27.5
0.5
15
0
0
5
10
15
20
FRACTION NO.
10^3 [^{3}H] dpm/fraction
% Sucrose
1.0
0.75
0.50
0.25
Turbidity - A_{330}
0.4
0.2
P_i μmol/ fraction
80
40
15
0
Protein μg/ fraction

appeared only on the top of the gradient.

Thus the results of the control experiments given in Fig. 8 and Fig. 9 indicate that incorporation of $(Na^+ + K^+)$-ATPase into liposomes can be achieved successfully by detergent depletion using the gel filtration procedure. The effect of this technique on the specific activity of $(Na^+ + K^+)$-ATPase was therefore investigated. The specific activity of beef brain $(Na^+ + K^+)$-ATPase at various stages during reconstitution into egg phosphatidylcholine liposomes is shown in Table 3. It is clear that reconstituted enzyme (i.e. 48-void volume) retained about 86% of the initial total ATPase activity. The specific activity of Mg^{++}-ATPase had decreased from 14.1 (native enzyme) to 6.9 (reconstituted enzyme) μmol ATP hydrolyzed/mg protein/hr which represented a 51% loss of enzyme activity. On the other hand reconstituted preparation regained 91% of the initial ouabain inhibitable ATPase [i.e. $(Na^+ + K^+)$-ATPase] activity. It is also apparent that the % ATPase sensitive to inhibition by ouabain had increased somewhat from 87% (native enzyme) to 93% (reconstituted enzyme).

In contrast to the $(Na^+ + K^+)$-ATPase reconstituted into liposomes (Table 3), the final material obtained from the control experiment (i.e. in the absence of exogenous lipid) was devoid of $(Na^+ + K^+)$-ATPase activity. The results of this experiment are summarized in Table 4 where it can be seen that the reconstituted preparation retained only 6% of the initial total ATPase activity. However, as seen in the "test" experiment (Table 3) there was a significant loss (about 68%) of Mg^{++}-ATPase activity. Similarly, ouabain inhibitable ATPase activity had decreased from 92.9 to 3.1 μmol ATP hydrolyzed/mg protein/hr which

TABLE 3

INCORPORATION OF $(Na^+ + K^+)$-ATPase INTO PHOSPHATIDYLCHOLINE LIPSOMES BY COLUMN CHROMATOGRAPHY TECHNIQUE: EFFECT ON SPECIFIC ACTIVITY*

	Specific activity**			
	Total ATPase	Mg^{++} ATPase	O.I. ATPase†	% ATPase sensitive to ouabain
Enzyme	114.2 ± 10.9	14.1 ± 1.5	100.1 ± 9.5	87 ± 0.5
(Enzyme/Lipid/DOC)#	44.5 ± 5.7	6.6 ± 0.7	38.3 ± 5.2	85 ± 1.3
G-50 void volume	88.7 ± 9.8	8.2 ± 0.8	80.5 ± 9.9	90 ± 1.5
4-8 void volume (Reconstituted enzyme)	98.3 ± 5.1	6.9 ± 0.5	91.4 ± 4.6	93 ± 0.3

* Results are based on five separate experiments which were performed at 4°C. Values given are Mean ± SEM.

** µmol ATP hydrolyzed/mg protein/hr at 37°C.

† Ouabain inhibitable ATPase.

Enzyme/lipid/detergent mixture, before gel filtration on Sephadex G-50.

TABLE 4

INCORPORATION OF ($Na^+ + K^+$)-ATPase INTO PHOSPHATIDYLCHOLINE LIPOSOMES BY COLUMN CHROMATOGRAPHY TECHNIQUE: (CONTROL EXPERIMENT): EFFECT ON SPECIFIC ACTIVITY*

	Specific activity**			
	Total ATPase	Mg^{++} ATPase	O.I. ATPase†	% ATPase sensitive to ouabain
Enzyme	103.9 ± 4.7	11.0 ± 1.4	92.9 ± 3.9	89 ± 0.9
(Enzyme/DOC)#	14.0 ± 2.4	5.3 ± 1.0	8.7 ± 1.9	61 ± 7.2
G-50 void volume	7.7 ± 2.1	3.3 ± 0.3	4.4 ± 2.1	45 ± 15.1
4-B void volume	6.6 ± 0.8	3.5 ± 0.7	3.1 ± 0.5	47 ± 7.1

* Results are based on four separate experiments which were performed at 4°C. Values given are Mean ± SEM.

** µmol ATP hydrolyzed/mg protein/hr at 37°C.

† Ouabain inhibitable ATPase.

Enzyme/detergent mixture, before gel filtration on Sephadex G-50.

represented about a 97% loss of enzyme activity. In addition, the % ATPase sensitive to ouabain inhibition had decreased from 89% to 47%.

The morphological integrity of the deoxycholate extracted ($Na^+ + K^+$)-ATPase before and after reconstitution into liposomes was studied by electron microscopy. Fig. 13 shows an electron micrograph of beef brain ($Na^+ + K^+$)-ATPase that had been treated with deoxycholate. The same enzyme preparation was reconstituted into egg lecithin liposomes by the gel-filtration technique and an electron micrograph of the final material is shown in Fig. 14. From the results of Fig. 13 it is clear that the native enzyme preparation had a vesicular appearance; the vesicles appeared to range from 500-1000 nm in diameter. In addition, the native enzyme preparation showed several multilamellar aggregates and fragmented membranes. In contrast, average size of the unilamellar vesicles seen in the reconstituted preparation (Fig. 14) was one order of magnitude lower than those of the native enzyme preparation (Fig. 13). Thus, it is clear that while the reconstituted preparations of ($Na^+ + K^+$)-ATPase had a primarily unilamellar structure, these vesicles were heterogeneous in size as they ranged from 50-100 nm in diameter.

E. Effect of Ouabain on Reconstituted Enzyme Preparations

(1) Beef Brain ($Na^+ + K^+$)-ATPase

The results presented so far indicate that ($Na^+ + K^+$)-ATPase can be reconstituted into phosphatidylcholine vesicles by a gel filtration technique with almost complete restoration of enzyme activity. However, these results do not indicate directly whether or not reconstituted enzyme preparations have retained the cardiac glycoside receptor character-

Fig. 13. Electron micrograph of deoxycholate treated beef brain $(Na^+ + K^+)$-ATPase. A microsomal fraction enriched in $(Na^+ + K^+)$-ATPase was prepared from cerebral cortex of beef and treated with 0.1% deoxycholate as described in Materials and Methods. Enzyme preparation was negatively stained with 1% phosphotungstate and observed in a JEM-7A electron microscope. The bar indicates 1000 nm. The enzyme preparation contains membrane fragments, vesiculated membrane fragments and multi-lamellar aggregates. The vesiculated membrane fragments appear to range between 500-1000 nm in diameter.

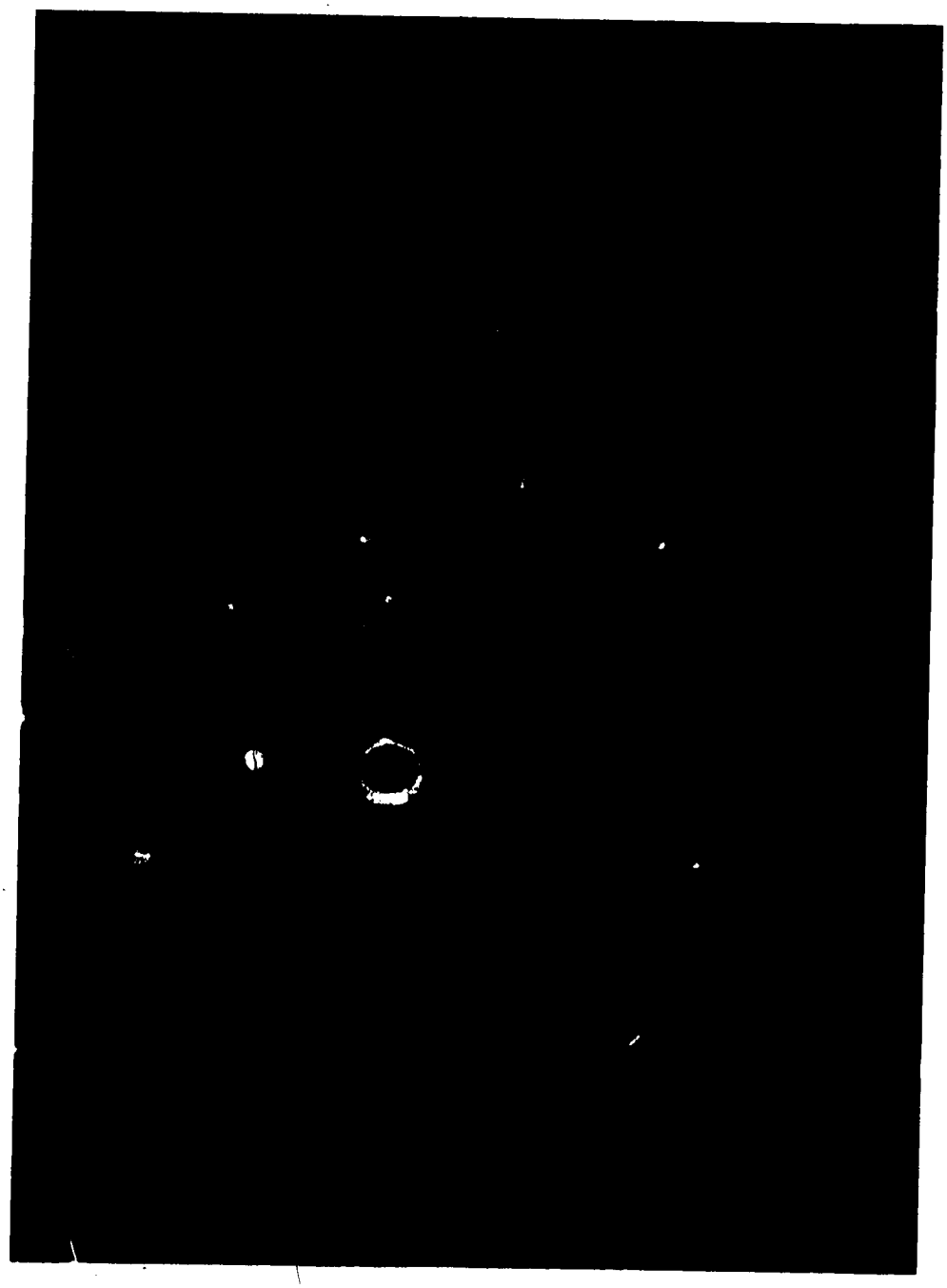
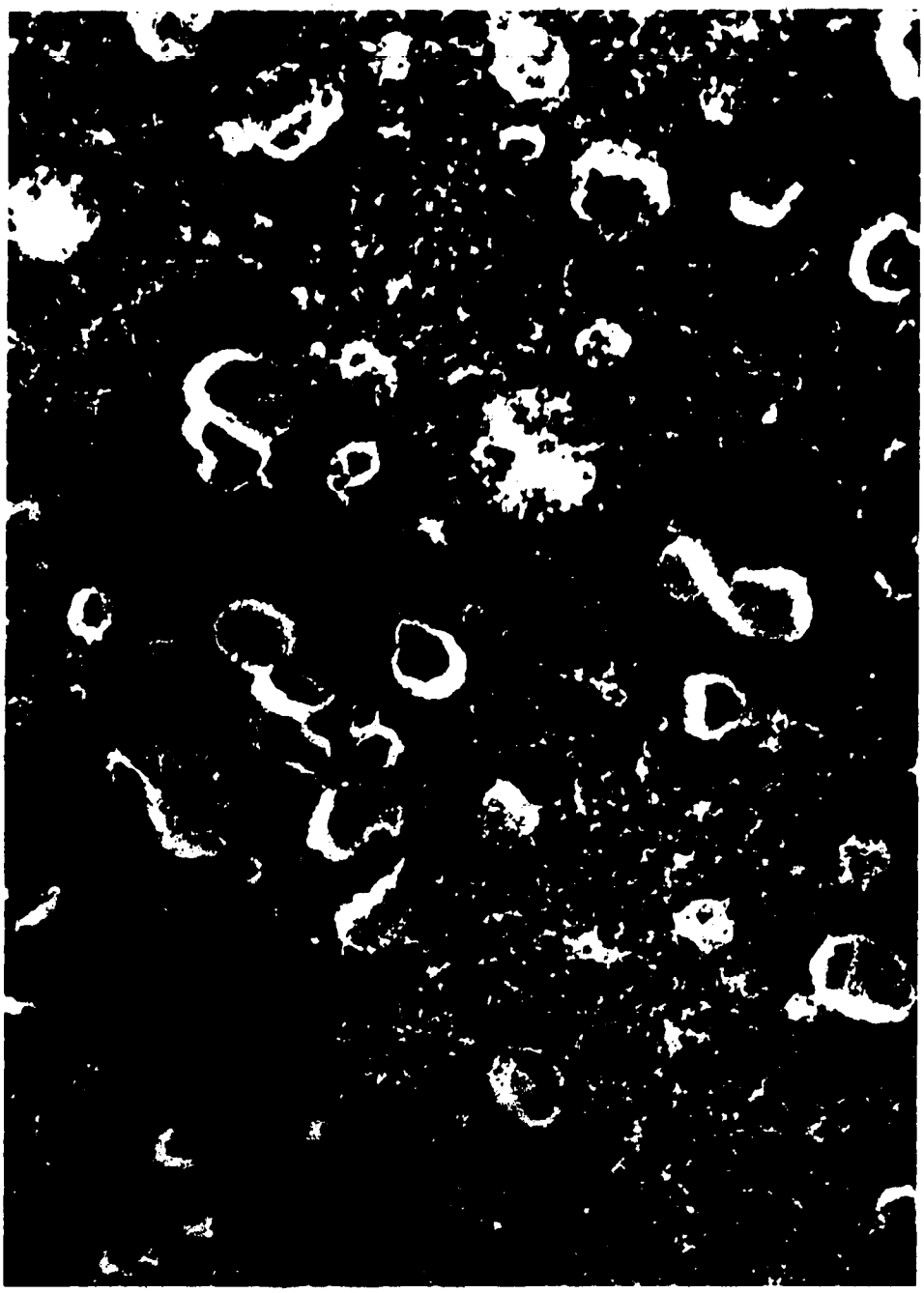


Fig. 14. Electron micrograph of beef brain $(Na^+ + K^+)$-ATPase reconstituted into egg-phosphatidylcholine liposomes. The liposomes containing $(Na^+ + K^+)$-ATPase were prepared by detergent depletion using gel filtration technique, as described in Materials and Methods. Reconstituted enzyme preparation was negatively stained with 1% phosphotungstate and observed in a JEM-7A electron microscope. The bar indicates 100 nm. Reconstituted preparation exhibits a structure of unilamellar vesicles, ranging between 50-100 nm in diameter. These vesicles are one order of magnitude smaller than those seen in native enzyme preparation (Fig. 13).



istics of the native enzyme. Thus, the effect of this method of reconstitution on the ouabain receptor properties of $(Na^+ + K^+)$-ATPase was investigated. In the first experiments the $(Na^+ + K^+)$-ATPase from beef brain was reconstituted into liposomes prepared from a total lipid extract of beef brain enzyme and the effect of this procedure on the specific activity of $(Na^+ + K^+)$-ATPase is shown in Table 5. It is clear from these results that the reconstituted preparation retained about 65% of the initial total ATPase activity. Similarly, there was about a 35% loss of $(Na^+ + K^+)$-ATPase when enzyme from beef brain was reintroduced into a lipid bilayer composed of lipid extracted from beef brain $(Na^+ + K^+)$-ATPase. In addition, the Mg^{++}-ATPase activity of the preparation had decreased from 13.0 (native enzyme) to 6.7 (reconstituted enzyme) μmol ATP hydrolyzed/mg protein/hr. This decrease in activity represented about a 50% loss of the initial activity. The cardiac glycoside receptor characteristics of reconstituted enzyme preparations were studied by constructing dose-response curves to inhibition by ouabain. The construction of the mean dose-response curve and the calculation of the mean ID_{50} value for ouabain was carried out as described in Materials and Methods. Fig. 15 shows the mean dose-response curve to inhibition by ouabain of beef brain $(Na^+ + K^+)$-ATPase reconstituted into beef lipid liposomes. Here, the % inhibition of $(Na^+ + K^+)$-ATPase activity was plotted against the log concentration of ouabain. The mean ID_{50} value ($4.05 \pm 0.37 \times 10^{-7}$M) to inhibition by ouabain of this reconstituted system did not differ significantly from the mean ID_{50} value obtained with the native enzyme ($3.06 \pm 0.44 \times 10^{-7}$M; Fig. 2). A similar observation has been made when endogenous lipids of $(Na^+ + K^+)$-ATPase isolated

TABLE 5

INCORPORATION OF BEEF BRAIN ($Na^+ + K^+$)-ATPase INTO BEEF LIPID LIPOSOMES: EFFECT ON SPECIFIC ACTIVITY*

	Specific activity**			
	Total ATPase	Mg^{++} ATPase	O.I. ATPase†	% ATPase sensitive to ouabain
Enzyme (before reconstitution)	107.8 ± 21.4	13.0 ± 4.6	95.4 ± 17.2	89 ± 1.8
Reconstituted enzyme (Beef-E/beef lipid)	69.8 ± 9.5	6.7 ± 1.1	63.1 ± 8.7	90 ± 1.0

* Results are based on four separate experiments. Values given are Mean ± SEM.

** µmol ATP hydrolyzed/mg protein/hr at 37°C.

† Ouabain inhibitable ATPase.

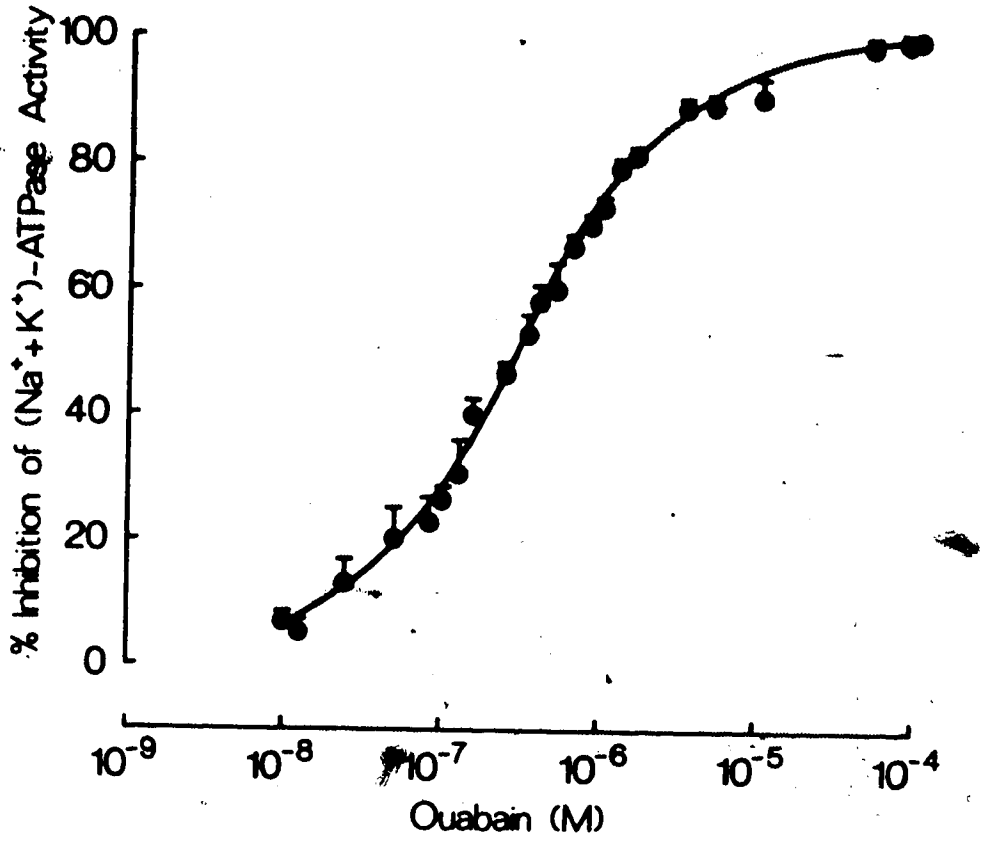


Fig. 15. Dose-response curve of ouabain inhibition of $(Na^+ + K^+)$-ATPase from beef brain reconstituted into beef lipid liposomes. Enzyme activity was determined at 37°C in the presence of 2 mM Mg^{++}, 20 mM K^+, 80 mM Na^+ plus variable concentrations of ouabain as indicated. 100% inhibition of $(Na^+ + K^+)$-ATPase occurred when the ouabain inhibited enzyme activity was similar to that basal activity which was observed in the absence of 80 mM Na^+. The points represent Mean ± SEM of 5 determinations. The value for one-half maximal inhibition (ID_{50}) was calculated by computer regression analysis and is $4.05 \pm 0.37 \times 10^{-7}$M.

from beef brain were serially substituted with lipids extracted from beef brain enzyme. The preparation of serially substituted enzyme was carried out as described in Materials and Methods. In brief, beef brain $(Na^+ + K^+)$-ATPase was added to a beef lipid/detergent mixture. The resultant mixture was then gel filtered on Sephadex G-50. The ATPase-liposomes eluted in the void volume were concentrated by centrifugation and reintroduced to a beef lipid/detergent mixture for the second time. The resultant mixture was gel filtered on Sephadex G-50 and the ATPase-liposomes eluted in the void volume were then exposed to a beef lipid/detergent mixture for the third time. The resultant enzyme/lipid/detergent mixture was gel filtered on Sephadex G-50 and the material eluted in the void volume was further fractionated on a column of Sepharose 4B-CL. The ATPase-liposomes eluted in the void volume from this column were then concentrated by centrifugation and assayed for activity as before. The mean dose-response curve to ouabain inhibition of this serially substituted enzyme preparation is shown in Fig. 16. It is clear that there was no significant difference between the mean ID_{50} values of native enzyme ($3.06 \pm 0.44 \times 10^{-7}$M; Fig. 2) and serially substituted enzyme ($2.65 \pm 0.05 \times 10^{-7}$M; Fig. 16). However, the repeated exposures to detergent/lipid mixtures appeared to affect the specific activity of the serially substituted enzyme, in that the final preparation retained only 26% of the initial ouabain sensitive ATPase activity (Table 6). These results obtained from ouabain dose-response curves, indicated that while there is a loss of specific activity, the reconstituted enzyme retained its native cardiac glycoside receptor characteristics and the experimental procedure had no effect on the interaction of ouabain with

TABLE 6

SERIAL SUBSTITUTION OF ENDOGENOUS LIPIDS OF BEEF BRAIN $(Na^+ + K^+)$-ATPase WITH BEEF LIPID LIPOSOMES: EFFECT ON SPECIFIC ACTIVITY*

	Specific activity**			
	Total ATPase	Mg^{++} ATPase	O.I. ATPase†	% ATPase sensitive to ouabain
Enzyme (before reconstitution)	163.1 ± 16.5	17.7 ± 2.4	145.4 ± 14.7	89 ± 0.8
Reconstituted enzyme (Beef-E/beef lipid serial subst.)	40.7 ± 11.8	3.8 ± 1.0	37.2 ± 10.7	92 ± 1.3

* Results are based on four separate experiments. Values given are Mean ± SEM.

** µmol ATP hydrolyzed/mg protein/hr at 37°C.

† Ouabain inhibitable ATPase.

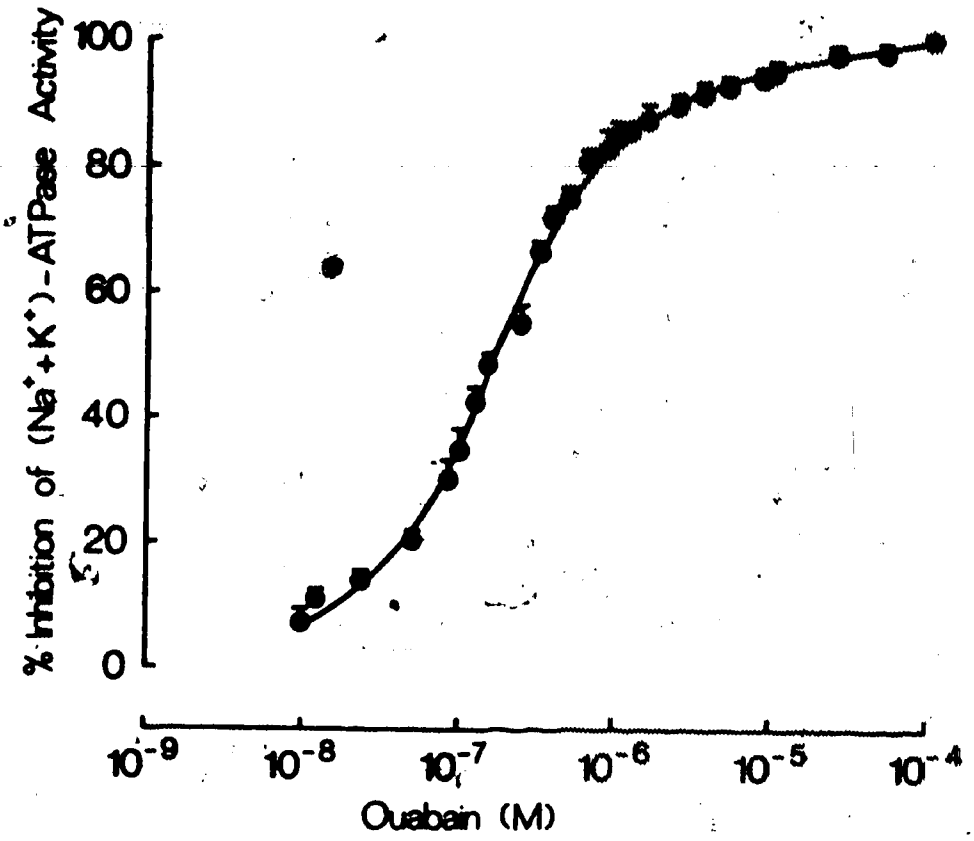


Fig. 16. Serial substitution of endogenous lipids of beef brain $(Na^+ + K^+)$-ATPase with beef lipid liposomes: Dose-response curve to inhibition by ouabain. Enzyme activity was determined at 37°C in the presence of 2 mM Mg^{++}, 20 mM K^+, 80 mM Na^+ plus variable concentrations of ouabain as indicated. 100% inhibition of $(Na^+ + K^+)$-ATPase occurred when the ouabain inhibited enzyme activity was similar to that basal activity which was observed in the absence of 80 mM Na^+. The points represent Mean ± SEM of 3 determinations. The value for one-half maximal inhibition (ID_{50}) was calculated by computer regression analysis and is $2.65 \pm 0.05 \times 10^{-7}$M.

the reconstituted enzyme system. The effect of phosphatidylcholine (egg) on the interaction of ouabain with beef brain ($Na^+ + K^+$)-ATPase was also studied. Table 7 and Fig. 17 summarize the results obtained with an enzyme preparation from beef brain that had been reconstituted into egg lecithin liposomes. It is clear from these results that mean ID_{50} value ($5.06 \pm 0.24 \times 10^{-7}$M) of ($Na^+ + K^+$)-ATPase reconstituted into phosphatidyl liposomes did not differ significantly from that obtained with the control preparation ($4.05 \pm 0.37 \times 10^{-7}$M; Fig. 15). From the results shown in Table 7, it can be seen that reconstituted preparations retained about 73% of the initial total ATPase activity. The Mg^{++}-ATPase indicated a 60% loss of activity and the ($Na^+ + K^+$)-ATPase was decreased from 112.9 (native enzyme) to 87.4 (reconstituted enzyme), μmol ATP hydrolyzed/mg protein/hr, which represented only a 23% loss of enzyme activity.

For the reasons discussed in the Rationale of this thesis, it was necessary to substitute the endogenous lipids of beef brain ($Na^+ + K^+$)-ATPase with lipids extracted from crab nerve ($Na^+ + K^+$)-ATPase and *vice versa*. Thus, ($Na^+ + K^+$)-ATPase from beef brain was reincorporated into liposomes prepared from the lipid extracted from a microsomal fraction enriched in crab nerve ($Na^+ + K^+$)-ATPase. The specific activity and mean dose-response curve to inhibition by ouabain of this "cross-over" reconstituted preparation are shown in Table 8 and Fig. 18 respectively. The data given in Table 8 demonstrate that reconstituted preparations retain only 35% of the initial total ATPase activity. The loss of Mg^{++}-ATPase activity was pronounced, as the reconstituted enzyme had only 20% of the initial Mg^{++}-ATPase activity. In contrast, 36% of the initial

TABLE 7

INCORPORATION OF BEEF BRAIN ($Na^+ + K^+$)-ATPase INTO EGG PHOSPHATIDYLCHOLINE LIPOSOMES: EFFECT ON SPECIFIC ACTIVITY*

	Specific activity**			
	Total ATPase	Mg^{++} ATPase	O.I. ATPase†	% ATPase sensitive to ouabain
Enzyme (before reconstitution)	128.5 ± 8.0	15.5 ± 1.3	112.9 ± 6.7	88 ± 0.3
Reconstituted enzyme (Beef-E/Egg PC)	93.7 ± 2.9	6.3 ± 0.4	87.4 ± 2.5	93 ± 0.3

* Results are based on three separate experiments. Values given are Mean ± SEM.

** µmol ATP hydrolyzed/mg protein/hr at 37°C.

† Ouabain inhibitable ATPase.

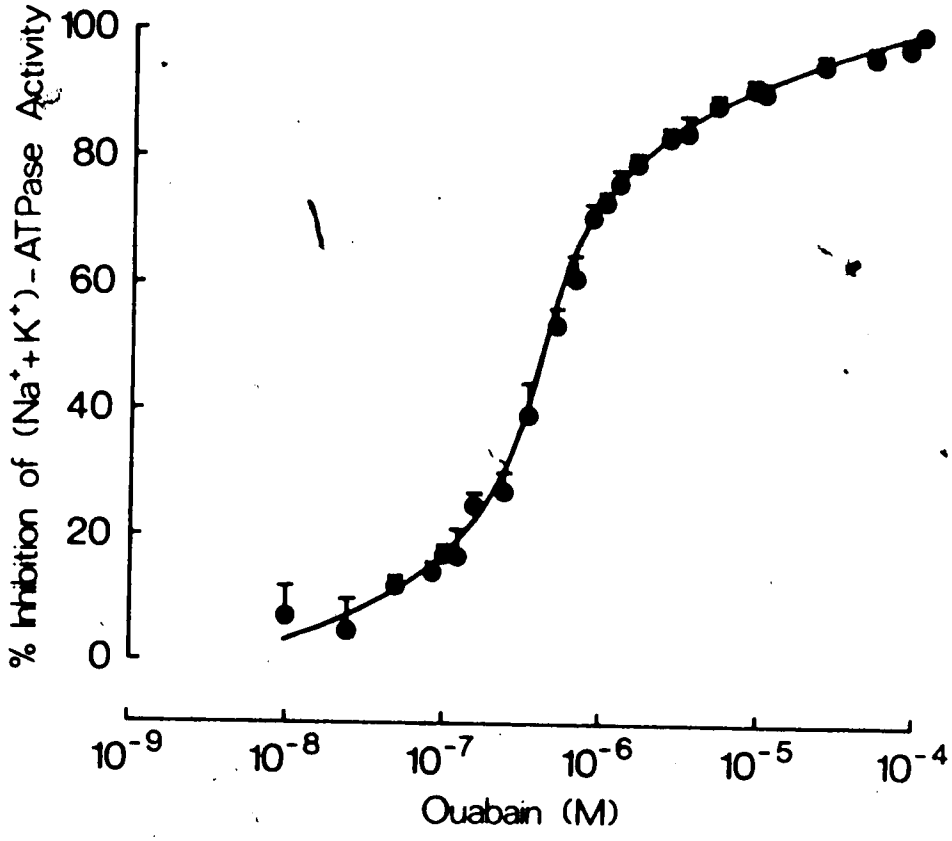


Fig. 17. Dose-response curve of ouabain inhibition of $(Na^+ + K^+)$-ATPase from beef brain, reconstituted into egg phosphatidylcholine liposomes. Enzyme activity was determined at 37°C in the presence of 2 mM Mg^{++}, 20 mM K^+, 80 mM Na^+ plus variable concentrations of ouabain as indicated. 100% inhibition of $(Na^+ + K^+)$-ATPase occurred when the ouabain inhibited enzyme activity was similar to that basal activity which was observed in the absence of 80 mM Na^+. The points represent Mean ± SEM of 3 determinations. The value for one-half maximal inhibition (ID_{50}) was calculated by computer regression analysis and is $5.06 \pm 0.24 \times 10^{-7}$M.

$(Na^+ + K^+)$-ATPase activity was present in the reconstituted preparation. It can be seen from Fig. 18 that the interaction of ouabain with beef brain $(Na^+ + K^+)$-ATPase reconstituted into crab lipid liposomes was markedly different to that observed with the enzyme preparation reconstituted with lipids extracted from beef brain $(Na^+ + K^+)$-ATPase. There was now a significant increase ($P < 0.01$) in the mean ID_{50} value to ouabain inhibition of the reconstituted enzyme ($1.42 \pm 0.19 \times 10^{-6}$M) as compared to the mean ID_{50} value from the respective control ($4.05 \pm 0.37 \times 10^{-7}$M; Fig. 15). Moreover, the maximal inhibition of enzyme activity of reconstituted preparation was observed at a much higher concentration of ouabain (1×10^{-3}M) when compared to the control preparation (1×10^{-4}M). Thus, the shape of the dose-response curve had changed from a steep curve, in the control experiments (Fig. 15) to a more shallow curve (Fig. 18). Similar but even more pronounced effects were observed when the endogenous lipids of beef brain $(Na^+ + K^+)$-ATPase were serially replaced with lipids extracted from crab nerve $(Na^+ + K^+)$-ATPase. Table 9 and Fig. 19 show the results of this latter experiment. The reconstituted preparation now retained only 24% of the initial total ATPase activity. The $(Na^+ + K^+)$-ATPase had decreased from 138.2 (before reconstitution) to 36.3 (reconstituted enzyme) µmol ATP hydrolyzed/mg protein/hr which represented a 74% loss of enzyme activity. The Mg^{++}-ATPase showed a 88% loss of activity. The mean dose-response curve to ouabain inhibition of this preparation is shown in Fig. 19. It is clear that the mean ID_{50} value of the serially substituted preparation ($2.62 \pm 0.21 \times 10^{-6}$M) is markedly different ($P < 0.01$) from that obtained from the respective control experiment ($2.65 \pm 0.05 \times 10^{-7}$M; Fig. 16). There is about one

TABLE 8

INCORPORATION OF BEEF BRAIN ($Na^+ + K^+$)-ATPase INTO CRAB LIPID LIPOSOMES: EFFECT ON SPECIFIC ACTIVITY*

	Specific activity**			
	Total ATPase	Mg^{++} ATPase	O.I. ATPase†	% ATPase sensitive to ouabain
Enzyme (before reconstitution)	149.0 ± 16.7	17.4 ± 3.4	131.6 ± 14.9	88 ± 2.1
Reconstituted enzyme (Beef-E/Crab lipid)	52.1 ± 1.1	3.5 ± 0.7	47.9 ± 1.1	93 ± 1.2

* Results are based on four separate experiments. Values given are Mean ± SEM.

** µmol ATP hydrolyzed/mg protein/hr at 37°C.

† Ouabain inhibitable ATPase.

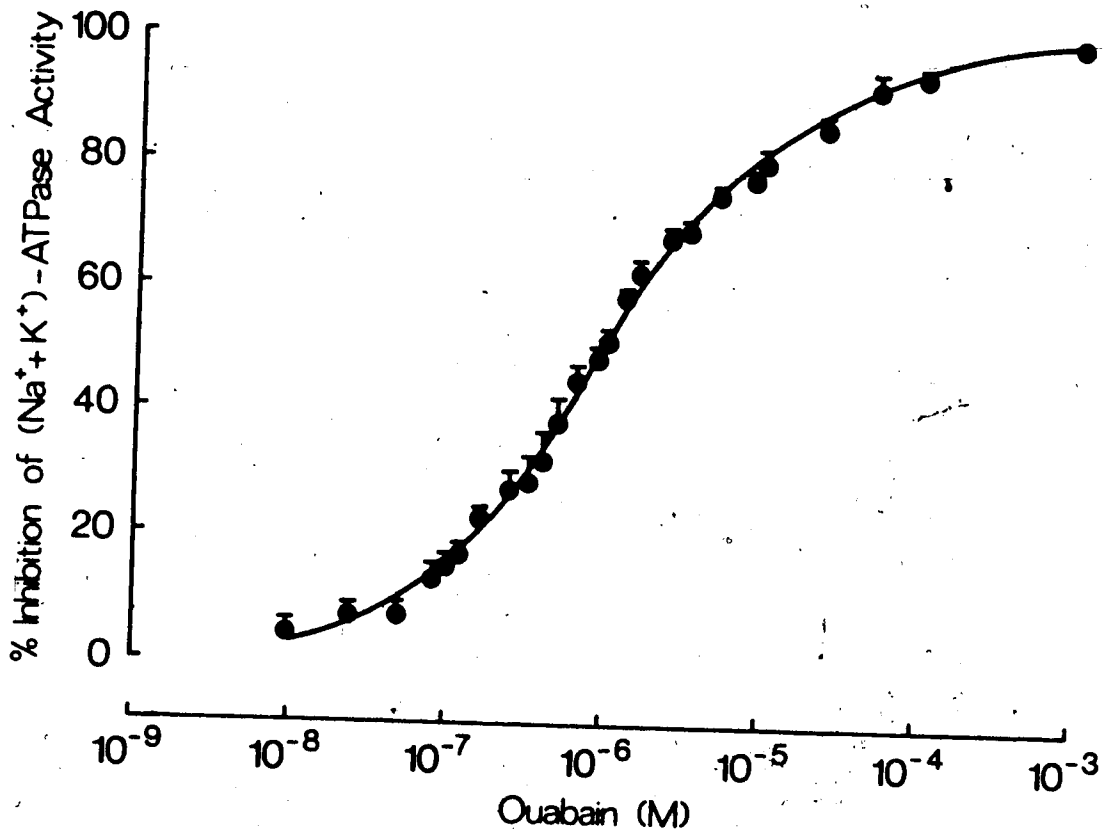


Fig. 18. Dose-response curve of ouabain inhibition of $(Na^+ + K^+)$-ATPase from beef brain reconstituted into crab lipid liposomes. Enzyme activity was determined at 37°C in the presence of 2 mM Mg^{++}, 20 mM K^+, 80 mM Na^+ plus variable concentrations of ouabain as indicated. 100% inhibition of $(Na^+ + K^+)$-ATPase occurred when the ouabain inhibited enzyme activity was similar to that basal activity which was observed in the absence of 80 mM Na^+. The points represent Mean ± SEM of 4 determinations. The value for one-half maximal inhibition (ID_{50}) was calculated by computer regression analysis and is 1.42 ± 0.19 x 10^{-6}M.

TABLE 9

SERIAL SUBSTITUTION OF ENDOGENOUS LIPIDS OF BEEF BRAIN ($Na^+ + K^+$)-ATPase WITH CRAB LIPID LIPOSOMES: EFFECT ON SPECIFIC ACTIVITY*

	Specific activity**			
	Total ATPase	Mg^{++} ATPase	O.I. ATPase†	% ATPase sensitive to ouabain
Enzyme (before reconstitution)	158.8 ± 15.4	20.6 ± 2.6	138.2 ± 12.9	87 ± 1.2
Reconstituted enzyme (Beef-E/crab lipid serial subst.)	38.7 ± 5.1	2.4 ± 0.4	36.3 ± 4.9	93 ± 1.2

* Results are based on four separate experiments. Values given are mean ± SEM.

**µmol ATP hydrolyzed/mg protein/hr at 37°C.

† Ouabain inhibitable ATPase.

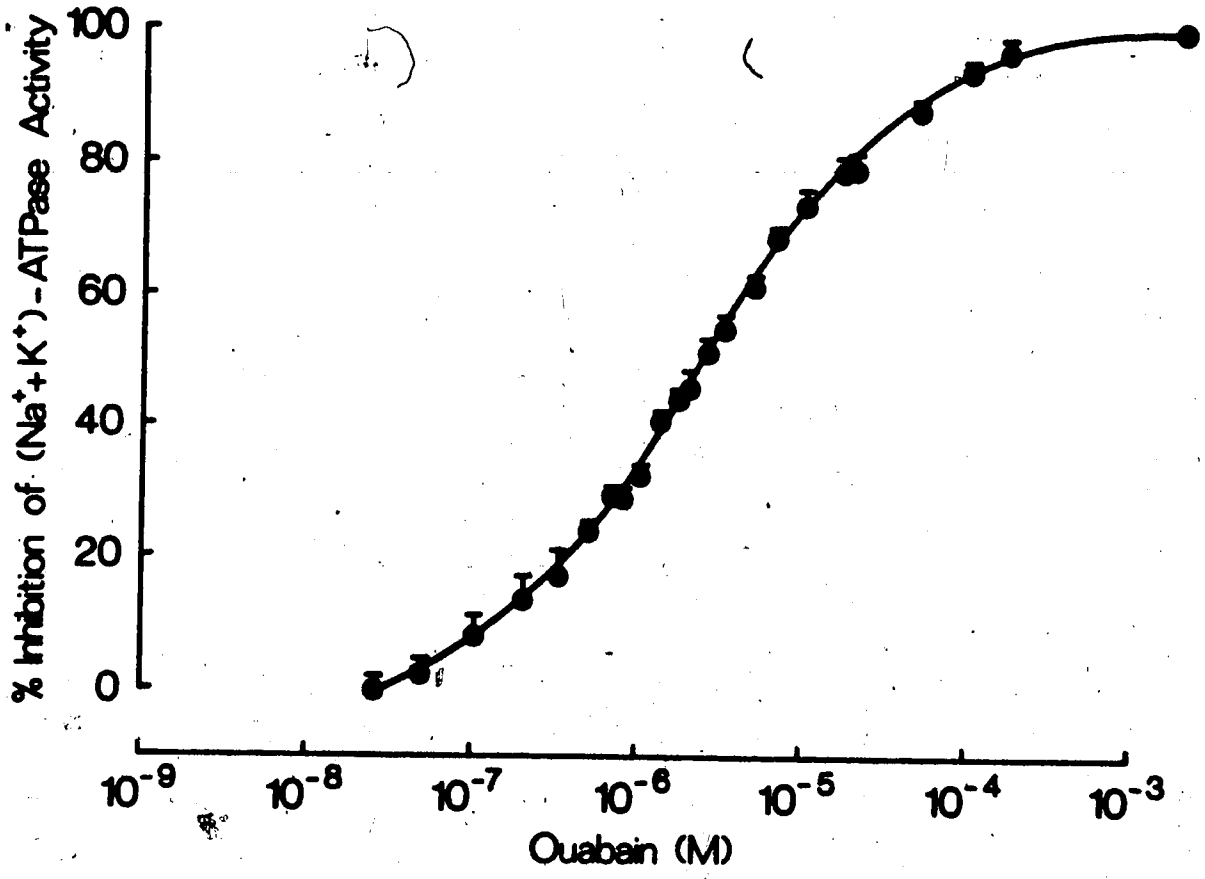


Fig. 19. Serial substitution of endogenous lipids of beef brain $(Na^+ + K^+)$-ATPase with crab lipid liposomes. Dose-response curve to inhibition by ouabain. Enzyme activity was determined at 37°C in the presence of 2 mM Mg^{++}, 20 mM K^+, 80 mM Na^+ plus variable concentrations of ouabain as indicated. 100% inhibition of $(Na^+ + K^+)$-ATPase occurred when the ouabain inhibited enzyme activity was similar to that basal activity which was observed in the absence of 80 mM Na^+. The points represent Mean ± SEM of 5 determinations. The value for one-half maximal inhibition (ID_{50}) was calculated by computer regression analysis and is $2.62 \pm 0.21 \times 10^{-6}$M.

Fig. 20. Electron micrograph of beef brain ($Na^+ + K^+$)-ATPase reconstituted into crab lipid liposomes. The endogenous lipids of beef brain enzyme were serially substituted with lipids extracted from crab nerve ($Na^+ + K^+$)-ATPase as described in Materials and Methods. Reconstituted preparation was negatively stained with 1% phosphotungstate and observed in a JEM-7A electron microscope. The bar indicates 100 nm. Reconstituted preparation exhibits a structure of unilamellar vesicles ranging between 50-100 nm in diameter.

log unit difference in the ID_{50} values, between these two preparations. Moreover, the maximal inhibition to ouabain was only achieved at 1 x 10^{-3}M concentration of drug compared to 1 x 10^{-4}M with the control preparations. It is also apparent that the shape of the dose-response curve had also changed from a steep curve (Fig. 16) to a shallow curve in the reconstituted preparation (Fig. 19). An electron micrograph of this reconstituted preparation is shown in Fig. 20. It can be seen from this Figure that reconstituted preparations had a structure of primarily unilamellar vesicles ranging between 50-100 nm in diameter. It should be noted that the morphological appearance of this preparation is quite similar to that of phosphatidylcholine reconstituted liposomes given in Fig. 14.

(2) Crab Nerve ($Na^+ + K^+$)-ATPase

The ($Na^+ + K^+$)-ATPase prepared from crab axons were reconstituted into liposomes of desired lipid composition as described in Materials and Methods. Firstly, to serve as a control, the enzyme was reconstituted into liposomes prepared from lipids extracted from crab nerve ($Na^+ + K^+$)-ATPase. The specific activity of crab nerve ($Na^+ + K^+$)-ATPase before and after reconstitution into lipid vesicles is shown in Table 10. From these results it is evident that reconstitution of crab nerve ATPase into crab liposomes resulted in a 33% loss of total ATPase activity. The reconstituted preparation retained about 74% of the initial ($Na^+ + K^+$)-ATPase activity. In contrast, the loss of Mg^{++}-ATPase activity was more dramatic, since it had decreased from 2.6 (native enzyme) to 0.6 (reconstituted enzyme) μmol ATP hydrolyzed/mg protein/hr. This decrease represented a 77% loss of activity of this enzyme.

TABLE 10

INCORPORATION OF CRAB NERVE ($Na^+ + K^+$)-ATPase INTO CRAB LIPID LIPOSOMES: EFFECT ON SPECIFIC ACTIVITY*

	Specific activity**			
	Total ATPase	Mg^{++} ATPase	O.I. ATPase†	% ATPase sensitive to ouabain
Enzyme (before reconstitution)	20.4 ± 4.1	2.6 ± 1.0	17.6 ± 2.6	87 ± 3.7
Reconstituted enzyme (Crab-E/crab lipid)	13.7 ± 0.8	0.6 ± 0.1	13.0 ± 0.8	95 ± 0.3

* Results are based on three separate experiments. Values given are Mean ± SEM.

** µmol ATP hydrolyzed/mg protein/hr at 37°C.

† Ouabain inhibitable ATPase.

Because the fall in Mg^{++}-ATPase activity was not paralleled by a similar decrease in ouabain sensitive $(Na^+ + K^+)$-ATPase, the resultant enzyme preparation had an increased sensitivity (%) to ouabain inhibition (95% for the reconstituted enzyme compared to 87% for the native preparation). Fig. 21 illustrates the mean dose-response curve to ouabain inhibition of this reconstituted preparation. The mean value for one-half maximal inhibition by ouabain was $1.78 \pm 0.21 \times 10^{-4}$M which is not different from that of the native enzyme ($1.51 \pm 0.09 \times 10^{-4}$M; Fig. 3). A similar result was obtained when crab nerve $(Na^+ + K^+)$-ATPase was reconstituted into phosphatidylcholine (egg) liposomes. The results of this latter experiment are summarized in Table 11 and Fig. 22. It is clear from the results shown in Table 11 that reconstituted preparations retained 98% of the initial total ATPase activity. The ouabain inhibitable ATPase activity of this enzyme preparation remained the same before and after reconstitution. The mean value obtained for one-half maximal inhibition to ouabain of reconstituted preparations was $1.57 \pm 0.30 \times 10^{-4}$M. This value did not differ significantly from that of the respective control ($1.78 \pm 0.21 \times 10^{-4}$M; Fig. 21).

The interaction of ouabain with crab nerve $(Na^+ + K^+)$-ATPase protein which was incorporated into a lipid bilayer composed of lipid extracted from beef brain $(Na^+ + K^+)$-ATPase was investigated. The ATPase from crab nerve was reconstituted into beef lipid liposomes as described in Materials and Methods. The specific activity of this preparation before and after reconstitution is shown in Table 12. It can be seen that reconstituted preparations retained 45% of the initial total ATPase activity. The specific activity of $(Na^+ + K^+)$-ATPase was decreased from

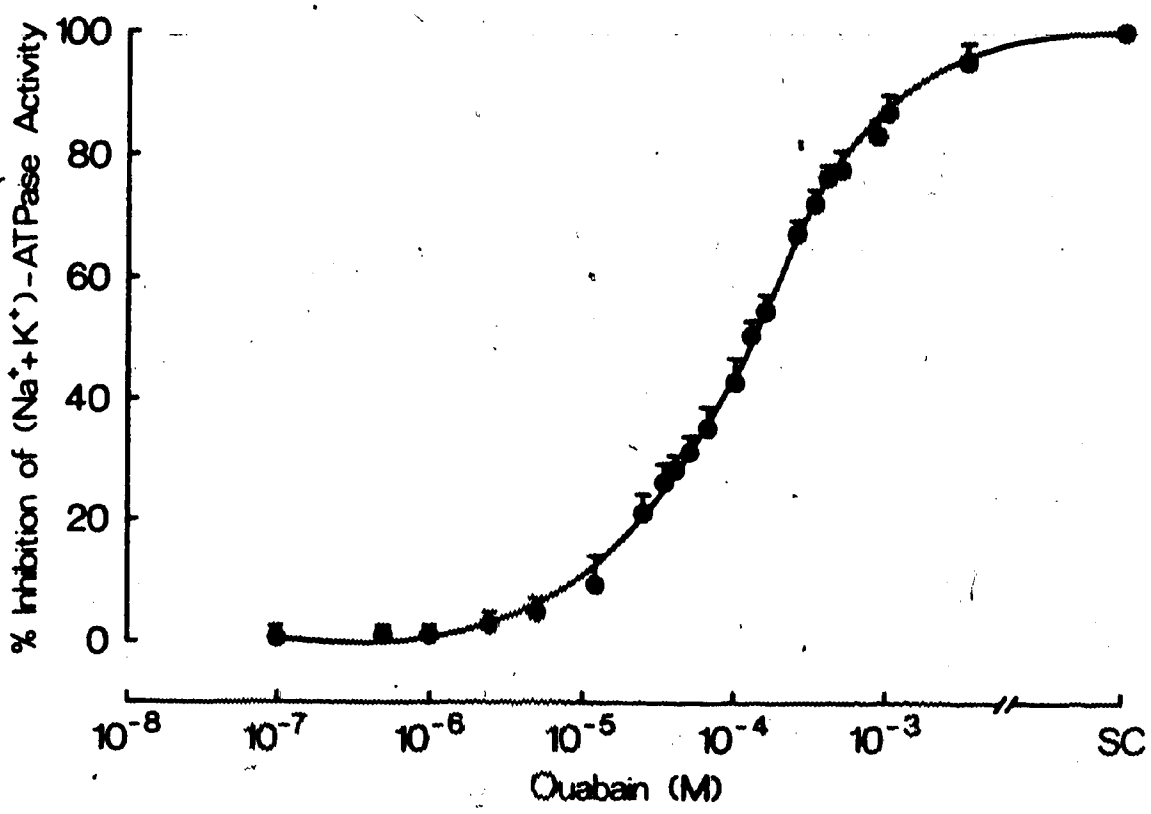


Fig. 21. Dose-response curve of ouabain inhibition of $(Na^+ + K^+)$-ATPase from crab nerve reconstituted into crab lipid liposomes. Enzyme activity was determined at 37°C in the presence of 2 mM Mg^{++}, 20 mM K^+, 80 mM Na^+ plus variable concentrations of ouabain as indicated. 100% inhibition of $(Na^+ + K^+)$-ATPase occurred when the ouabain inhibited enzyme activity was similar to that basal activity which was observed in the absence of 80 mM Na^+. SC indicates saturating concentration of ouabain. The points represent Mean ± SEM of 3 determinations. The value for one-half maximal inhibition (ID_{50}) was calculated by computer regression analysis and is $1.78 \pm 0.21 \times 10^{-4}$M.

TABLE 11

INCORPORATION OF CRAB NERVE ($Na^+ + K^+$)-ATPase INTO EGG PHOSPHATIDYLCHOLINE LIPOSOMES: EFFECT ON SPECIFIC ACTIVITY*

	Specific activity**			
	Total ATPase	Mg^{++} ATPase	O.I. ATPase†	% ATPase sensitive to ouabain
Enzyme (before reconstitution)	16.1 ± 0.8	1.8 ± 0.2	14.4 ± 0.8	90 ± 0.9
Reconstituted enzyme (crab-E/egg PC)	15.8 ± 3.2	1.5 ± 0.3	14.4 ± 2.8	90 ± 0.9

* Results are based on four separate experiments. Values given are Mean ± SEM.

** μmol ATP hydrolyzed/mg protein/hr at 37°C.

† Ouabain inhibitable ATPase.

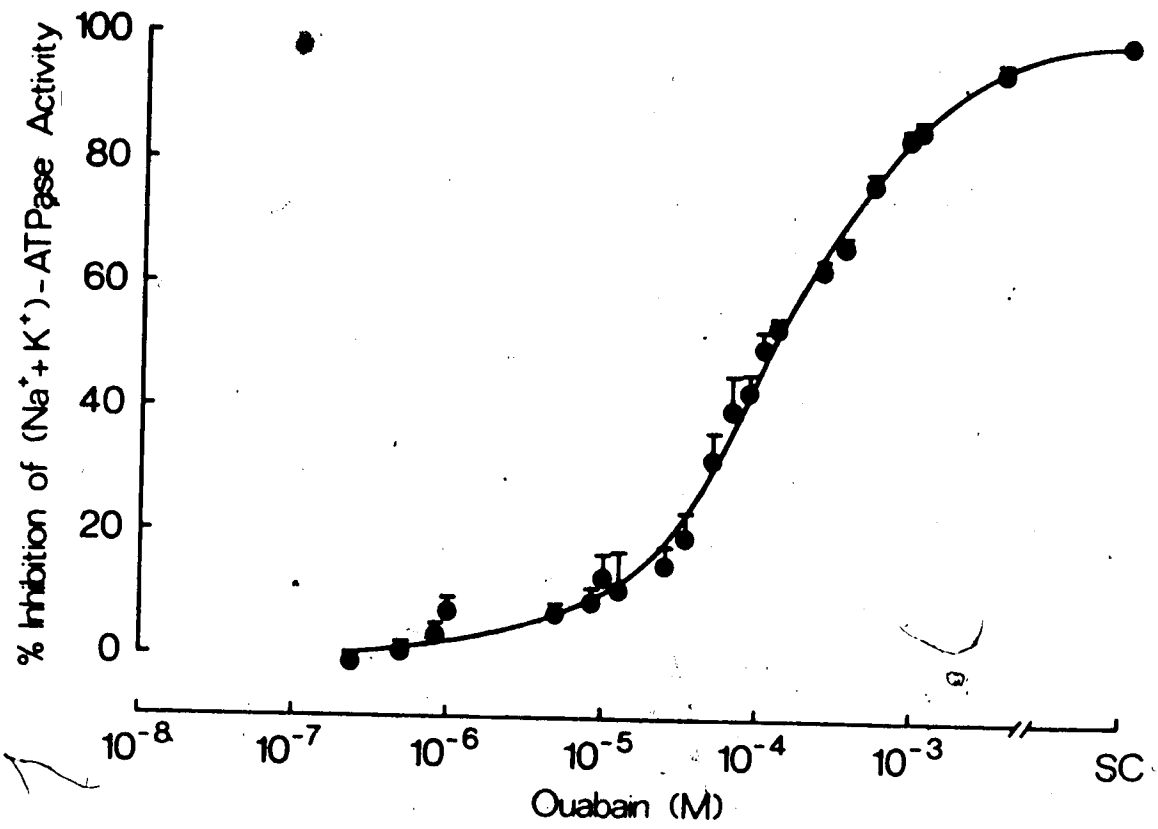


Fig. 22. Dose response curve of ouabain inhibition of $(Na^+ + K^+)$-ATPase from crab nerve reconstituted into egg phosphatidylcholine liposomes. Enzyme activity was determined at 37°C in the presence of 2 mM Mg^{++}, 20 mM K^+, 80 mM Na^+ plus variable concentrations of ouabain as indicated. 100% inhibition of $(Na^+ + K^+)$-ATPase occurred when the ouabain inhibited enzyme activity was similar to that basal activity which was observed in the absence of 80 mM Na^+. SC indicates saturating concentration of ouabain. The points represent Mean ± SEM of 4 separate determinations. The value for one-half maximal inhibition (ID_{50}) was calculated by computer regression analysis and is $1.57 \pm 0.30 \times 10^{-4}$M.

13.9 (native enzyme) to 5.9 (reconstituted enzyme) µmol ATP hydrolyzed/ mg protein/hr which represented a 58% loss of activity. Surprisingly, although the Mg^{++}-ATPase activity was reduced by 47% this was not accompanied by an increase in the % ouabain sensitivity of the preparation, as this parameter remained unchanged (86% and 89% in the reconstituted and native enzyme preparations respectively). Fig. 23 represents the mean dose-response curve to inhibition by ouabain of reconstituted preparations. When compared with the respective control (Fig. 21) it can be seen that the ouabain interaction properties of reconstituted preparations had altered. The mean ID_{50} value of the reconstituted enzyme ($5.40 \pm 0.87 \times 10^{-5}$M) was significantly lower than that of the respective control ($1.78 \pm 0.21 \times 10^{-4}$M; Fig. 21). In addition the maximal inhibition of reconstituted enzyme was observed at a much lower concentration of ouabain compared to the control preparation. It had decreased from the saturating concentration (about 2.0×10^{-2}M) to 3×10^{-3}M. This latter effect became more pronounced when an enzyme preparation from crab nerve was reconstituted into beef lipid liposomes by the serial substitution method. The experiment was performed as described in Materials and Methods. Table 13 shows the specific activity of this preparation before and after reconstitution into liposomes. It can be seen that the reconstituted preparation retained 38% of the initial ouabain inhibitable ATPase activity. The Mg^{++}-ATPase activity of the preparation was decreased by 46% from 2.6 (native enzyme) to 1.4 (reconstituted enzyme) µmol ATP hydrolyzed/mg protein/hr. However this decrease in Mg^{++}-ATPase was not accompanied by an increase in the % ATPase sensitive to inhibition by ouabain; it was in fact decreased from 88%

TABLE 12

INCORPORATION OF CRAB NERVE ($Na^+ + K^+$)-ATPase INTO BEEF LIPID LIPOSOMES: EFFECT ON SPECIFIC ACTIVITY*

	Specific activity**			
	Total ATPase	Mg^{++} ATPase	O.I. ATPase†	% ATPase sensitive to ouabain
Enzyme (before reconstitution)	15.4 ± 0.7	1.7 ± 0.1	13.9 ± 0.9	89 ± 1.4
Reconstituted enzyme (crab-E/beef lipid)	6.9 ± 0.8	0.9 ± 0.04	5.9 ± 0.8	86 ± 1.2

* Results are based on four separate experiments. Values given are Mean ± SEM.

** µmol ATP hydrolyzed/mg protein/hr at 37°C.

† Ouabain inhibitable ATPase.

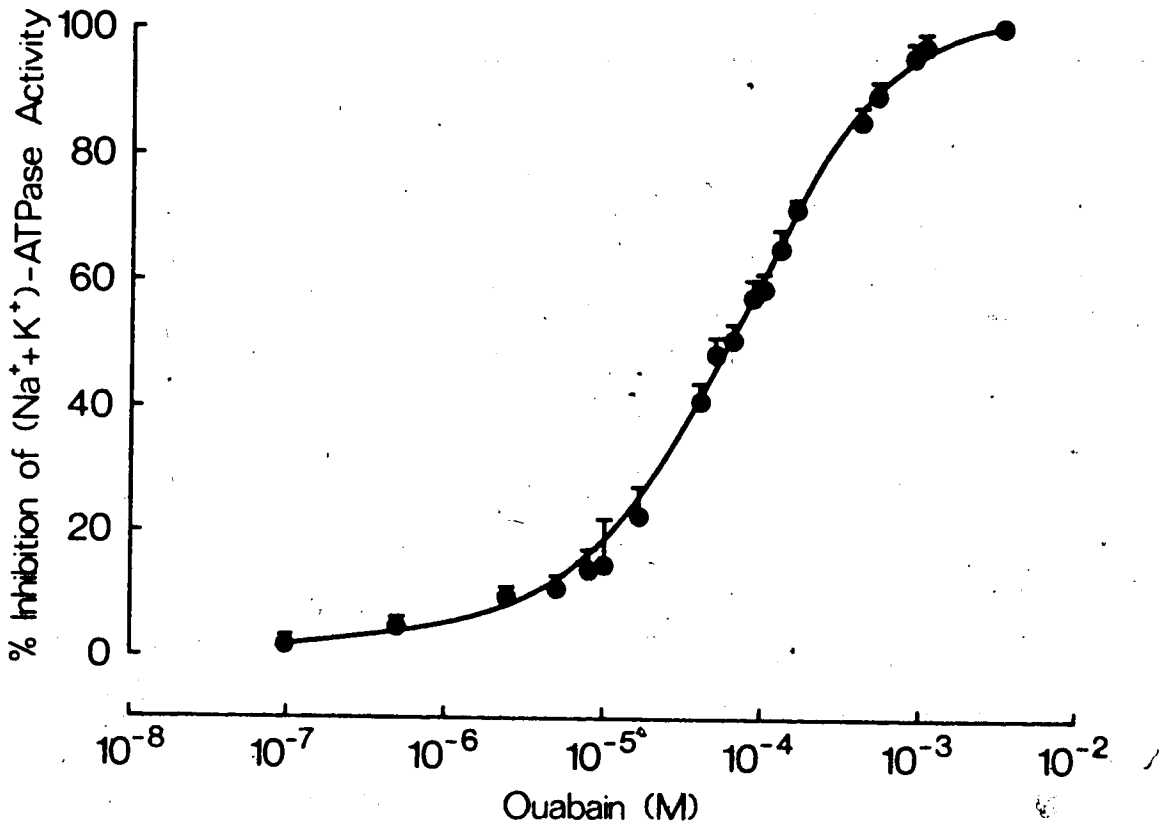


Fig. 23. Dose-response curve of ouabain inhibtion of $(Na^+ + K^+)$-ATPase from crab nerve, reconstituted into beef lipid liposomes. Enzyme activity was determined at 37°C in the presence of 2 mM Mg^{++}, 20 mM K^+, 80 mM Na^+ plus variable concentrations of ouabain as indicated. 100% inhibition of $(Na^+ + K^+)$-ATPase occurred when the ouabain inhibited enzyme activity was similar to that basal activity which was observed in the absence of 80 mM Na^+. The points represent Mean ± SEM of 5 determinations. The value for one-half maximal inhibition (ID_{50}) was calculated by computer regression analysis and is $5.40 \pm 0.87 \times 10^{-5}$M.

(native enzyme) to 81% (reconstituted enzyme). The mean dose-response curve to inhibition by ouabain of the reconstituted preparations is shown in Fig. 24. The mean value obtained for one-half maximal inhibition by ouabain was $5.14 \pm 0.33 \times 10^{-5}$M which is significantly lower ($P < 0.01$) than that of the control preparations (Fig. 3 and Fig. 21). However, the maximal inhibition of $(Na^+ + K^+)$-ATPase activity of the reconstituted preparations was observed at a much lower concentration of ouabain (1×10^{-3}M) compared to the control preparations which required saturating concentrations of ouabain (2.0×10^{-2}M) for complete inhibition. It is also evident that the shape of the dose-response curve of the reconstituted preparations was different to those of control preparations. It had changed from a shallow curve to a more steep curve. The morphological integrity of reconstituted preparations was studied by electron microscopy and is illustrated in Fig. 25. It is clear that this preparation was quite similar to those for phosphatidylcholine liposomes (Fig. 14) and for beef brain $(Na^+ + K^+)$-ATPase reconstituted in crab lipid liposomes (Fig. 20), in that it had a structure of primarily unilamellar vesicles ranging between 50-100 nm in diameter.

(3) Summary of Effects of Ouabain

The results obtained with the reconstituted enzyme preparations are summarized in Tables 14 and 15. Table 14 shows the effect of membrane lipid composition on the interaction of ouabain with beef brain $(Na^+ + K^+)$-ATPase. It can be seen that reincorporation of beef enzyme into crab lipid liposomes resulted in a decreased sensitivity of the reconstituted enzyme to inhibition by ouabain compared to the control prepara-

TABLE 13

SERIAL SUBSTITUTION OF ENDOGENOUS LIPIDS OF CRAB NERVE ($Na^+ + K^+$)-ATPase WITH BEEF LIPID LIPOSOMES: EFFECT ON SPECIFIC ACTIVITY*

	Specific activity**			
	Total ATPase	Mg^{++} ATPase	O.I. ATPase†	% ATPase sensitive to ouabain
Enzyme (before reconstitution)	18.7 ± 3.3	2.6 ± 1.2	16.2 ± 2.2	88 ± 2.8
Reconstituted enzyme (Crab-E/beef lipid-serial subst.)	7.9 ± 1.8	1.4 ± 0.3	6.2 ± 1.4	81 ± 3.7

* Results are based on four separate experiments. Values given are Mean ± SEM.

** µmol ATP hydrolyzed/mg protein/hr at 37°C.

† Ouabain inhibitable ATPase.

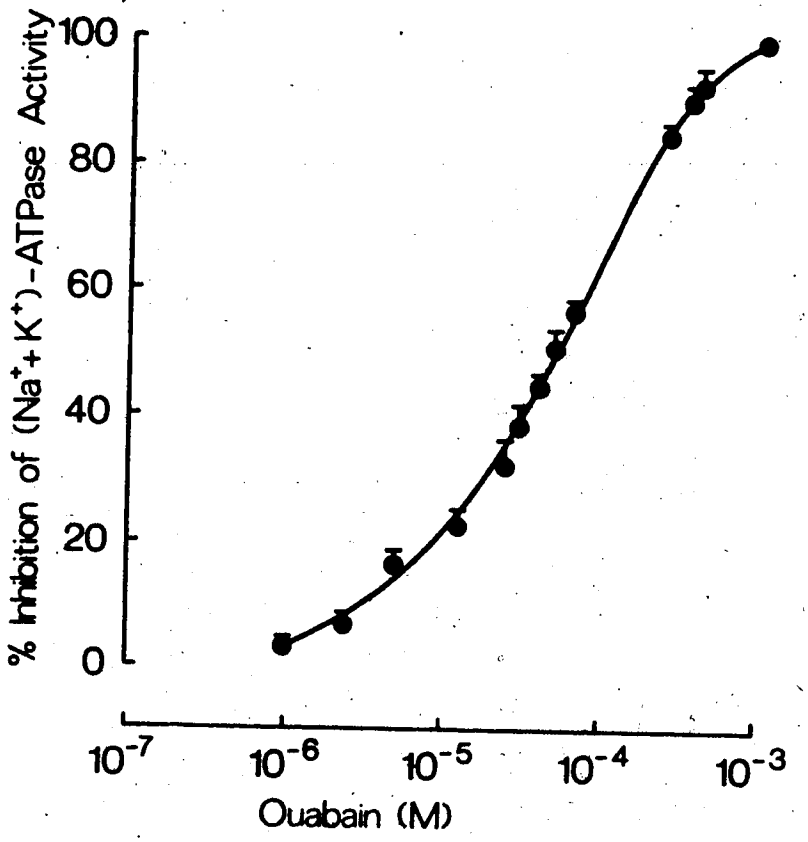


Fig. 24. Serial substitution of endogenous lipids of crab nerve $(Na^+ + K^+)$-ATPase with beef lipid liposomes. Dose response curve to inhibition by ouabain. Enzyme activity was determined at 37°C in the presence of 2 mM Mg^{++}, 20 mM K^+, 80 mM Na^+ plus variable concentrations of ouabain as indicated. 100% inhibition of $(Na^+ + K^+)$-ATPase occurred when the ouabain inhibited enzyme activity was similar to that basal activity which was observed in the absence of 80 mM Na^+. The points represent Mean ± SEM of 4 determinations. The value for one-half maximal inhibition (ID_{50}) was calculated by computer regression analysis and is $5.14 \pm 0.33 \times 10^{-5}$M.

Fig. 25. Electron micrograph of crab nerve ($Na^+ + K^+$)-ATPase reconstituted into beef lipid liposomes. The endogenous lipids of crab nerve enzyme were serially substituted with lipid extracted from beef brain ($Na^+ + K^+$)-ATPase as described in Materials and Methods. Reconstituted preparation was negatively stained with 1% phosphotungstate and observed in a JEM-7A electron microscope. The bar indicates 100 nm. Reconstituted preparation exhibits a structure of unilamellar vesicles, ranging between 50-100 nm in diameter.

tions. This was evident by the significantly increased ($P < 0.01$) mean ID_{50} value to ouabain inhibition. This effect became more pronounced when the beef enzyme was reconstituted into crab lipid liposomes by the serial substitution method. On the other hand incorporation of beef brain enzyme into egg PC liposomes did not result in an alteration in the ouabain interaction properties of the native enzyme.

Table 15 summarizes the results obtained with reconstituted preparations of $(Na^+ + K^+)$-ATPase from crab nerve. It is clear that the $(Na^+ + K^+)$-ATPase protein had maintained its native characteristics to ouabain inhibition after reconstitution into egg phosphatidylcholine liposomes. However, when crab nerve enzyme was introduced into a beef lipid environment, the ouabain inhibitory properties had changed, in that the reconstituted preparations displayed a significantly lower ($P < 0.01$) mean ID_{50} value to inhibition by ouabain compared to the control preparations. However, this effect did not become more pronounced when the endogenous lipids of crab nerve enzyme were serially substituted with beef lipid liposomes prepared from lipid extracted from the beef brain $(Na^+ + K^+)$-ATPase.

TABLE 14

EFFECT OF LIPID COMPOSITION OF MEMBRANE ON THE INHIBITION OF BEEF BRAIN $(Na^+ + K^+)$-ATPase BY OUABAIN: SUMMARY OF RESULTS

Enzyme preparation	n	ID_{50}-ouabain (M)		
A. Beef-Enz.	4	$3.06 \pm 0.44 \times 10^{-7}$	-	(Fig. 2)
B. Beef-E/beef lipid	5	$4.05 \pm 0.37 \times 10^{-7}$	a	(Fig.15)
C. Beef-E/beef lipid (serial subst.)	3	$2.65 \pm 0.05 \times 10^{-7}$	b	(Fig.16)
D. Beef-E/egg PC	3	$5.06 \pm 0.24 \times 10^{-7}$	c	(Fig.17)
E. Beef-E/crab lipid	4	$1.42 \pm 0.19 \times 10^{-6}$	d	(Fig.18)
F. Beef-E/crab lipid (serial subst.)	5	$2.62 \pm 0.21 \times 10^{-6}$	e	(Fig.19)

a. Not significantly different from A.

b. Not significantly different from either A or B.

c. Not significantly different from respective control (B).

d. Significantly different ($P < 0.01$) from respective control (B).

e. Significantly different ($P < 0.01$) from respective control (C) and from E.

TABLE 15

EFFECT OF LIPID COMPOSITION OF MEMBRANE ON THE INHIBITION OF CRAB NERVE $(Na^+ + K^+)$-ATPase BY OUABAIN: SUMMARY OF RESULTS

Enzyme preparation	n	ID_{50}-ouabain (M)		
A. Crab Enz.	4	$1.51 \pm 0.09 \times 10^{-4}$	-	(Fig. 3)
B. Crab-E/crab lipid	3	$1.78 \pm 0.21 \times 10^{-4}$	a	(Fig.21)
C. Crab-E/egg PC	4	$1.57 \pm 0.30 \times 10^{-4}$	b	(Fig.22)
D. Crab-E/beef lipid	5	$5.40 \pm 0.87 \times 10^{-5}$	c	(Fig.23)
E. Crab-E/beef lipid (serial subst.)	4	$5.14 \pm 0.33 \times 10^{-5}$	d	(Fig.24)

a. Not significantly different from A.

b. Not significantly different from respective control (B).

c. Significantly different ($P < 0.01$) from respective control (B).

d. Significantly different ($P < 0.01$) from B, but not significantly different from D.

F. Effect of Lipid Fluidity on Ouabain Sensitivity of $(Na^+ + K^+)$-ATPase

Lipids from biological membranes undergo a thermotropic phase transition from a liquid crystalline state to a gel state as the temperature is lowered through the transition region. The transition arises from a cooperative freezing of the hydrocarbon chains located in the interior of the bilayer (Chapman, 1975). At temperatures above the phase transition temperature (T_c), membrane lipids are considered to be predominantly in a fluid, liquid crystalline phase. At temperatures below the T_c, membrane lipids are thought to be in a solid or gel phase. Within the phase transition lipids are in a mixed phase (Shimshick and McConnell, 1973; Chapman, 1975). The phase properties of biological membranes can be modified by several ways. For instance, an increase in the average length of the fatty acyl chains or a decrease in the ratio of unsaturated/saturated fatty acid moieties would raise the transition temperature (Steim *et al.*, 1969; Chapman, 1975). In addition, alteration of the cholesterol content of cell membranes will modify the molecular packing of the phospholipid acyl chains (Papahadjopoulos *et al.*, 1973; Papahadjopoulos, 1976). Moreover, changes in the distribution of polar head groups of the negatively charged phospholipids of biomembranes, or allowing these groups to interact with divalent cations which tend to stabilize the bilayers could also markedly alter the phase transition temperatures (Kimelberg and Papahadjopoulos, 1971; Trauble and Eibl, 1974; Verkleij *et al.*, 1974; Jacobson and Papahadjopoulos, 1975; Sandermann, 1978).

The degree of membrane fluidity could give rise to a number of important effects. A consequence of the phase transition is that the rate

of rotational and/or translational diffusions of protein molecules in the membrane increase by several orders of magnitude on going from the gel to liquid-crystalline state (Poo and Cone, 1974; Papahadjopoulos, 1976; Kuo and Wade, 1979). More recent studies indicate that alteration of lipid fluidity also modulates the availability of protein substituents at the membrane surfaces (Borochov and Shinitzky, 1976; Shinitzky and Rivnay, 1977; Borochov *et al.*, 1979). These temperature induced changes in the lipid structure alter the kinetics of membrane bound enzymes and thus can affect various physiological functions (McMurchie *et al.*, 1973). As discussed in the Introduction of this thesis, there is now much evidence to indicate that the physical properties of membrane lipids are vitally important for the proper function of $(Na^+ + K^+)$-ATPase enzyme system. Optimum enzyme activity was observed when the membrane lipids were in a fluid state. This, is readily demonstrated by the presence of breaks or discontinuities in Arrhenius plots of enzyme activity that correlate with lipid phase separations (Kimelberg and Papahadjopoulos, 1974; Papahadjopoulos, 1976; Palatini *et al.*, 1977).

From the results presented so far it is clear that the inhibition of membrane bound $(Na^+ + K^+)$-ATPase by ouabain is strongly modulated by the membrane lipid composition. The information discussed above indicates that changes in membrane lipid fluidity alters the function of $(Na^+ + K^+)$-ATPase enzyme system. Thus, it seems possible that interaction of specific inhibitors (such as ouabain) with membrane bound $(Na^+ + K^+)$-ATPase is influenced by the physical state of membrane lipids. In an attempt to explain the lipid modulation of ouabain sensitivity of $(Na^+ + K^+)$-ATPase, the effect of changes in the fludity of the membrane lipid

phase (in relation to the thermal transition) on the ouabain inhibition of beef brain ($Na^+ + K^+$)-ATPase was investigated. This was accomplished by employing a series of synthetic phosphatidylcholines of defined fatty acid composition.

The lipids studied include, dioleoyl ($18:1_{cis}/18:1_{cis}$)-phosphatidylcholine (DOPC)—T_c, -22°C (Phillips *et al.*, 1969; Ladbrooke and Chapman, 1969; de Kruyff *et al.*, 1973; Chapman, 1975); 1-palmito,2-oleoyl ($16:0/18:1_{cis}$)-phosphatidylcholine—T_c, -5°C (Ververgaert *et al.*, 1973; de Kruyff *et al.*, 1973; Seelig and Waespe-Sarcevic, 1978); dimyristoyl (14:0/14:0)-phosphatidylcholine (DMPC)—T_c, +23°C (Chapman *et al.*, 1967; Ladbrooke and Chapman, 1969; Phillips *et al.*, 1969; Chapman, 1975); dipalmitoyl (16:0/16:0)-phosphatidylcholine (DPPC)—T_c, +41°C (Ladbrooke and Chapman, 1969; Ververgaert *et al.*, 1973; Chapman, 1975) and distearoyl (18:0/18:0)-phosphatidylcholine (DSPC)—T_c, +58°C (Chapman *et al.*, 1967; Ladbrooke and Chapman, 1969; Phillips *et al.*, 1969; Phillips *et al.*, 1972; Ververgaert *et al.*, 1973; Chapman *et al.*, 1975).

(1) Beef Brain ($Na^+ + K^+$)-ATPase/Dimyristoyl-PC

The ($Na^+ + K^+$)-ATPase from beef brain was reconstituted into DMPC liposomes and assayed for activity as described in Materials and Methods. The specific activity of ($Na^+ + K^+$)-ATPase before and after reconstitution into DMPC vesicles is shown in Table 16. It is clear from these results that the reconstituted preparation retained about 73% of the initial Total ATPase activity. Similarly, there was about a 32% loss of ($Na^+ + K^+$)-ATPase activity, when enzyme was introduced into DMPC liposomes. In contrast, the Mg^{++}-ATPase activity of the preparation had

TABLE 16

INCORPORATION OF BEEF BRAIN ($Na^+ + K^+$)-ATPase INTO DIMYRISTOYL PHOSPHATIDYLCHOLINE (DMPC) LIPOSOMES: EFFECT ON SPECIFIC ACTIVITY*

	Specific activity**			
	Total ATPase	Mg^{++} ATPase	O.I. ATPase†	% ATPase sensitive to ouabain
Enzyme (before reconstitution)	194.7 ± 8.5	27.1 ± 1.8	167.6 ± 8.2	86 ± 1.1
Reconstituted enzyme (Beef-E/DMPC)	142.5 ± 3.7	28.6 ± 2.4	113.8 ± 4.2	80 ± 1.6

* Results are based on four separate experiments. Values given are Mean ± SEM.

** µmol ATP hydrolyzed/mg protein/hr at 37°C.

† Ouabain inhibitable ATPase.

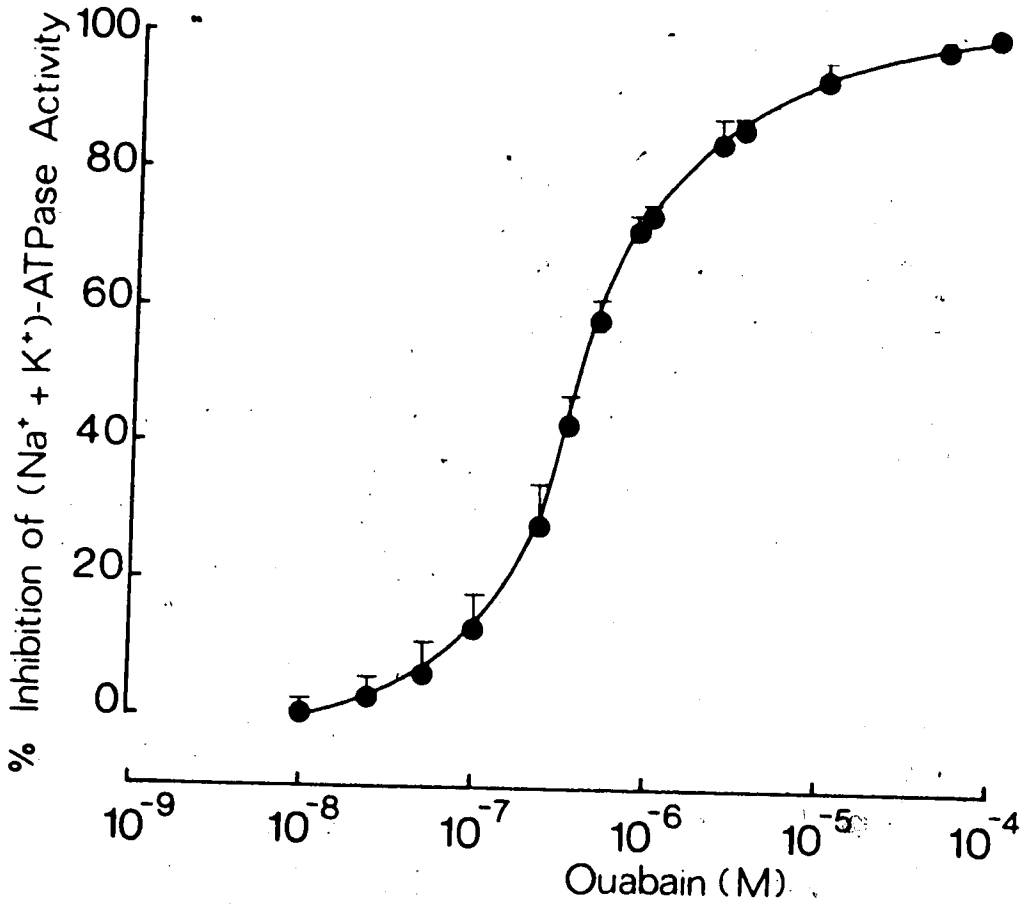


Fig. 26 Dose-response curve of ouabain inhibition of $(Na^+ + K^+)$-ATPase from beef brain reconstituted into dimyristoyl phosphatidylcholine (DMPC) liposomes. Enzyme activity was determined at 37°C in the presence of 2 mM Mg^{++}, 20 mM K^+, 80 mM Na^+ plus variable concentrations of ouabain as indicated. 100% inhibition of $(Na^+ + K^+)$-ATPase occurred when the ouabain inhibited enzyme activity was similar to that basal activity which was observed in the absence of 80 mM Na^+. The points represent Mean ± SEM of 4 determinations. The value for one-half maximal inhibition (ID_{50}) was calculated by computer regression analysis and is $4.03 \pm 0.45 \times 10^{-7}$M.

remained unchanged before (27.1 μmol ATP hydrolyzed/mg protein/hr) and after (28.6 μmol ATP hydrolyzed/mg protein/hr) reconstitution. The % ouabain sensitivity of the preparation was slightly decreased from 86% (before reconstitution) to 80% (after reconstitution). The cardiac glycoside receptor characteristics of reconstituted enzyme preparations were studied by constructing dose-response curves to inhibition by ouabain. The construction of dose-response curves and the calculation of the mean ID_{50} value for ouabain was carried out as described in Materials and Methods. The mean dose-response curve to inhibition by ouabain of beef brain $(Na^+ + K^+)$-ATPase reconstituted into DMPC liposomes is shown in Fig. 26. Here, the % inhibition of $(Na^+ + K^+)$-ATPase activity was plotted against the log concentration of ouabain. The mean ID_{50} value ($4.03 \pm 0.45 \times 10^{-7}$M) to inhibition by ouabain of this reconstituted preparation did not differ significantly from the mean ID_{50} value obtained with the respective control — Beef-Enzyme/beef-lipid ($4.05 \pm 0.37 \times 10^{-7}$M; Fig. 15).

(2) Beef Brain $(Na^+ + K^+)$-ATPase/1-palmito,2-oleoyl-PC

Table 17 shows the specific activity of $(Na^+ + K^+)$-ATPase preparation before and after reconstitution into 1-palmito,2-oleoyl-PC vesicles. The reconstitution of ATPase protein into liposomes was achieved by gel filtration technique as described in Materials and Methods. It can be seen that reconstitution of $(Na^+ + K^+)$-ATPase into this mixed-lecithin system, had resulted in a 32% loss of initial total ATPase activity. The $(Na^+ + K^+)$-ATPase activity was also decreased from 129.1 (before reconstitution) to 84:9 (reconstituted enzyme) μmol ATP hydrolyzed/mg protein/hr.

TABLE 17

INCORPORATION OF BEEF BRAIN ($Na^+ + K^+$)-ATPase INTO 1-PALMITO,2-OLEOYL PHOSPHATIDYLCHOLINE LIPOSOMES: EFFECT ON SPECIFIC ACTIVITY*

	Specific activity**			
	Total ATPase	Mg^{++} ATPase	O.I. ATPase†	% ATPase sensitive to ouabain
Enzyme (before reconstitution)	150.1 ± 20.1	20.9 ± 3.0	129.1 ± 17.1	86 ± 0.1
Reconstituted enzyme (Beef-E/1-palmito,2-oleoyl-PC)	102.7 ± 3.9	17.8 ± 0.6	84.9 ± 3.6	83 ± 0.7

* Results are based on four separate experiments. Values given are Mean ± SEM.

** µmol ATP hydrolyzed/mg protein/hr at 37°C.

† Ouabain inhibitable ATPase.

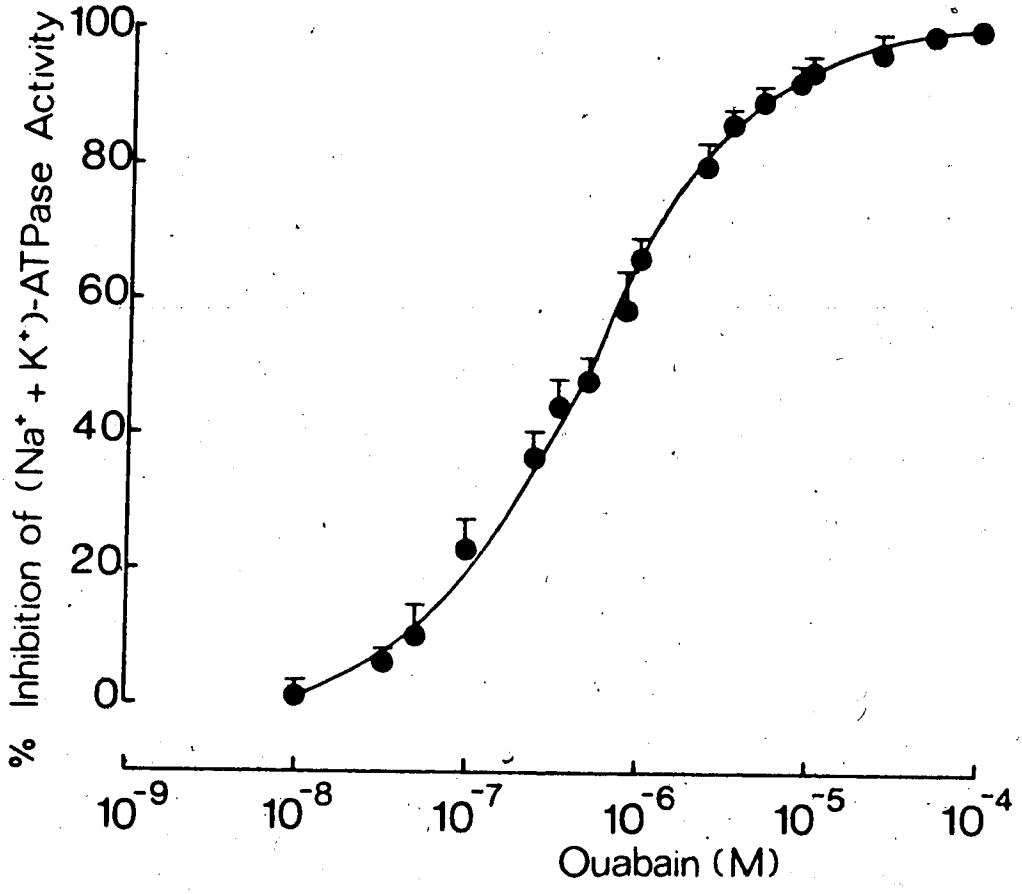


Fig. 27 Dose-response curve of ouabain inhibition of $(Na^+ + K^+)$-ATPase from beef brain reconstituted into 1-palmito, 2-oleoyl phosphatidylcholine liposomes. Enzyme activity was determined at 37°C in the presence of 2 mM Mg^{++}, 20 mM K^+, 80 mM Na^+ plus variable concentrations of ouabain as indicated. 100% inhibition of $(Na^+ + K^+)$-ATPase occurred when the ouabain inhibited enzyme activity was similar to that basal activity which was observed in the absence of 80 mM Na^+. The points represent Mean ± SEM of 4 determinations. The value for one-half maximal inhibition (ID_{50}) was calculated by computer regression analysis and is $5.12 \pm 0.55 \times 10^{-7}$M.

This decrease in activity represented about a 34% loss of the initial ouabain inhibitable ATPase activity which was comparable to that observed with the DMPC reconstituted system. The Mg^{++}-ATPase activity had decreased slightly from 20.9 (before reconstitution) to 17.8 (reconstituted enzyme) μmol ATP hydrolyzed/mg protein/hr, and there was no change in the % ATPase sensitive to inhibition by ouabain. Fig. 27 shows the mean dose-response curve to inhibition by ouabain of beef brain enzyme reconstituted into 1-palmito,2-oleoyl-PC liposomes. The mean ID_{50} value obtained for one-half maximal inhibition to ouabain of this reconstituted system was $5.12 \pm 0.55 \times 10^{-7}$M. This value did not differ significantly from that of the control preparation ($4.05 \pm 0.37 \times 10^{-7}$M; Fig. 15).

(3) Beef Brain ($Na^+ + K^+$)-ATPase/Dioleoyl-PC

The effect of increased bilayer fluidity on the interaction of ouabain with ($Na^+ + K^+$)-ATPase was studied by using dioleoyl phosphatidylcholine liposomes. Table 18 and Fig. 28 summarize the results obtained with an enzyme preparation that had been reconstituted into DOPC liposomes. From the results shown in Table 18 it can be seen that the reconstituted preparation had increased total ATPase activity compared to the native enzyme preparation. The total ATPase activity had increased from 144.9 (native enzyme) to 168.4 (reconstituted enzyme) μmol ATP hydrolyzed/mg protein/hr. This represented about a 16% increase of the initial total ATPase activity. Similarly the reconstituted preparation had an increased (about 14%) ($Na^+ + K^+$)-ATPase activity (123.0 and 140.5 μmol ATP hydrolyzed/mg protein/hr in the native and reconstituted enzyme preparations respectively). However, these increases in enzyme activity were

TABLE 18

INCORPORATION OF BEEF BRAIN ($Na^+ + K^+$)-ATPase INTO DIOLEOYL PHOSPHATIDYLCHOLINE (DOPC) LIPOSOMES: EFFECT ON SPECIFIC ACTIVITY*

	Specific activity**			
	Total ATPase	Mg^{++} ATPase	O.I. ATPase†	% ATPase sensitive to ouabain
Enzyme (before reconstitution)	144.9 ± 18.2	21.8 ± 1.9	123.0 ± 16.5	85 ± 0.8
Reconstituted enzyme (Beef-E/DOPC)	168.4 ± 13.6	23.8 ± 0.3	140.5 ± 13.6	86 ± 1.3

* Results are based on four separate experiments. Values given are Mean ± SEM.

** µmol ATP hydrolyzed/mg protein/hr at 37°C.

† Ouabain inhibitable ATPase.

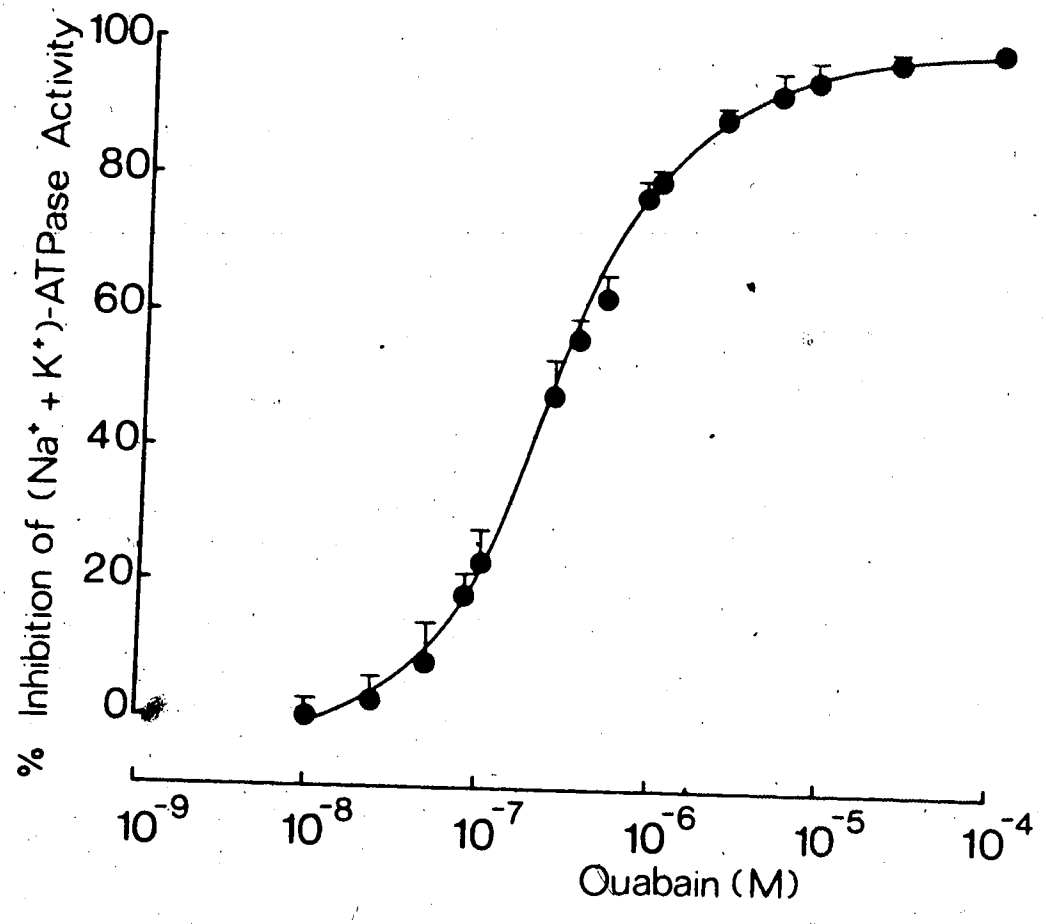


Fig. 28 Dose-response curve of ouabain inhibition of $(Na^+ + K^+)$-ATPase from beef brain reconstituted into dioleoyl phosphatidylcholine (DOPC) liposomes. Enzyme activity was determined at 37°C in the presence of 2 mM Mg^{++}, 20 mM K^+, 80 mM Na^+ plus variable concentrations of ouabain as indicated. 100% inhibition of $(Na^+ + K^+)$-ATPase occurred when the ouabain inhibited enzyme activity was similar to that basal activity which was observed in the absence of 80 mM Na^+. The points represent Mean ± SEM of 4 determinations. The value for one-half maximal inhibition (ID_{50}) was calculated by computer regression analysis and is $3.46 \pm 0.53 \times 10^{-7}$M.

not significant at the 1% level. The Mg^{++}-ATPase activity and the % ATPase sensitive to ouabain inhibition of the preparation was unchanged before and after reconstitution. Fig. 28 shows the mean dose-response curve to inhibition by ouabain of this DOPC reconstituted system. The mean value obtained for one-half maximal inhibition by ouabain was 3.46 ± 0.53 x 10^{-7}M, which was similar to the mean ID_{50} value of the control preparation (4.05 ± 0.37 x 10^{-7}M; Fig. 15).

(4) Beef Brain (Na^+ + K^+)-ATPase/Dipalmitoyl-PC

Table 19 and Fig. 29 summarize the results obtained with an enzyme preparation from beef brain that had been reconstituted into DPPC liposomes. The data given in Table 19 demonstrate that reconstituted preparation retained only 42% of the initial total ATPase activity. Similarly, the ouabain inhibitable ATPase activity was decreased from 152.8 (before reconstitution) to 54.2 (reconstituted enzyme), μmol ATP hydrolyzed/mg protein/hr which represented a 65% loss of enzyme activity. The loss of Mg^{++}-ATPase activity was not significant, as the reconstituted enzyme had 90% of the initial Mg^{++}-ATPase activity. Since the loss of (Na^+ + K^+)-ATPase activity was not paralleled by a similar reduction in the Mg^{++}-ATPase activity, the % ouabain sensitivity of the preparation had reduced from 88% (native enzyme) to 74% (reconstituted enzyme). The mean dose-response curve to ouabain inhibition of this DPPC reconstituted system is shown in Fig. 29. It can be seen that there was no significant difference between the mean ID_{50} values of control preparation (4.05 ± 0.37 x 10^{-7}M; Fig. 15) and DPPC reconstituted enzyme (6.45 ± 0.47 x 10^{-7}M; Fig. 29).

TABLE 19

INCORPORATION OF BEEF BRAIN ($Na^+ + K^+$)-ATPase INTO DIPALMITOYL PHOSPHATIDYLCHOLINE (DPPC) LIPOSOMES: EFFECT ON SPECIFIC ACTIVITY*

	Specific activity**			
	Total ATPase	Mg^{++} ATPase	O.I.† ATPase	% ATPase sensitive to ouabain
Enzyme (before reconstitution)	173.5 ± 7.4	20.6 ± 1.4	152.8 ± 7.8	88 ± 1.0
Reconstituted enzyme (Beef-E/DPPC)	72.7 ± 3.6	18.5 ± 1.3	54.2 ± 2.7	74 ± 1.2

* Results are based on three separate experiments. Values given are Mean ± SEM.

** µmol ATP hydrolyzed/mg protein/hr at 37°C.

† Ouabain inhibitable ATPase.

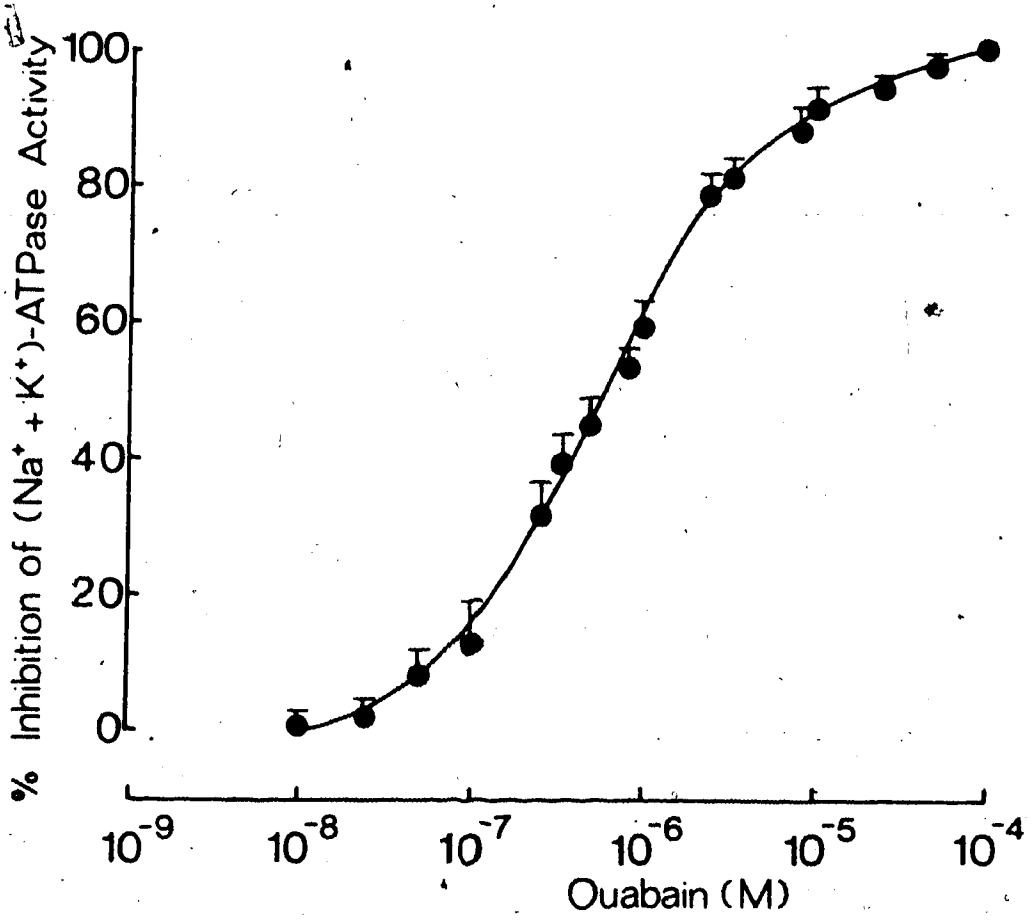


Fig. 29 Dose-response curve of ouabain inhibition of $(Na^+ + K^+)$-ATPase from beef brain, reconstituted into dipalmitoyl phosphatidylcholine (DPPC) liposomes. Enzyme activity was determined at 37°C in the presence of 2 mM Mg^{++}, 20 mM K^+, 80 mM Na^+ plus variable concentrations of ouabain as indicated. 100% inhibition of $(Na^+ + K^+)$-ATPase occurred when the ouabain inhibited enzyme activity was similar to that basal activity which was observed in the absence of 80 mM Na^+. The points represent Mean ± SEM of 3 determinations. The value for one-half maximal inhibition (ID_{50}) was calculated by computer regression analysis and is $6.45 \pm 0.47 \times 10^{-7}$M.

TABLE 20

SERIAL SUBSTITUTION OF ENDOGENOUS LIPIDS OF BEEF BRAIN ($Na^+ + K^+$)-ATPase WITH DIPALMITOYL PHOSPHATIDYLCHOLINE (DPPC) LIPOSOMES: EFFECT ON SPECIFIC ACTIVITY*

	Specific activity**			
	Total ATPase	Mg^{++} ATPase	O.I. ATPase†	% ATPase sensitive to ouabain
Enzyme (before reconstitution)	185.1 ± 17.9	24.3 ± 2.6	160.8 ± 16.1	87 ± 1.0
G-50 void volume after 1st substitution	73.4 ± 8.2	17.0 ± 2.3	58.4 ± 6.4	77 ± 2.0
G-50 void volume after 2nd substitution	34.1 ± 6.9	9.0 ± 1.5	25.1 ± 5.5	73 ± 1.4
G-50 void volume after 3rd substitution	13.1 ± 0.9	3.2 ± 0.2	9.9 ± 0.9	71 ± 1.0
4-B void volume (Reconstituted enzyme)	6.1 ± 0.5	2.6 ± 0.3	3.4 ± 0.5	51 ± 6.0

* Results are based on three separate experiments. Values given are Mean ± SEM.

** µmol ATP hydrolyzed/mg protein/hr at 37°C.

† Ouabain inhibitable ATPase.

The effect of serial substitution of the endogenous lipids of beef brain $(Na^+ + K^+)$-ATPase with DPPC liposome was also investigated. The preparation of serially substituted enzyme was carried out as described in Materials and Methods. The results of this experiment are summarized in Table 20. It is clear that this serially substituted preparation retained only 3% of the initial total ATPase activity. Similarly, the activity of $(Na^+ + K^+)$-ATPase was drastically reduced from 160.8 (native enzyme) to 3.4 (serially substituted enzyme) μmol ATP hydrolyzed/mg protein/hr which represented about a 98% loss of activity. The very low $(Na^+ + K^+)$-ATPase activity and decreased % ouabain sensitivity had ruled out the study of ouabain interaction properties (by constructing a mean dose-response curve) of this serially substituted preparation.

(5) Beef Brain $(Na^+ + K^+)$-ATPase/Distearoyl-PC

The effect of decreased bilayer fluidity on the ouabain inhibition of $(Na^+ + K^+)$-ATPase was investigated by using DSPC liposomes. The $(Na^+ + K^+)$-ATPase was reconstituted into DSPC liposomes as described in Materials and Methods. The specific activity of this ATPase preparation before and after reconstitution into DSPC lipid vesicles is shown in Table 21. From these results it is evident that the reconstituted preparation retained only 27% of the initial total ATPase activity. The $(Na^+ + K^+)$-ATPase activity had decreased from 160.1 (native enzyme) to 32.5 (reconstituted enzyme) μmol ATP hydrolyzed/mg protein/hr which represented an 80% loss of enzyme activity. In contrast, the reconstituted preparation retained about 82% of the initial Mg^{++}-ATPase activity. Since the loss of $(Na^+ + K^+)$-ATPase activity was not paralleled by a

TABLE 21

INCORPORATION OF BEEF BRAIN ($Na^+ + K^+$)-ATPase INTO DISTEAROYL PHOSPHATIDYLCHOLINE (DSPC) LIPOSOMES: EFFECT ON SPECIFIC ACTIVITY*

	Specific activity**			
	Total ATPase	Mg^{++} ATPase	O.I. ATPase†	% ATPase sensitive to ouabain
Enzyme (before reconstitution)	180.9 ± 8.4	20.8 ± 1.9	158.9 ± 8.4	88 ± 0.9
Reconstituted enzyme (Beef-E/DSPC)	49.6 ± 1.4	17.1 ± 1.6	32.5 ± 1.7	68 ± 2.5

* Results are based on four separate experiments. Values given are Mean ± SEM.

** µmol ATP hydrolyzed/mg protein/hr at 37°C.

† Ouabain inhibitable ATPase.

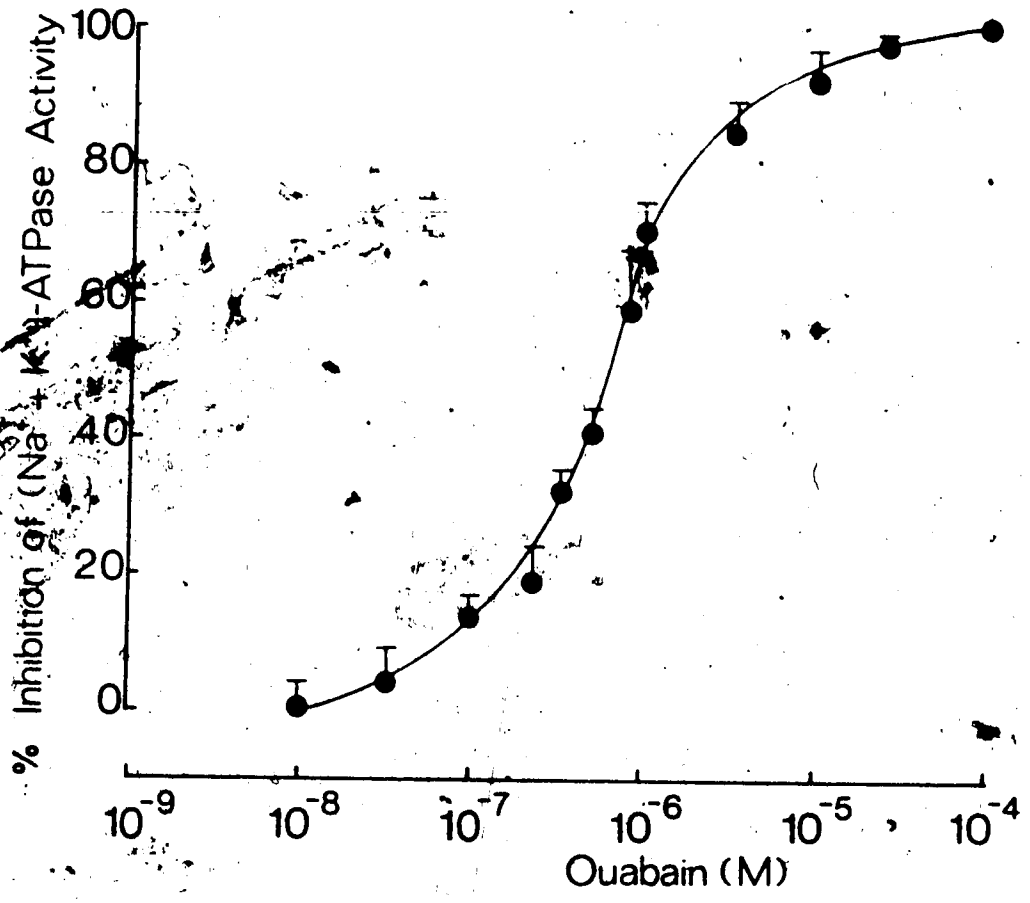


Fig. 30 Dose-response curve of ouabain inhibition of $(Na^+ + K^+)$-ATPase from beef brain, reconstituted into distearoyl phosphatidylcholine (DSPC) liposomes. Enzyme activity was determined at 37°C in the presence of 2 mM Mg^{++}, 20 mM K^+, 80 mM Na^+ plus variable concentrations of ouabain as indicated. 100% inhibition of $(Na^+ + K^+)$-ATPase occurred when the ouabain inhibited enzyme activity was similar to that basal activity which was observed in the absence of 80 mM Na^+. The points represent Mean ± SEM of 4 determinations. The value for one-half maximal inhibition (ID_{50}) was calculated by computer regression analysis and is $7.26 \pm 1.15 \times 10^{-7}$M.

similar reduction in the Mg^{++}-ATPase activity, the % ouabain sensitivity of the preparation had decreased from 88% (native enzyme) to 68% (reconstituted enzyme). Fig 30 illustrates the mean dose-response curve to inhibition by ouabain of this DSPC reconstituted preparation. The mean value for one-half maximal inhibition by ouabain was $7.26 \pm 1.15 \times 10^{-7}$M. This value did not differ significantly from that of the respective control ($4.05 \pm 0.37 \times 10^{-7}$M; Fig. 15).

The effect of serial substitution of endogenous lipids of beef brain $(Na^+ + K^+)$-ATPase with DSPC liposomes was also investigated. The experiment was performed as described in Materials and Methods. Table 22 summarizes the results of this experiment. It can be seen that this serially substituted preparation retained little or no total ATPase activity. The total ATPase activity had decreased from 201.6 (native enzyme) to 3.4 (serially substituted enzyme) μmol ATP hydrolyzed/mg protein/hr, which represented over 98% loss of initial total ATPase activity. Similarly, the serial replacement of endogenous lipids of $(Na^+ + K^+)$-ATPase protein with DSPC liposomes had resulted in more or less a complete loss of $(Na^+ + K^+)$-ATPase activity, as it was decreased from 178.0 (native enzyme) to 0.9 (serially substituted enzyme) μmol ATP hydrolyzed/mg protein/hr.

(6) Summary of Effects of Fluidity

The results obtained with beef brain $(Na^+ + K^+)$-ATPase reconstituted into a series of synthetic phosphatidylcholines with defined fatty acid composition are summarized in Table 23. As mentioned above the phase transition temperatures [which is a measure of the 'fluidity' of the lipid structure] of these synthetic lecithins were ranged from -22°C to

TABLE 22

SERIAL SUBSTITUTION OF ENDOGENOUS LIPIDS OF BEEF BRAIN ($Na^+ + K^+$)-ATPase WITH DISTEAROYL PHOSPHATIDYLCHOLINE (DSPC) LIPOSOMES: EFFECT ON SPECIFIC ACTIVITY*

	Specific activity**			
	Total ATPase	Mg^{++} ATPase	O.I. ATPase†	% ATPase sensitive to ouabain
Enzyme (before reconstitution)	201.6 ± 5.2	23.6 ± 1.7	178.0 ± 3.6	88 ± 0.7
G-50 void volume after 1st substitution	71.3 ± 9.1	18.3 ± 0.4	52.9 ± 9.2	73 ± 3.1
G-50 void volume after 2nd substitution	33.5 ± 1.9	10.9 ± 0.9	22.6 ± 2.7	67 ± 4.5
G-50 void volume after 3rd substitution	8.9 ± 0.4	3.3 ± 0.3	5.6 ± 0.4	59 ± 1.9
4-B void volume (Reconstituted enzyme)	3.4 ± 0.4	2.5 ± 0.5	0.9 ± 0.1	34 ± 5.0

* Results are based on three separate experiments. Values given are mean ± SEM.

** µmol ATP hydrolyzed/mg protein/hr at 37°C.

† Ouabain inhibitable ATPase.

+58°C. It can be seen that incorporation of beef ($Na^+ + K^+$)-ATPase into synthetic lecithin liposomes did not alter the ouabain interaction properties of the reconstituted systems compared to the control preparation.

TABLE 23

EFFECT OF LIPID FLUIDITY OF MEMBRANE ON THE INHIBITION OF BEEF BRAIN $(Na^+ + K^+)$-ATPase BY OUABAIN: SUMMARY OF RESULTS

Enzyme preparation	n	ID_{50}-Ouabain (M)		
A. Beef-E/beef lipid	5	$4.05 \pm 0.37 \times 10^{-7}$	-	(Fig. 15)
B. Beef-E/DMPC	4	$4.0 \pm 0.45 \times 10^{-7}$	*	(Fig. 26)
C. Beef-E/1-palmito, 2-oleoyl-PC	4	$5.12 \pm 0.55 \times 10^{-7}$	*	(Fig. 27)
D. Beef-E/DOPC	4	$3.46 \pm 0.53 \times 10^{-7}$	*	(Fig. 28)
E. Beef-E/DPPC	3	$6.45 \pm 0.47 \times 10^{-7}$	*	(Fig. 29)
F. Beef-E/DSPC	4	$7.26 \pm 1.15 \times 10^{-7}$	*	(Fig. 30)

* Not significantly different from A.

DISCUSSION

IV. DISCUSSION

A. General

As mentioned in the rationale section of this presentation the present study was based on the observation that the $(Na^+ + K^+)$-ATPase isolated from poikilotherms has a markedly reduced sensitivity to inhibition by ouabain compared to similar enzyme preparations from homeotherms. The aim of the present investigation was to study the reason(s) underlying this phenomenon. In the present study both the axons from the cold water crab (*Cancer magister*) and the cortex from beef brain (*Bos taurus*) were employed as the sources of $(Na^+ + K^+)$-ATPase from poikilotherms and homeotherms respectively.

B. Control Preparations

(1) Specific Activity

The results shown in Table 1 and Fig. 1 indicated that although beef brain $(Na^+ + K^+)$-ATPase can be activated significantly by deoxycholate treatment, no such effect was observed with crab nerve $(Na^+ + K^+)$-ATPase. The precise mechanism by which deoxycholate causes an activation of $(Na^+ + K^+)$-ATPase is not known. However, it was thought that the detergent treatment results in a removal of extraneous proteins as well as certain more labile lipids (Jørgensen, 1975). If this is the case, then the lack of activation of $(Na^+ + K^+)$-ATPase from crab nerve by deoxycholate extraction may perhaps be due to one or both of the following reasons.

a) The enzyme preparations isolated from crab axons were more "pure", in that they contained little or no extraneous proteins.

b) It is possible that the lipids of crab nerve enzyme preparations

are more "tightly packed" than those of beef brain ($Na^+ + K^+$)-ATPase and thus not easily removed by detergent treatment.

Intuitively, it seems more likely that lack of activation by deoxycholate of crab nerve ($Na^+ + K^+$)-ATPase is due to the fact that these preparations contained little or no extraneous proteins. In fact, it has been reported by Jørgensen and Skou (1971) and Jørgensen (1972) that the activation of rabbit kidney ($Na^+ + K^+$)-ATPase by DOC was due to the removal of extraneous proteins, as determined by SDS-gel electrophoresis.

However, the activation of beef brain ($Na^+ + K^+$)-ATPase by detergent extraction can also be explained as follows. The untreated enzyme preparation contained both active (right side-out) and non-active (inside-out) membrane vesicles. The detergent treatment had resulted in the "opening" of these membrane vesicles, allowing the ready access of substrate to the ($Na^+ + K^+$)-ATPase active sites and hence caused an activation of the vesicle population which was inside-out (non-active). Therefore, the increase in beef brain ($Na^+ + K^+$)-ATPase activity by deoxycholate may perhaps be due to the exposure of these latent enzyme active sites. If this speculation is correct then the number of ouabain binding sites must also be increased as a result of the detergent extraction. Indeed, the exposure of additional ouabain binding sites after detergent extraction has been reported by Charnock *et al.* (1977). Thus, a good correlation between the enzyme specific activity and the amount of [^{3}H]-ouabain bound at equilibrium at 37°C, exists (Erdmann and Schoner, 1973, 1974; Charnock *et al.*, 1977).

If the above explanation is correct then the lack of activation of crab nerve ($Na^+ + K^+$)-ATPase by deoxycholate extraction should be due

to one or both of the following reasons.

a) The majority (if not all) of the membrane vesicles contained in the untreated enzyme preparation were oriented right side-out (active).

b) Instead of non-leaky membrane vesicles, the untreated enzyme preparation contained non-vesiculated membrane fragments or perhaps the membrane vesicles were leaky enough to allow the ready passage of enzyme substrate into the inside-out (non-active) vesicles.

It is possible that these characteristics of untreated membrane fractions enriched in $(Na^+ + K^+)$-ATPase from crab axons are related to its membrane lipid composition. However, since no ouabain binding studies on crab nerve $(Na^+ + K^+)$-ATPase, before and after extraction with deoxycholate have been carried out, these explanations remain speculative.

(2) Inhibition by Ouabain

The results confirmed that there is a marked difference between the ID_{50} values to inhibition by ouabain of the two different types of enzyme preparations from beef brain and crab nerve. The polyacrylamide gel electrophoretic patterns of the two enzyme proteins appeared to be very similar (Fig. 4 and Fig. 5). Although a major structural difference between the two proteins was not found in the present study it should be remembered that the gel electrophoresis technique has several drawbacks. For example, different gel systems are known to differ in their resolving power and the sensitivity of staining of proteins (Dahl and Hokin, 1974). Therefore, it remains possible that a relatively small but

decisive structural difference between the two $(Na^+ + K^+)$-ATPase from beef and crab exists which was not resolved by the gel system employed in the present study. Since a detailed study was not carried out, the present study does not allow the unequivocal conclusion that the structure of two enzyme proteins are identical. Therefore, although it seems unlikely, a minor structural difference may be present between the two proteins and this may in part be responsible for the decreased ouabain sensitivity observed in crab $(Na^+ + K^+)$-ATPase.

Theoretically, the observed difference in ID_{50} values for ouabain inhibition may also be explained by assuming the presence of an endogenous inhibitor of $(Na^+ + K^+)$-ATPase. This is a possibility since it has been reported by Zachowski *et al.* (1977) and Lelievre *et al.* (1979) that $(Na^+ + K^+)$-ATPase isolated from murine plasmocytoma MOPC 173 ascites cells contains an endogenous inhibitor which was easily removed by exposure to 1 mM EDTA. The inhibitor has been isolated and found to be a phospholipid free protein with a molecular weight of 30,000-34,000 daltons. The incubation of EDTA treated membrane fractions in the presence of this low molecular weight protein and exogenous Ca^{++} restored the original resistance to ouabain inhibition (Lelievre *et al.*, 1979). However, the presence of such an inhibitor in enzyme preparations employed in the present study is very unlikely since,

a) All membrane fractions enriched in $(Na^+ + K^+)$-ATPase were prepared in buffered sucrose solutions which contained either 1 mM (beef brain) or 2 mM (crab nerve) EDTA.

b) When enzyme preparations were treated with detergent, the incubation mixture again contained EDTA.

Thus, since the "inhibitor" has been reported to be solubilized upon exposure to EDTA the above procedure(s) should result in the removal of the inhibitor. However, even after these treatments the value for one-half maximal inhibition to ouabain of the two enzyme preparations differed by more than two log units. Therefore, it is clear that the decreased ouabain sensitivity of crab nerve ($Na^+ + K^+$)-ATPase cannot be readily explained by the presence of an EDTA sensitive "endogenous inhibitor".

(3) Lipid Composition

The results indicated that crab nerve membrane fractions enriched in ($Na^+ + K^+$)-ATPase contained increased amount of long chain polyunsaturated fatty acids compared to membrane enzyme preparations from beef brain. In addition crab nerve enzyme contained higher amount of cholesterol than the beef brain ($Na^+ + K^+$)-ATPase. Cholesterol is known to modulate the lipid fluidity of membranes causing lipids to be less fluid above the phase transition (T_c) temperature, whereas below the T_c cholesterol has been reported to increase the membrane lipid fluidity (Chapman *et al.*, 1979). According to Papahadjopoulos *et al.* (1973), cholesterol may be needed to provide a generally stable membrane framework but seems to be excluded from the lipid micro-environment of the ($Na^+ + K^+$)-ATPase. It has also been reported that the immediate lipid environment of integral membrane enzyme systems such as Ca^{++}-ATPase, contain little or no cholesterol (Warren *et al.*, 1975; Kimelberg, 1976). Thus in agreement with the observation of Charnock and Bashford (1975) it is possible that at any given temperature the immediate lipid

environment of crab nerve ($Na^+ + K^+$)-ATPase may be more fluid than the lipids adjacent to the beef brain ($Na^+ + K^+$)-ATPase. The difference in membrane lipid composition and its resultant effect on lipid fluidity may well be responsible for the decreased sensitivity of crab nerve ($Na^+ + K^+$)-ATPase to inhibition by ouabain. Therefore, the possible lipid modulation of ouabain interaction with ($Na^+ + K^+$)-ATPase was investigated by the reconstitution experiments discussed below.

C. Reconstitution into Liposomes

(1) General

Although the incorporation of membrane proteins into lipid vesicles can be achieved by the "sonication method" the loss of activity that accompanies the sonication process severely limits the use of this method for the reconstitution of membrane enzyme systems. The results summarized in Fig. 6 indicated that sonication of ($Na^+ + K^+$)-ATPase caused complete loss of enzyme activity within a short period of time. Thus, an alternative method for the reconstitution of ($Na^+ + K^+$)-ATPasé into liposomes had to be found. The incorporation of membrane proteins into lipid vesicles can be accomplished by several detergent depletion methods. These methods rely on the solubilization of lipids by detergent (usually deoxycholate) and the subsequent removal of the detergent by either gel filtration or dialysis: liposomes form spontaneously during this latter stage of the procedure. In addition the reconstitution of membrane proteins by detergent depletion using a gel filtration procedure has been reported for at least three different membrane proteins. They include, hog kidney aminopeptidase (Wa[illegible] *et al.*, 1976),

human acetylcholinesterase (Hall and Brodbeck, 1978) and $T_{(1s)}$ hydrophobic peptide from MN-glycoprotein (Allen *et al.*, 1980).

Several workers (Hall and Brodbeck, 1978; Allen *et al.*, 1980) have reported that detergent depletion using gel filtration is a better method for the reconstitution of membrane proteins, as there is a more complete removal of detergent by gel filtration of Sephadex G-50 than could be obtained by dialysis. Thus, the potential advantages of a column chromatography technique for membrane reconstitution over other methods such as dialysis include,

a) Detergent can be removed very easily and effectively.

b) The percentage incorporation of enzyme protein into liposome can be much higher than that achieved by dialysis method [a 100% incorporation of acetylcholinesterase by gel filtration method has been reported by Hall and Brodbeck (1978)].

c) Final material can be achieved within a relatively short period of time (a few hours compared to several days by dialysis procedure).

(2) Reconstitution

The results of this study demonstrated that the reconstitution of $(Na^+ + K^+)$-ATPase into liposomes can be successfully achieved with almost complete restoration of enzyme activity by the gel filtration technique. The passage of enzyme/deoxycholate/lipid mixture through a column of Sephadex G-50 resulted in the incorporation of $(Na^+ + K^+)$-ATPase into the bilayer structure of liposomes. This step also resulted in complete removal of deoxycholate. The separation of detergent from ATPase-

liposomes was followed by using ^{14}C labelled deoxycholate. Hall and Brodbeck reported that Sephadex G-50 gel filtration of lipid/detergent/protein mixture resulted in 99.97% removal of the detergent from the lipid. According to Allen *et al.* (1980) liposomes prepared by gel filtration method may retain as few as 10 molecules of deoxycholate per 1000 molecules of phospholipid after one column passage. However, in the present study it was found that the ATPase-liposomes contained no detectable amount of [^{14}C]-deoxycholate.

The ($Na^+ + K^+$)-ATPase used in the preliminary experiments was labelled with [^3H]-ouabain. This enzyme-inhibitor complex was formed as a method for tracking the ($Na^+ + K^+$)-ATPase protein (in a "mixture" of impure proteins). The void volume of Sephadex G-50 contained liposomes and enzyme-ouabain complex (Fig. 7; fractions 14-19) whereas deoxycholate was eluted at a much later stage (Fig. 7; fractions 31-52). This is not surprising since the gel filtration is based on the separation of molecules according to size. Therefore both liposomes and ($Na^+ + K^+$)-ATPase having a molecular weight above the exclusion limit of Sephadex G-50 (about 38,000 daltons) appeared in the void volume. In contrast, the elution of free ouabain which was not bound to ($Na^+ + K^+$)-ATPase was retarded due to its low molecular weight. Thus, the second tritium peak which was much larger in size was due to free [^3H]-ouabain (Fig. 7; fractions 26-52).

Since 100% incorporation of ($Na^+ + K^+$)-ATPase into liposomes was not achieved, the void volume of Sephadex G-50 (Fig. 7; fractions 14-19) contained incorporated enzyme as well as free enzyme protein which was not incorporated into the lipid bilayer structure of liposomes. However,

based on the total protein content, 70-80% incorporation of $(Na^+ + K^+)$-ATPase into liposomes was achieved in the present study. In addition to the incorporated and non-incorporated enzyme the Sephadex G-50 void volume contained free liposomes which did not contain any protein. The concentration by centrifugation of Sephadex G-50 void volume resulted in the separation of free liposomes from the free and incorporated enzyme. Upon centrifugation the $(Na^+ + K^+)$-ATPase that had been incorporated into liposomes and free $(Na^+ + K^+)$-ATPase were sedimented as a firm pellet, while free liposomes stayed in the supernatant. Subsequently the free enzyme was separated from the incorporated enzyme by fractionating the concentrated Sephadex G-50 void volume on a column of Sepharose 4B-CL. The $(Na^+ + K^+)$-ATPase incorporated into liposomes was eluted in the void volume (Fig. 8; fractions 12-24) whereas the free enzyme was eluted from the Sepharose column at a later stage (Fig. 8; fractions 32-40).

The incorporation of $(Na^+ + K^+)$-ATPase protein into liposomes had resulted in a marked increase in the size of the $(Na^+ + K^+)$-ATPase compared to the free enzyme protein molecule. These ATPase-liposomes were large enough to exceed the exclusion limit of Sepharose 4B-CL and thus eluted in the void volume. In contrast, the free enzyme protein was trapped in the gel beads and eluted at a later stage. Therefore, an excellent separation of ATPase-liposomes from free $(Na^+ + K^+)$-ATPase was achieved. However, another possibility was that $(Na^+ + K^+)$-ATPase was eluted in the Sepharose void volume not because it had been incorporated into lipid bilayer vesicles but merely due to the aggregation of $(Na^+ + K^+)$-ATPase protein. The aggregated enzyme proteins may be able to

exceed the exclusion limit of Sepharose 4B-CL and thus would be eluted in the void volume. This latter possibility was ruled out and the incorporation of $(Na^+ + K^+)$-ATPase into liposomes was established by density gradient centrifugation of Sepharose 4B-CL void volume on sucrose. The co-appearance of liposomes and enzyme-ouabain complex only on the top of the gradient demonstrated that the enzyme protein had been incorporated into lipid bilayer vesicles.

The relatively broad distribution of ATPase-liposomes eluted in the void volume (Fig. 8; fractions 12-24) indicated that they were heterogeneous in size. This observation was later confirmed by electron microscopy which revealed that ATPase-liposomes had a structure of primarily unilamellar vesicles ranging between 50-100 nm in diameter. These vesicles were of similar size to those previously described by other workers (Wacker *et al.*, 1976; Hall and Brodbeck, 1978).

Aggregation of $(Na^+ + K^+)$-ATPase was seen when the exogenous phospholipid was left out from the enzyme/deoxycholate mixture. The chromatography of enzyme/detergent mixture over a Sephadex G-50 column (control experiment) had produced elution profiles of enzyme-ouabain complex, free ouabain and deoxycholate which were all very similar (Fig. 10) to those seen in the "test" experiment (Fig. 7). However, since exogenous phospholipid was not used the total P_i content present in the Sephadex G-50 void volume (Fig. 10; fractions 13-18) was much less than that in the test experiment (Fig. 7). The fractionation of concentrated Sephadex G-50 void volume on a column of Sepharose 4B-CL also resulted in an elution profile of enzyme-ouabain complex (Fig. 11) similar to that of the "test" experiment (Fig. 8). For instance the Sepharose void volume

(Fig. 11; fractions 12-17) indicated the presence of enzyme-ouabain complex. In addition fractions [illegible] also appeared to contain the enzyme-ouabain complex which represented the non-incorporated (free) enzyme. However, since no exogenous phospholipid was used, the enzyme protein eluted at the void volume (Fig. 11; fractions 12-17) cannot be due to $(Na^+ + K^+)$-ATPase that had been incorporated into liposomes. However, this can be explained by assuming that in the absence of exogenous lipid, the $(Na^+ + K^+)$-ATPase protein had formed aggregates which were large enough to be excluded from the Sepharose 4B-CL column. This speculation was confirmed from the results of the sucrose density gradient centrifugation experiment (Fig. 12). In contrast to the $(Na^+ + K^+)$-ATPase reconstituted into liposomes (Figs. 7, 8 and 9) the final material obtained from the control experiment (Fig. 10 and Fig. 11) appeared to have an increased density (Fig. 12). The enzyme-ouabain complex had travelled deeper into the sucrose gradient compared to the $(Na^+ + K^+)$-ATPase reconstituted into liposomes which appeared only on the top of the gradient (Fig. 9). These results indicated that in the absence of exogenous lipid the $(Na^+ + K^+)$-ATPase had formed aggregates. The aggregation had resulted in increased size and density of $(Na^+ + K^+)$-ATPase which in turn resulted in the penetration of enzyme protein deeper into the sucrose gradient, compared to ATPase-liposomes.

(3) Orientation

It was observed that externally added ouabain caused a complete inhibition of $(Na^+ + K^+)$-ATPase activity of ATPase-liposomes. In addition reconstituted preparations exhibited similar ID_{50} values to inhibition

by ouabain compared to the native enzyme preparations. Therefore, it could be assumed that the majority of the $(Na^+ + K^+)$-ATPase molecules had oriented right side-out (i.e. ouabain binding site is on the out side) in these liposomes. According to Hall and Brodbeck (1978), reconstitution of acetylcholinesterase into lecithin liposomes by gel filtration of Sephadex G-50 had resulted in the location of about 80% of the enzyme protein in the out side of liposomes (i.e. right side-out). However, another serious possibility is that enzyme protein was oriented in both ways (i.e. right side out and inside out) but externally added ouabain was still effective in inhibiting the enzyme activity because the vesicles were leaky. This would allow the quick diffusion of external ouabain and enzyme substrates into the vesicles. Similar leakage experiments were carried out, the exact orientation of $(Na^+ + K^+)$-ATPase protein in these ATPase-liposomes is not known.

(4) Effect on Specific Activity

The results of preliminary experiments had indicated that $(Na^+ + K^+)$-ATPase can be incorporated into egg phosphatidylcholine liposomes with only 10-20% loss of initial enzyme activity. The exogenous phospholipid appeared to protect the enzyme against denaturation from deoxycholate. This was evident from the results of control experiments since in the absence of exogenous phospholipids, the final material was devoid of $(Na^+ + K^+)$-ATPase activity.

The $\%(Na^+ + K^+)$-ATPase activity retained in the reconstituted enzyme preparations was found to be related to the amount of deoxycholate used to solubilize the exogenous lipid. When somewhat higher amounts of

deoxycholate were required to dissolve the lipid film, this was paralleled by an increased loss of $(Na^+ + K^+)$-ATPase activity. Similarly, when the $(Na^+ + K^+)$-ATPase was reconstituted into liposomes by the serial substitution method, the % loss of $(Na^+ + K^+)$-ATPase activity was much higher than that observed after a single substitution. It should be recalled that this serial substitution procedure involved the exposure of $(Na^+ + K^+)$-ATPase to deoxycholate/lipid mixtures not once, but three times. Although the exact mechanism by which deoxycholate inactivates the $(Na^+ + K^+)$-ATPase is not known, it is possible that higher concentrations of detergent result in the dissociation of the two polypeptide subunits of the $(Na^+ + K^+)$-ATPase protein.

In all instances, the reconstitution of $(Na^+ + K^+)$-ATPase into liposomes resulted in a decrease in the associated Mg^{++}-ATPase activity. In many instances this was not accompanied by a decrease in the $(Na^+ + K^+)$-ATPase activity to a similar extent. Thus, the final effect was a reconstituted enzyme preparation with an increased sensitivity to inhibition by ouabain (on a percentage basis) compared to the native enzyme. This decrease in Mg^{++}-ATPase content during the reconstitution procedure may be explained as follows. The exposure of membrane fractions enriched in $(Na^+ + K^+)$-ATPase to lipid-deoxycholate mixture may result in the extraction of "loosely bound" Mg^{++}-ATPase protein. Thus, the subsequent steps which involved the gel filtration and centrifugation procedures would result in the separation of this "extracted" Mg^{++}-ATPase from $(Na^+ + K^+)$-ATPase protein. Therefore, the reconstituted enzyme preparations exhibited less Mg^{++}-ATPase activity compared to native enzyme preparations.

D. Effect of Ouabain on Reconstituted Preparations

The information presented in the Introduction section of this dissertation indicated that there is much conflicting evidence in the literature as to the role of phospholipids in ouabain binding to $(Na^+ + K^+)$-ATPase. However, it is now generally agreed that the lipids of biological membranes exert a regulatory effect upon the behaviour of many integral membrane enzyme systems such as $(Na^+ + K^+)$-ATPase. The results presented in this thesis suggest that membrane lipids are capable of modulating the ouabain sensitivity of $(Na^+ + K^+)$-ATPase - that is the drug/receptor properties of this important system. For example, the results indicated that the method used for the reconstitution of $(Na^+ + K^+)$-ATPase into liposomes had no effect on the cardiac glycoside receptor properties of this enzyme system. Therefore, beef brain $(Na^+ + K^+)$-ATPase when reconstituted into beef lipid liposomes yielded an ID_{50} value for inhibition by ouabain similar to that of the native enzyme (Fig. 2, Fig. 15, and Fig. 16). Similarly, crab nerve $(Na^+ + K^+)$-ATPase reconstituted into a matrix of crab lipid was found to have similar cardiac glycoside receptor properties as the native enzyme (Fig. 3 and Fig. 21).

The possible lipid modulation of ouabain sensitivity of $(Na^+ + K^+)$-ATPase was sought by incorporating both beef brain and crab nerve enzyme preparations into phosphatidylcholine (egg) liposomes. Although the reconstituted preparations retained 80-90% of the activity of native enzyme preparations, it was observed that egg phosphatidylcholine had no effect in altering the ouabain inhibitory properties of $(Na^+ + K^+)$-ATPase from both beef and crab. Therefore, it is clear that activation of lipid depleted $(Na^+ + K^+)$-ATPase by a particular phospholipid does

not necessarily mean that it has a modulating effect upon the ouabain sensitivity of the $(Na^+ + K^+)$-ATPase enzyme system. In other words, a phospholipid may be effective in reactivating the lipid depleted enzyme but may completely be without effect in altering the cardiac glycoside receptor properties of the native $(Na^+ + K^+)$-ATPase preparation.

The role of PC in reactivating the lipid depleted $(Na^+ + K^+)$-ATPase has been reported by other workers. Palatini *et al.* (1977) reported that PC was without effect in restoring the activity of lipid depleted $(Na^+ + K^+)$-ATPase unless negative charges were introduced into the liposome bilayer. Similarly, Vandersloot *et al.* (1978) found phosphatidylcholine to be effective only if cholate was present during reconstitution. They speculated that the presence of cholate induces the necessary negative charge on the lipid bilayer structure thus making PC effective. Conversely, Hilden and Hokin reported that PC can replace endogenous lipids of $(Na^+ + K^+)$-ATPase maintaining the coupled transport of Na^+ and K^+. The observations reported in this thesis that $(Na^+ + K^+)$-ATPase can be incorporated into phosphatidylcholine liposomes, in an active form are in agreement with those of Hilden and Hokin (1976). Therefore, phosphatidylcholine may be effective in replacing the endogenous phospholipids of $(Na^+ + K^+)$-ATPase maintaining the enzyme activity or alternatively these results may be due to one or both of the following reasons.

a) The reconstituted preparations retained relatively high amounts of endogenous phospholipids.

b) The ATPase-liposomes contained small amounts of deoxycholate which induced the necessary negative charge on the lipid

structure, thus making PC effective.

Although it is unlikely that the results reported in this thesis were due to one or both of the above factors, further experiments are needed to completely rule out these possibilities.

However, the results of biological "cross-over" experiments strongly suggest that the ouabain sensitivity of $(Na^+ + K^+)$-ATPase is regulated by the membrane lipids. The beef brain $(Na^+ + K^+)$-ATPase which was more than 100 fold more sensitive to ouabain inhibition than the crab enzyme, became less sensitive to ouabain inhibition when it was introduced into a crab lipid environment. Moreover, when the endogenous lipids of beef enzyme were replaced with crab lipid liposomes by the serial substitution method, the shift in the dose-response curve was even more pronounced (Fig. 18 and Fig. 19). The ID_{50} value of this latter reconstituted preparation differed from the ID_{50} value of control preparation by almost one log unit (Fig. 16 and Fig. 19). In addition, crab nerve $(Na^+ + K^+)$-ATPase reconstituted into beef lipid liposomes was found to be more sensitive to ouabain inhibition than the native enzyme (Fig. 21, Fig. 23 and Fig. 24). From these observations the following conclusions can be made,

a) The first shift seen in the dose-response curve to ouabain inhibition of single substituted enzyme preparations may be due to the change in the bulk lipid.

b) The beef brain $(Na^+ + K^+)$-ATPase may contain an annular lipid[4] layer which is not easily replaceable. However, this layer of endogenous lipid can be replaced with exogenous lipids by the serial substitution method.

c) Therefore, the more pronounced effects seen when endogenous lipids of beef brain enzyme were serially substituted with crab lipids may perhaps represent the removal of the annular lipid layer of beef brain ($Na^+ + K^+$)-ATPase.

Many investigators believe that integral proteins of cell membranes have a closely associated layer of lipid adjacent to them. Various terms have been used to describe this layer of lipid including "boundary layer" lipid (Jost *et al.*, 1973), "halo" lipid (Trauble and Overath, 1973; Stier and Sackmann, 1973) and "annular" lipid (Warren *et al.*, 1975). All these workers suggested that a rigid or immobilised lipid shell exists separating the integral proteins from the adjacent bulk lipid pool. One can assume that the primary function of such a lipid annulus is to seal the protein into the bilayer and the annulus may, depending upon the protein, either segregate lipids essential for activity (Houslay *et al.*, 1975) or exclude inhibitory lipids (Warren *et al.*, 1975). Hesketh (1976) suggested that this lipid layer excludes cholesterol. In addition the rate of exchange between annular lipid and bulk lipid is thought to be slow, even when the bulk lipid is fluid. However, in a recent review Chapman *et al.* (1979) argued that recent NMR experiments do not support the concept of boundary layers of lipids or annular lipids which lead to long lived "lipid shells" which regulate enzyme actfvity. In addition Chapman and his colleaugues cautioned that ESR data showing an "immobile component" may well be due to other effects such as high microviscosity of trapped lipid, the specific attachment of the ESR probe to the protein or formation of aggregates of high protein to lipid content (Pink and Chapman, 1979; Chapman *et al.*, 1979; Rice *et al.*,

1979). Thus, although the current status of the existence of such lipid annulus around intrinsic proteins is debatable, the observations reported in this thesis may be explained by assuming the presence of such boundary layer of lipid.

The cardiac glycoside receptor properties of crab nerve ($Na^+ + K^+$)-ATPase changed when this enzyme was introduced into a beef lipid environment. The reconstituted preparations became more sensitive to inhibition by ouabain. However, unlike the observation with the beef brain ($Na^+ + K^+$)-ATPase, serial substitution of endogenous lipids of crab enzyme with beef lipid liposomes did not result in a shift in the dose-response curve to ouabain inhibition (Fig. 23 and Fig. 24). This may perhaps be due to either,

a) The "annular" lipids of crab nerve ($Na^+ + K^+$)-ATPase are more tightly bound than those of beef brain enzyme and thus not replaceable by even the serial substitution method.

OR

b) The "annular" lipid layer of crab nerve enzyme is more loosely bound than those of beef enzyme, thus single substitution results in a complete replacement of the endogenous lipids.

Since no lipid analysis studies were done on these single and serially substituted preparations, the answer remains speculative.

It is known that the membrane lipids of poikilotherms contain relatively high proportion of unsaturated fatty acids and thus would be more disordered than the lipids in membranes of homeotherms (Richardson and Tappel, 1962). A similar observation was made in the present study as there was a marked increase in the polyunsaturated fatty acids content of crab nerve ($Na^+ + K^+$)-ATPase compared to enzyme from beef brain ($Na^+ + K^+$)-ATPase (Table 2b). Therefore, it should be correct to assume that beef brain ($Na^+ + K^+$)-ATPase when reconstituted into crab lipid liposomes was embedded in a more fluid environment than the native enzyme. Similarly, crab nerve enzyme when introduced into a less fluid lipid environment than the native preparation (i.e. by reconstituting into beef lipid liposomes), became more sensitive to inhibition by ouabain. Thus, the ouabain sensitivity of ($Na^+ + K^+$)-ATPase may be related to the "fluidity" of the membrane in that as the fluidity of the membrane is increased the sensitivity to inhibition by ouabain is decreased, and *vice versa*. On the other hand it seems very likely that the interaction of ouabain with ($Na^+ + K^+$)-ATPase is dependent on the length of the fatty acyl chain of phospholipid surrounding the protein.

E. Effects of Lipid Fluidity

(1) General

The results discussed so far strongly suggest that membrane lipids are capable of modulating the ouabain interaction properties of ($Na^+ + K^+$)-ATPase enzyme system. One membrane property which might be expected to influence the behaviour of membrane bound enzymes is phospholipid phase state or fluidity. Evidence that membrane receptors, enzymes and

transport proteins are affected by lipid fluidity has been reported (Papahadjopoulos *et al.*, 1973; Shattil *et al.*, 1975; Warren *et al.*, 1975; Wiley and Cooper, 1975; Shinitzky and Inbar, 1976). Fluidity is a descriptive term used to express the relative motional freedom of the membrane lipid molecules and protein substituents. It conveys the idea that the overall structure of the membrane is dynamic rather than static (Singer and Nicolson, 1972).

It has been reported that in biological membranes the degree of "fluidity" is a direct reflection of the transition temperature of the lipids (Chapman, 1975). The appropriate phase transition of lipids will therefore determine the correct fluidity of a cell membrane and also the correct lipid phase separation characteristics. These in turn can affect membrane elasticity, the insertion, aggregation and diffusional movements of the protein and lipid components as well as permeability characteristics (Papahadjopoulos *et al.*, 1974; Trauble and Eible, 1974; Chapman, 1975; Sinensky *et al.*, 1979a). From the information discussed above it seems possible that the ouabain sensitivity of $(Na^+ + K^+)$-ATPase is modulated by the "fluidity" of the membrane lipids. Thus the effects of changes in membrane lipid fluidity on ouabain inhibition of beef brain $(Na^+ + K^+)$-ATPase was studied by using a series of synthetic phosphatidylcholines with defined fatty acid composition.

(2) Effect on Specific Activity

The results reported suggest that degree of membrane fluidity can determine the catalytic activity of $(Na^+ + K^+)$-ATPase. There appears to be a direct relationship between the phase transition temperature

of membrane lipids on the one hand and ouabain inhibitable ATPase activity on the other (Fig. 31). There was no loss of $(Na^+ + K^+)$-ATPase occurred when enzyme protein was reconstituted into two cis unsaturated phosphatidylcholine (DOPC; T_c, -22°C) liposomes (Table 18). Similarly, enzyme preparations reconstituted into either 1-palmito,2-oleoyl-PC (T_c, -5°C) or DMPC (T_c, +23°C) retained about 70% of initial $(Na^+ + K^+)$-ATPase activity. In contrast, reconstitution of ATPase protein into either DPPC (T_c, +41°C) or DSPC (T_c, +68°C) liposomes resulted in a considerable loss of enzyme activity, as the reconstituted preparations retained only 20-35% of the initial $(Na^+ + K^+)$-ATPase activity (Tables 19 and 21). The decreased % ouabain sensitivity observed with either DPPC (Table 19) or DSPC (Table 21) reconstituted preparations was due to the fact that these phospholipids selectively decreased the activity of $(Na^+ + K^+)$-ATPase without affecting the associated Mg^{++}-ATPase activity. Therefore, these reconstituted preparations had a decreased % ATPase sensitive to ouabain compared to the native enzyme preparations (Tables 19 and 21).

All the ATPase assays were carried out at 37°C. Therefore it would be correct to assume that at the assay temperature DOPC, 1-palmito-2-oleoyl-PC and DMPC reconstituted systems all had completed their phase transitions and existed only in the liquid-crystalline state while DPPC and DSPC reconstituted systems were in the gel state. It is evident from the data presented that an appropriate fluid environment is essential for the proper maintenance of the ouabain sensitive ATPase activity. In the absence of proper lipid milieu, such as the case of either DPPC or DSPC reconstituted preparations at 37°C, the state of enzyme molecule

cholesterol levels can alter the surface labelling or exposure of both intrinsic and extrinsic membrane proteins (Borochov and Shinitzky, 1976; Shinitzky and Rivnay, 1977; Borochov *et al.*, 1979). Although it is unlikely, it is possible that the increased cholesterol content of the membrane is responsible for the decreased ouabain sensitivity observed with crab nerve ($Na^+ + K^+$)-ATPase. Therefore, further experiments are needed to rule out the possible role of cholesterol in modulating the ouabain sensitivity of ($Na^+ + K^+$)-ATPase. Unfortunately, preliminary experiments (not reported here) indicated that only very limited changes in cholesterol content of liposomes was possible without destroying the integrity of the reconstituted preparations. From the information discussed in the Introduction of this thesis it is evident that the polar head group of phospholipid molecule plays a major role in the reactivation of lipid-deficient ($Na^+ + K^+$)-ATPase. Maximum reactivation was achieved with phospholipid molecules bearing a net negative charge. However, the influence of the polar head group of the phospholipid molecules on the ouabain interaction properties of ($Na^+ + K^+$)-ATPase has not been reported. Although it is unlikely, the possibility exists that a difference in the net charge of the lipid matrix is responsible for the observed decreased sensitivity of crab nerve ($Na^+ + K^+$)-ATPase to inhibition by ouabain.

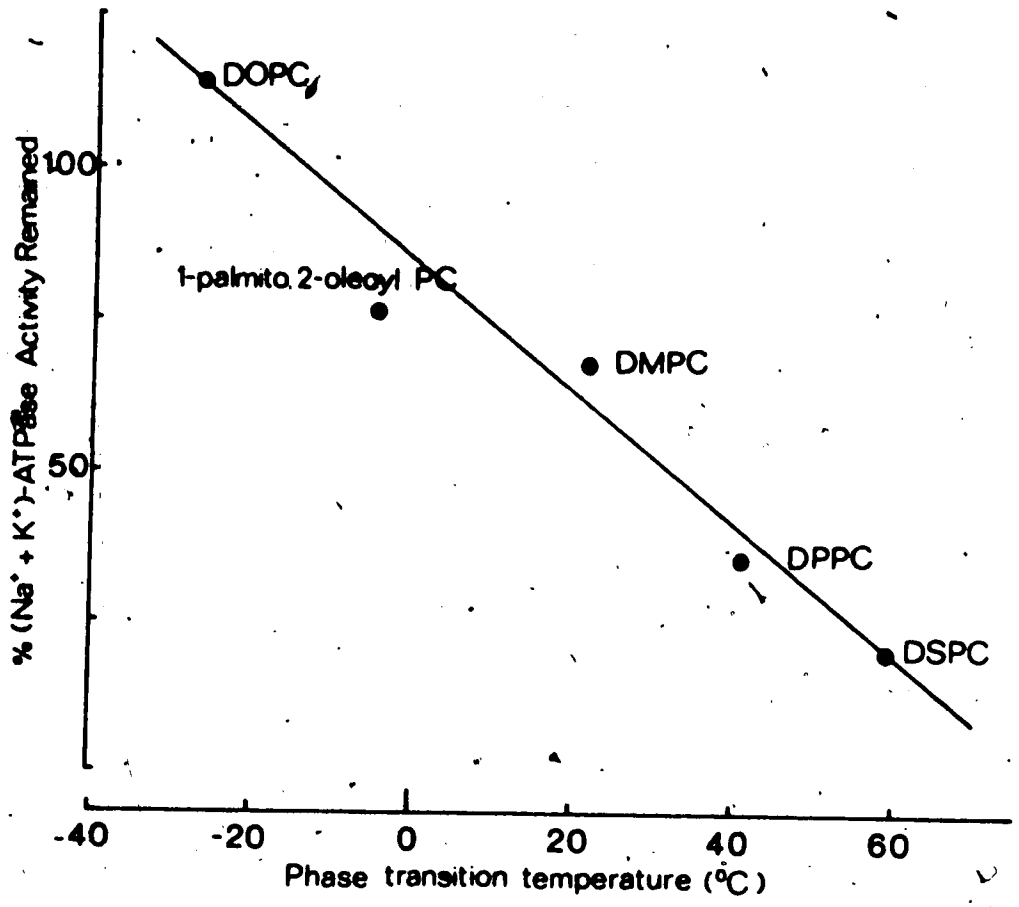


Fig. 31. Correlation between the effect of membrane lipid fluidity and the specific activity of beef brain ($Na^+ + K^+$)-ATPase reconstituted into various synthetic phosphatidylcholine liposomes.

becomes such that it would not support the $(Na^+ + K^+)$-stimulated hydrolysis of ATP. Gel state of lipid milieu would produce a hindrance on conformational changes required for catalytic activity. This was further supported by the observation that serially substituted preparations of $(Na^+ + K^+)$-ATPase with either DPPC or DSPC, retained little or no ouabain sensitive ATPase activity (Tables 20 and 22). Although some direct inhibition of $(Na^+ + K^+)$-ATPase protein by deoxycholate is present, this alone will not account for the complete loss of $(Na^+ + K^+)$-ATPase activity of these serially substituted preparations. This is evident from the fact that the endogenous lipids of beef brain enzyme can be serially replaced by either beef or crab lipid liposomes without complete loss of enzyme activity (Tables 6 and 9).

The ability of $(Na^+ + K^+)$-ATPase protein to function (although at much reduced rates), below the T_c of surrounding lipids, as in the case with DPPC or DSPC reconstituted systems (Tables 19 and 21) can be explained as follows. As mentioned earlier, the $(Na^+ + K^+)$-ATPase protein may be surrounded by a lipid annulus which is not easily replaceable. Therefore, after single substitution with either DPPC or DSPC the ATPase protein may phase separate with more fluid lipid (i.e. with its lipid annulus) at 37°C, which was below the phase transition temperature of the bulk lipid pool of these reconstituted preparations. It can be speculated that *in situ* the immediate lipid environment of beef brain $(Na^+ + K^+)$-ATPase is more fluid than the bulk lipid and requires a lower temperature for the transition. The idea of a more fluid enzyme microenvironment is also supported by the finding of Grisham and Barnett that a highly purified $(Na^+ + K^+)$-ATPase had a more fluid membrane than the

original microsomes. In addition, increased fluidity of the lipid micro-environment of ATPase protein compared to bulk lipids has been reported for the rat basolateral membrane ($Na^+ + K^+$)-ATPase, (Brasitus and Schachter, 1980).

The annular lipid shell of beef brain ($Na^+ + K^+$)-ATPase appears to be replaceable with exogenous phospholipids by the serial substitution method. The replacement of this annular lipid layer with a phospholipid which exists in the gel state results in a complete loss of ($Na^+ + K^+$)-ATPase activity. Therefore, the loss of activity of serially substituted preparations of beef brain ($Na^+ + K^+$)-ATPase with either DPPC or DSPC would be due to the apparent lack of fluidity of the lipid micro-environment of ATPase protein.

In contrast with ($Na^+ + K^+$)-ATPase, the associated Mg^{++}-ATPase activity of reconstituted preparations remained unchanged, before and after reconstitution into liposomes composed of defined fatty acid composition. The decrease in Mg^{++}-ATPase activity observed in serially substituted preparations (Tables 20 and 22) is most likely to be due to a direct effect of deoxycholate upon the protein. Thus, these results suggest that membrane bound Mg^{++}-ATPase is relatively insensitive to changes in membrane lipid fluidity. In accord with this hypothesis Gordon *et al.* (1980) reported that the membrane bound Mg^{++}-ATPase is insensitive to changes in the lipid fluidity achieved by the local anaesthetic, benzyl alcohol.

As reported by several other investigators (Kimelberg and Papahadjopoulos, 1972; Charnock *et al.*, 1973; Papahadjopoulos *et al.*, 1973; Kimelberg and Papahadjopoulos, 1974; Simensky *et al.*, 1979b; Gordon *et al.*, 1980), the results discussed so far clearly suggest that the

physical state of the phospholipids or hydrocarbon fluidity of the membrane is an important determinant in the activity of $(Na^+ + K^+)$-ATPase. The necessity of a fluid state of membrane lipid matrix for optimal activity of $(Na^+ + K^+)$-ATPase is not surprising since this enzyme system is involved in the transport of cations across cell membranes and thus expected to possess extensive conformational mobility. The fluidity of the acyl chains of phospholipids in the membrane provides the required motional freedom allowing the $(Na^+ + K^+)$-ATPase protein to undergo conformational changes associated with its activity. According to Gordon *et al.* (1980) the changes in bulk membrane lipids would be expected to act on all membrane bound enzyme proteins, and the selectivity of this effect would lie at the level of the protein and its shell of annular lipid. These speculations may perhaps be supported by the observations reported in this thesis, since $(Na^+ + K^+)$-ATPase and Mg^{++}-ATPase were found to be affected differently to changes in the membrane lipid fluidity.

The results of this study support prior reports (Kimelberg, 1976; Kimelberg, 1977) that suggest that physiological conditions, such as drug treatments, hormone treatments and changes in membrane lipid composition - that can produce alterations in membrane fluidity can have marked effects on rates of enzyme catalysis and hence normal cell function.

(3) Effect on Inhibition by Ouabain

The results which have been discussed so far indicated that the activity of beef brain $(Na^+ + K^+)$-ATPase was sensitive to the physical state of membrane phospholipids. However, the ouabain interaction properties of this enzyme system appeared to be independent of the bulk lipid fluidity. This was evident from the fact that $(Na^+ + K^+)$-ATPase from beef brain reconstituted into various synthetic lecithin systems (T_c, ranging from -22 to +58°C), retained its native cardiac glycoside receptor properties (Table 23). However, these studies do not rule out the possibility that the inhibition by ouabain of $(Na^+ + K^+)$-ATPase is modulated by the fluidity of its lipid annulus. This is a possibility, since these studies were performed only with single substituted enzyme preparations. Attempts to prepare active preparations of beef brain $(Na^+ + K^+)$-ATPase reconstituted into either DPPC or DSPC liposomes, by the serial substitution method were unsuccessful (Tables 20 and 22).

Farias and coworkers claimed that fluoride inhibition of membrane bound rat erythrocyte $(Na^+ + K^+)$-ATPase and acetylcholinesterase are dependent on the fluidity of the membrane lipid phase and that changes in this fluidity affect the allosteric properties of these enzyme systems (Morero *et al.*, 1972; Bloj *et al.*, 1973; Farias *et al.*, 1975). However, a more recent report from Van Deenen's group indicates that inactivation of acetylcholinesterase from human erythrocytes by fluoride is not dependent on the fluidity of the surrounding membrane environment (Frenkel *et al.*, 1980). In contrast, the enzyme activity of acetylcholinesterase was found to be strongly modulated by its hydrophobic environment. These findings of Frenkel *et al.* are very similar to the

observations reported in this thesis for the beef brain ($Na^+ + K^+$)-ATPase.

The results of the "cross over" experiments (Figures 18, 19 and 23), clearly suggest that membrane lipid composition can modulate the sensitivity of ($Na^+ + K^+$)-ATPase to inhibition by ouabain. However, the ouabain interaction properties of beef brain ($Na^+ + K^+$)-ATPase were unchanged when the fluidity of the lipid matrix was varied with synthetic lecithins (Table 23). Thus, the lipid modulation of ouabain sensitivity of ($Na^+ + K^+$)-ATPase must be due to some other property(ies) of the lipid bilayer.

It has been reported that ouabain binding sites on ($Na^+ + K^+$)-ATPase may exist at least in two different environments (Inagaki *et al.*, 1974; Hansen, 1976; Charnock *et al.*, 1977; Frickle and Klaus, 1977; Schoner *et al.*, 1977; Van-Alstyre, 1978; Rhee and Hokin, 1979). The difference in the ID_{50} values to ouabain of beef brain and crab nerve ($Na^+ + K^+$)-ATPase may be explained by assuming two classes of ouabain binding sites. If this is the case, then one site may be located on the surface of the membrane and therefore easily accessible to ouabain. However, if there is a second population of ouabain binding sites which are located in the core of the membrane then these sites may or may not be accessible to ouabain, depending on the length of the fatty acyl chain of phospholipid adjacent to ($Na^+ + K^+$)-ATPase protein. Phospholipids with relatively short fatty acyl chains may allow the easy access of ouabain to these "deep" binding sites. Conversely, phospholipids with longer chain lengths may hinder (perhaps by phospholipid induced conformational changes of the ATPase protein) the accessibility of ouabain to these binding sites. In addition, increased fluidity of the

fatty acyl chains may perhaps support this "hindering" effect. These speculations are in accordance with the results obtained from the lipid analysis of membrane fractions enriched in beef brain and crab nerve $(Na^+ + K^+)$-ATPase which indicated that lipids from crab nerve enzyme preparation contained more long chain fatty acids compared to beef brain $(Na^+ + K^+)$-ATPase. It can be calculated from the results given in Table 2b that in beef brain enzyme only 3% of total saturates contributed towards long chain (from 20:0 onwards) fatty acids. In contrast 33% of total saturates of lipids of $(Na^+ + K^+)$-ATPase from crab nerve contained fatty acids with longer chain lengths. Among the unsaturates, 56% of the total unsaturated fatty acids of beef enzyme were consisted of longer chain lengths, while in the crab enzyme this value had increased up to 71%. Therefore, it is clear that the enzyme from crab nerve is markedly rich in fatty acids with longer chain lengths and contained more polyunsaturated fatty acids compared to the lipids of beef brain enzyme. The regulation of both enzyme activity and sensitivity to inhibitors by the chain length and the degree of unsaturation of phospholipid acyl chains has been reported for at least one membrane bound enzyme system, namely the mitochondrial ATPase complex (Bruni *et al.*, 1975; Pitotti *et al.*, 1980). Therefore, it seems likely that the chain length and the degree of unsaturation of phospholipid acyl chains of the lipid microenvironment of $(Na^+ + K^+)$-ATPase play a role in the modulation of its cardiac glycoside receptor properties.

It should be recalled that membrane preparations from crab axons contained higher amounts of cholesterol compared to enzyme preparations from beef brain. It has been reported that changes in membrane

F. Summary

The observations reported in this thesis can be summarized as follows.

a) The $(Na^+ + K^+)$-ATPase enzyme system can be incorporated into liposomes of desired lipid composition by the gel chromatography technique.

b) The ouabain interaction properties of $(Na^+ + K^+)$-ATPase are strongly modulated by the membrane lipid composition.

c) The ouabain sensitive ATPase activity is dependent on the physical state of membrane phospholipids. The bulk lipid fluidity will, in general, regulate the $(Na^+ + K^+)$-ATPase activity. The $(Na^+ + K^+)$-ATPase enzyme system requires a fluid state of membrane lipids and a longer fatty acyl chain length to be fully active. In contrast the Mg^{++}-ATPase activity appears to be relatively insensitive to changes in the bulk lipid fluidity.

d) The bulk lipid fluidity does not appear to regulate the interaction of ouabain with the beef brain $(Na^+ + K^+)$-ATPase. These observations however do not eliminate the possibility that ouabain sensitivity of beef brain $(Na^+ + K^+)$-ATPase may perhaps be regulated by the "fluidity" of its lipid annulus.

e) It is speculated that the increased chain length and the degree of unsaturation of the phospholipid acyl chains or a phospholipid head group effect may be responsible for the decreased ouabain sensitivity observed with the crab nerve $(Na^+ + K^+)$-ATPase.

Although the results presented in this thesis indicated the decreased sensitivity to ouabain inhibition of crab nerve $(Na^+ + K^+)$-ATPase compared to enzyme from beef brain relies on the lipid component, the present study does not allow a precise interpretation. The fluidity of the lipid matrix was found not to affect the ouabain interaction properties of the beef brain $(Na^+ + K^+)$-ATPase. However, the effect of polar head group, increased chain length and degree of unsaturation of the fatty acyl chains of phospholipids on the ouabain inhibition of $(Na^+ + K^+)$-ATPase remain to be seen. It is quite possible that modulation of ouabain sensitivity of $(Na^+ + K^+)$-ATPase is due either to one of these factors or perhaps a combination of two or more factors. There is no doubt that the concept of lipid modulation of cardiac glycoside receptor properties of $(Na^+ + K^+)$-ATPase requires and deserves further investigation.

BIBLIOGRAPHY

BIBLIOGRAPHY

Abeles, A.L. (1969) Structure-activity relationship of several cardiotonic steroids with respect to inhibition of ion transport in frog muscle. J. Gen. Physiol. 54: 268-285.

Ahmed, K. and Judah, J.D. (1964) Preparation of lipoproteins containing cation dependent ATPase. Biochem. Biophys. Acta. 93: 603-613.

Akera, T. (1977) Membrane adenosine triphosphatase: a digitalis receptor? Science 198: 569-574.

Akera, T., Temma, K., Wiest, S.A. and Brody, T.M. (1978) Reduction of the equilibrium binding of cardiac glycosides and related compounds to Na^+, K^+-ATPase as a possible mechanism for the potassium-induced reversal of their toxicity. Naunyn-Schiniedeberg's Arch. Pharmacol. 304: 157-165.

Albers, R.W. and Koval, G.J. (1966) Sodium-potassium-activated adenosine triphosphatase of *Electrophorus* electric organ. III. An associated potassium-activated neutral phosphatase. J. Biol. Chem. 241: 1896-1898.

Albers, R.W., Koval, G.J. and Seigel, G.J. (1968) Studies on the interaction of ouabain and other cardio-active steroids with sodium-potassium-activated adenosine triphosphatase. Mol. Pharmacol. 4: 324-336.

Albers, R.W., Rodriguez de Lores, G. and De Robertis, E. (1965) Sodium and potassium-activated ATPase and potassium activated p-nitrophenyl phosphatase - A comparison of their subcellular localizations in rat brain. Proc. Nat. Acad. Sci. (U.S.A.) 53: 557-564.

Alexander, D.R. and Rodnight, R. (1970) Separation at neutral pH of a ^{32}P labelled membrane protein associated with the ($Na^+ + Mg^{++}$) ion activated ATPase from ox brain. Biochem. J. 119: 44P.

Allen, J.C., Lindenmayer, G.E. and Schwartz, A. (1970) An allosteric explanation for quabain induced time dependent inhibition of sodium, potassium-adenosine triphosphatase. Arch. Biochem. Biophys. 141: 322-328.

Allen, J.C. and Schwartz, A. (1969) A possible biochemical explanation for the insensitivity of the rat to cardiac glycosides. J. Pharmacol. Exp. Ther. 168: 42-46.

Allen, J.C. and Schwartz, A. (1970) Effects of potassium, temperature and time on ouabain interaction with the cardiac $Na^+ + K^+$-ATPase. Further evidence supporting an allosteric site. J. Mol. Cell. Cardiol. 1: 39-45.

Allen, T.M., Romans, A.Y., Kercret, H. and Segrest, J.P. (1980) Detergent removal during membrane reconstitution. Biochim. Biophys. Acta. 601: 328-342.

Almeida, A.F. and Charnock, J.S. (1977) An electron spin probe study of ($Na^+ + K^+$)-ATPase-containing membranes. Biochim. Biophys. Acta 467: 19-28.

Aloia, R.C., Pengelley, E.T., Bolen, J.L. and Rouser, G. (1974) Changes in phospholipid composition in hibernating ground squirrel, *Citellis lateralis* and their relationship to membrane function at reduced temperature. Lipids 9: 939-999.

Anner, B.M., Lane, L.K., Schwartz, A. and Pitts, B.J.R. (1977) A reconstituted ($Na^+ + K^+$) pump in liposomes containing purified ($Na^+ + K^+$)-ATPase from kidney medulla. Biochim. Biophys. Acta 467: 340-345.

Askari, A. and Koyal, D. (1971) Studies on the partial reactions catalyzed by the ($Na^+ + K^+$)-activated ATPase. II. Effects of oligomycin and other inhibitors of the ATPase on the p-nitrophenylphosphatase. Biochim. Biophys. Acta 225: 20-25.

Atkinson, A., Hunt, S. and Lowe, A.G. (1968) Manganese activation of ($Na^+ + K^+$) dependent ATPase in pig brain microsomes. Biochim. Biophys. Acta 167: 469-472.

Atkinson, A. and Lowe, A.G. (1972) The pH dependence of the effects of Na^+, K^+ and ouabain on the ATPase activity of NaI-treated brain microsomes with Mg^{++}, Mn^{++}, Ca^{++} and Zn^{++} as divalent metal activators. Biochim. Biophys. Acta 266: 103-115.

Baker, P.F. (1972) Transport and metabolism of calcium ion in nerve. Prog. Biophys. Mol. Biol. 24: 177-223.

Banerjee, S.P., Dwosh, I.L., Khanna, V.K. and Sen, A.K. (1970) Solubilization of guinea pig kidney Na^+, K^+-ATPase with lubrol and Triton X-100. Biochim. Biophys. Acta. 211: 345-355.

Barnett, R.E. (1977) The Na^+-K^+-ATPase. In: Membrane Proteins vol 1: Membrane proteins and their interactions with lipid. Ed., R.A. Capaldi: Marcell Dekker Inc., N.Y. pp. 189-206.

Beauge, L.A. and Glynn, I.M. (1978) Commercial ATP containing traces of vanadate alters the response of Na, K-ATPase to external potassium. Nature 272: 551-552.

Blaker, W.D. and Moscatelli, E.A. (1978) The effect of hibernation on the lipids of brain myelin and microsomes in the syrian hamster. J. Neurochem. 31: 1513-1518.

Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911-917.

Bloj, B., Morero, R.D., Farias, R.N. and Trucco, R.E. (1973) Membrane lipid fatty acids and regulation of membrane bound enzymes: Allosteric behaviour of erythrocyte Mg^{++}-ATPase, ($Na^+ + K^+$)-ATPase and acetylcholinesterase from rats fed different fat-supplemented diets. Biochim. Biophys. Acta 311: 67-79.

Bonting, S.L. and Caravaggio, L.L. (1963) Studies on Na:K activated ATPase. V. Correlation of enzyme activity with cation flux in six tissues. Arch. Biochem. Biophys. 101: 37-46.

Borochov, H., Abbot, R.E., Schachter, D. and Shinitzky, M. (1979) Modulation of erythrocyte membrane proteins by membrane cholesterol and lipid fluidity. Biochemistry 18: 251-255.

Borochov, H. and Shinitzky, M. (1976) Vertical displacement of membrane proteins mediated by changes in micro-viscosity. Proc. Natl. Acad. Sci. (U.S.A.) 73: 4526-4530.

Bowler, K. and Duncan, C.J. (1970) The effect of copper on membrane enzymes. Biochim. Biophys. Acta 196: 116-119.

Brasitus, T.A. and Schachter, D. (1980) Lipid dynamics and lipid-protein interactions in rat enterocyte basolateral and microvillus membranes. Biochemistry 19: 2763-2769.

Britten, J.S. and Blank, M. (1968) Thallium activation of the ($Na^+ + K^+$)-activated ATPase of rabbit kidney. Biochim. Biophys. Acta 159: 160-166.

Bruni, A., Van Dijck, P.W.M. and De Gier, J. (1975) The role of phospholipid acyl chains in the activation of mitochondrial ATPase complex. Biochim. Biophys. Acta 406: 315-328.

Cantley, L.C., Josephson, L., Warner, R., Yanagisawa, M., Lechene, C., and Guidotti, G. (1977) Vanadate is a potent (Na,K)-ATPase inhibitor found in ATP derived from muscle. J. Biol. Chem. 252: 7421-7423.

Cantley, L.C. Jr., Resh, M.D. and Guidotti, G. (1978) Vanadate inhibits the red cell Na,K-ATPase from the cytoplasmic side. Nature 272: 552-554.

Chapman, D. (1975) Phase transitions and fluidity characteristics of lipids and cell membranes. Q. Rev. Biophysics 8: 185-235.

Chapman, D., Gomez-Fernandez, J.C. and Goni, F.M. (1979) Intrinsic protein-lipid interactions. FEBS Lett. 98: 211-223.

Chapman, D., Williams, R.M. and Ladbrooke, B.D. (1967) Physical studies of phospholipids. VI. Thermotropic and lyotropic mesomorphism of some 1,2 diacyl-phosphatidylcholines (Lecithins). Chem. Phys. Lipids 1: 445-475.

Charnock, J.S., Almeida, A.F. and To, R. (1975) Temperature-activity relationships of cation activation and ouabain inhibition of ($Na^+ + K^+$) ATPase. Arch. Biochim. Biophys. 167: 480-487.

Charnock, J.S. and Bashford, I.C. (1975) A fluorescent probe study of the lipid mobility of membranes containing sodium and potassium-dependent adenosine triphosphatase. Mol. Pharmacol. 11: 766-774.

Charnock, J.S., Cook, D.A., Almeida, A.F. and To, R. (1973) Activation energy and phospholipid requirements of membrane bound adenosine triphosphatase. Arch. Biochem. Biophys. 159: 393-399.

Charnock, J.S., Cook, D.A. and Casey, R. (1971) The role of cations and other factors on the apparent energy of activation of $(Na^+ + K^+)$-ATPase. Arch. Biochem. Biophys. 147: 323-329.

Charnock, J.S., Dryden, W.F., Skoog, C. and Lauzon, P.A. (1980b) Depression of the positive inotropic response to ouabain and noradrenaline by the myocardium of the ground squirrel (*Spermophilus richardsonii*) during hibernation. Comp. Biochem. Physiol. 65B: 675-680.

Charnock, J.S. and Opit, L.J. (1968) Membrane metabolism and ion transport. In: Biological Basis of Medicine. Academic Press, London & New York. vol 1: pp 69-103.

Charnock, J.S. and Post, R.L. (1963a) The preparation and properties of a cation stimulated adenosine-triphosphatase from guinea pig kidney cortex. Aust. J. Exp. Biol. Med. Sci. 41: 547-560.

Charnock, J.S. and Post, R.L. (1963b) Evidence of the mechanism of ouabain inhibition of cation transport. Nature 199: 910-911.

Charnock, J.S., Raison, J.K., Gibson, R.A. and McMurchie, E.J. (1980a) Differences in the thermal behaviour of myocardial membranes relative to hybernation. Submitted for publication.

Charnock, J.S., Rosenthal, A.L. and Post, R.L. (1963) A phosphorylation intermediate in the cation stimulated enzymic hydrolysis of ATP. Aust. J. Exp. Biol. & Med. Sci. 41: 675-686.

Charnock, J.S. and Simonson, L.P. (1977) Differential lipid control of $(Na^+ + K^+)$-ATPase in homeotherms and poikilotherms. Comp. Biochem. Physiol. 58B: 381-387.

Charnock, J.S. and Simonson, L.P. (1978) Variations in $(Na^+ + K^+)$-ATPase and Mg^{++}-ATPase activity of the ground squirrel brain during hibernation. Comp. Biochem. Physiol. 59B: 223-229.

Charnock, J.S., Simonson, L.P. and Almeida, A.F. (1977) Variation in sensitivity of the cardiac glycoside receptor characteristics of $(Na^+ + K^+)$-ATPase to lipolysis and temperature. Biochim. Biophys. Acta 465: 77-92.

Charnock, J.S., Simonson, L.P. and Dryden, W.F. (1980c) Seasonal variations in myocardial $(Na^+ + K^+)$-ATPase activity and $[^3H]$-ouabain binding in a hybernating mammal: Positive correlation with the pharmacological effects of ouabain. Comp. Biochem. Physiol. 65B: 681-686.

Chipperfield, A.R. and Whittam, R. (1973) Reconstitution of the sodium pump from protein and phosphatidyl serine: features of ouabain binding. J. Physiol. (Lond.) 230: 467-476.

Dahl, J.L. and Hokin, L.E. (1974) The sodium-potassium adenosine triphosphatase. Ann. Rev. Biochem. 43: 327-356.

De Caldenty, M.I. and Wheller, K.P. (1979) Requirement for negatively charged dispersions of phospholipid for interaction with lipid-depleted adenosine triphosphatase. Biochem. J. 177: 265-273.

De Kruyff, B., Demel, R.A., Slotboom, A.J., Van Deenen, L.L.M. and Rosenthal, A.F. (1973) The effect of the polar head group on the lipid-cholesterol interaction: A monolayer and differential scanning calorimetry study. Biochim. Biophys. Acta 307: 1-19.

De Pierre, J.W. and Ernster, L. (1977) Enzyme topology of intracellular membranes. Ann. Rev. Biochem. 46: 201-262.

De Pont, J.J.H.H.M., Van Prooijen-Van Eeden, A. and Bonting, S.L. (1973) Studies on ($Na^+ + K^+$) activated ATPase. XXXIV-phosphatidylserine not essential for ($Na^+ + K^+$)-ATPase activity. Biochim. Biophys. Acta 323: 487-494.

Donaldson, J., Minnich, J.L. and Barbeau (1972) Ouabain-induced seizures in rats: Regional and subcellular localization of ^3H-ouabain associated with Na^+-K^+-ATPase in brain. Can. J. Biochem. 50: 888-896.

Edidin, M. (1974) Rotational and translational diffusion in membranes. Ann. Rev. Bioeng. 3: 179-201.

Emmelot, P. and Bos, C.J. (1966) Studies on plasma membranes. III. Potassium-dependent p-nitrophenylphosphatase activity of plasma membranes isolated from rat liver. Biochim. Biophys. Acta 121: 375-385.

Erdmann, E. and Schoner, W. (1973) Ouabain-receptor interactions in ($Na^+ + K^+$)-ATPase preparations from different tissues and species: Determination of kinetic constants and dissociation constants. Biochim. Biophys. Acta. 307: 386-398.

Erdmann, E. and Schoner, W. (1974) Ouabain-receptor interactions in ($Na^+ + K^+$)-ATPase preparations. IV. The molecular structure of different cardioactive steroids and other substances and their affinity to the glycoside receptor. Naunyn-Schmiedeberg's Arch. Pharmacol. 283: 335-356.

Esmann, M., Skou, J.C. and Christiansen, C. (1979) Solubilization and molecular weight determinations of the ($Na^+ + K^+$)-ATPase from rectal glands of *Squalus acanthias*. Biochim. Biophys. Acta. 567: 410-420.

Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 10: 2606-2617.

Farias, R.N., Bloj, B., Morero, R.D., Sineriz, F. and Trucco, R.E. (1975) Regulation of allosteric membrane-bound enzymes through changes in membrane lipid composition. Biochim. Biophys. Acta. 415: 231-251.

Fenster, L.J. and Copenhaver, J.H. (1967) Phosphatidylserine requirement of ($Na^+ + K^+$)-activated ATPase from rat kidney and brain. Biochim. Biophys. Acta. 137: 406-408.

Fiske, C.H. and Subbarow, Y. (1925) The colorimetric determination of phosphorus. J. Biol. Chem. 66: 375-400.

Forbush, B. and Hoffman, J.F. (1979a) Direct photaffinity labelling of the primary region of the ouabain binding site of ($Na^+ + K^+$)-ATPase with [^{3}H] ouabain, [^{3}H] digitoxin, and [^{3}H] digitoxigenin. Biochim. Biophys. Acta 555: 299-306.

Forbush, B. III, and Hoffman, J.F. (1979b) Evidence that ouabain binds to the same large polypeptide chain of dimeric Na,K-ATPase that is phosphorylated from P_i. Biochemistry 18: 2308-2315.

Forbush, B. III, Kaplan, J.H. and Hoffman, J.F. (1978) Characterization of a new photoaffinity derivative of ouabain: Labelling of the large polypeptide and of a proteolipid component of the Na,K-ATPase. Biochemistry 17: 3667-3678.

Frenkel, E.J., Roelofsen, B., Brodbeck, U., Van Deenen, L.L.M. and Ott, P. (1980) Lipid-protein interactions in human erythrocyte-membrane acetylcholinesterase: Modulation of enzyme activity by lipids. Eur. J. Biochem. 109: 377-382.

Froehlich, J.P., Albers, R.W., Koral, G.I., Goebel, R. and Berman, M. (1976) Evidence for a new intermediate state in the mechanism of Na^+,K^+-adenosine triphosphatase. J. Biol. Chem. 251: 2186-2188.

Frickle, V. and Klaus, W. (1971) Evidence for two different Na^+ dependent [^{3}H] ouabain binding sites of a ($Na^+ + K^+$)-ATPase of guinea pig hearts. Br. J. Pharmacol. 61: 423-428.

Fujita, M., Nakao, T., Tashima, Y., Mizuno, N., Nagano, K., and Nakao, M. (1966) Potassium-ion stimulated p-nitrophenylphosphatase activity occurring in a highly specific adenosine triphosphatase preparation from rabbit brain. Biochim. Biophys. Acta. 117: 42-53.

Garrahan, P.J. and Glynn, I.M. (1967) The stoichiometry of the sodium pump. J. Physiol. 192: 217-235.

Garrahan, P.J., Pouchan, M.I. and Rega, A.F. (1969) Potassium activated phosphatase from human red blood cells. The mechanism of potassium activation. J. Physiol. (Lond.) 202: 305-327.

Garrahan, P.J. and Rega, A.F. (1972) Potassium activated phosphatase from human red blood cells. The effects of p-nitrophenylphosphate on cation fluxes. J. Physiol. (Lond.) 223: 595-617.

Gelbart, A. and Goldman, R.H. (1977) Correlation between microsomal Na,K-ATPase activity and ^{3}H-ouabain binding to heart tissue homogenates. Biochim. Biophys. Acta 481: 689-694.

Giotta, G.J. (1975) Native ($Na^+ + K^+$)-dependent adenosine triphosphatase has two trypsin-sensitive sites. J. Biol. Chem. 250: 5159-5164.

Giotta, G.J. (1976) Quaternary structure of ($Na^+ + K^+$)-dependent adenosine triphosphatase. J. Biol. Chem. 251: 1247-1252.

Glynn, I.M. (1962) Activation of adenosine triphosphatase activity in a cell membrane by external potassium and internal sodium. J. Physiol. (Lond.) 160: 18-19P.

Glynn, I.M. (1968) Membrane ATPase and cation transport. Br. Med. Bull. 24: 165-169.

Glynn, I.M., Hoffman, J.F. and Lew, V.L. (1971) Some partial reactions of the sodium pump. Phil. Trans. Roy. Soc. Lond. 262: 91-102.

Glynn, I.M. and Karlsih, S.J.D. (1975) The sodium pump. Ann. Rev. Physiol. 37: 13-55.

Goldin, S.M. (1977) Active transport of sodium and potassium ions by the sodium and potassium ion-activated adenosine triphosphatase from renal medulla. J. Biol. Chem. 252: 5630-5642.

Goldin, S.M. and Sweadner, K.J. (1975) Reconstitution of active transport by kidney and brain ($Na^+ + K^+$)-ATPase. Ann. N.Y. Acad. Sci. 264: 387-397.

Goldin, S.M. and Tong, S.W. (1974) Reconstitution of active transport catalyzed by the purified sodium and potassium ion stimulated adenosine triphosphatase from canine renal medulla. J. Biol. Chem. 249: 5907-5915.

Goldman, S.S. (1975) Cold resistance of the brain during hibernation. III. Evidence of a lipid adaptation. Ann. J. Physiol. 228: 834-838.

Goldman, S.S. and Albers, R.W. (1973) Sodium-potassium activated ATPase. The role of phospholipids. J. Biol. Chem. 248: 867-874.

Goldman, S.S. and Willis, J.S. (1973a) Cold resistance of the brain during hibernation. I. K^+ transport in cerebral cortex slices. Cryobiology 10: 212-217.

Goldman, S.S. and Willis, J.S. (1973b) Cold resistance of the brain during hibernation. II. Na-K-activated ATPase. Cryobiology 10: 218-224.

Goodman, S.L. and Wheeler, K.P. (1978) Ouabain binding to phospholipid dependent adenosine triphosphatase. Biochem. J. 169: 313-320.

Gordon, L.M., Sauerheber, R.D., Esgate, J.A., Dipple, I., Marchmont, R.J. and Houslay, M.D. (1980) The increase in bilayer fluidity of rat liver plasma membranes achieved by the local anaesthetic benzyl alcohol affects the activity of intrinsic membrane enzymes. J. Biol. Chem. 255: 4519-4527.

Grisham, C.M. and Barnett, R.E. (1973) The role of lipid-phase transitions in the regulation of the (sodium + potassium) adenosine triphosphatase. Biochemistry 12: 2635-2637.

Gruener, N. and Avi-Dor, Y. (1966) Temperature dependence of activation and inhibition of rat brain adenosine triphosphatase activated by sodium and potassium ions. Biochem. J. 100: 762-767.

Hall, E.R. and Brodbeck, U. (1978) Human erythrocyte membrane acetylcholine esterase. Incorporation into the lipid bilayer structure of liposomes. Eur. J. Biochem. 89: 159-167.

Hallam, C. and Whittam, R. (1975) The stimulation by phosphate of ouabain binding to the sodium pump and its dependence on the method of preparing the membranes. J. Physiol. (Lond.) 252: 84-85P.

Hansen, O. (1976) Non-uniform populations of G-strophanthin binding sites of ($Na^+ + K^+$)-activated ATPase. Apparent conversion to uniformity by K^+. Biochim. Biophys. Acta 443: 383-392.

Harris, W.E., Swanson, P.D. and Stahl, W.L. (1973) Ouabain binding sites and the ($Na^+ + K^+$)-ATPase of brain microsomal membranes. Biochim. Biophys. Acta 298: 680-689.

Hart, Jr., W.M. and Titus, E.O. (1973) Sulfhydryl groups of sodium-potassium transport adenosine triphosphatase. J. Biol. Chem. 248: 4674-4681.

Hegyvary, C. (1975) Covalent labelling of the digitalis binding compartment of plasma membranes. Mol. Pharmacol. 11: 588-594.

Hegyvary, C. and Post, R.L. (1969) Reversible inactivation of ($Na^+ + K^+$)-ATPase by removing and restoring phospholipids. In: Molecular Basis of Membrane Functions. ed. by D.C. Tosteson, pp. 519-528. Prentice-Hall, Inc., Englewood, Cliffs, New Jersey.

Hegyvary, C. and Post, R.L. (1971) Binding of ATP to Na,K-ATPase. J. Biol. Chem. 246: 5234-5240.

Hesketh, T.R., Smith G.A., Houslay, M.D., McGill, K.A., Bridsall, N.J.M., Metcalfe, J.C. and Warren, G.B. (1976) Annular lipids determine the ATPase activity of a calcium transport protein complexed with dipalmitoyllecithin. Biochemistry 15: 4145-4151.

Hilden, S. and Hokin, L.E. (1975) Active K^+ transport coupled to active Na^+ transport in vesicles reconstituted from purified Na and K ion activated ATPase from the rectal gland of *Squalus acanthias*. J. Biol. Chem. 250: 6296-6303.

Hilden, S. and Hokin, L.E. (1976) Coupled Na^+-K^+ transport in vesicles containing a purified (Na^+ + K^+)-ATPase and only phosphatidylcholine. Biochem. Biophys. Res. Commun. 69: 521-527.

Hilden, S., Rhee, H.M. and Hokin, L.E. (1974) Sodium transport by phospholipid vesicles, containing purified sodium and potassium ion-activated adenosine triphosphatase.

Hoffman, J.F. (1960) The link between metabolism and the active transport of Na in human red cell ghosts. Fed. Proc. 19: 127 (Abstr.).

Hoffman, J.F. (1962) Cation transport and structure of the red cell plasma membrane. Circulation 26: 1201-1213.

Hokin, L.E. and Dahl, J.L. (1972) The sodium-potassium adenosine-triphosphatase. In: Metabolic Pathways, Metabolic Transport, ed. by L.E. Hokin. vol VI. pp. 267-315. Academic Press - New York.

Hokin, L.E., Dahl, J.L., Deupree, J.D., Dixon, J.F., Hackney, J.F., and Perdue, J.F. (1973) Studies on the characterization of the sodium-potassium transport adenosine triphosphatase. X. Purification of the enzyme from the rectal gland of *Squalus acanthias*. J. Biol. Chem. 248: 2593-2605.

Hokin, L.E. and Hexum, T.D. (1972) Studies on the characterization of the sodium-potassium transport adenosine triphosphatase. IX. On the role of phospholipids in the enzyme. Arch. Biochem. Biophys. 151: 453-463.

Hokin, L.E. and Yoda, A. (1964) Inhibition by diisopropylfluorophosphate of a kidney transport adenosine triphosphatase by phosphorylation of a serine residue. Proc. Natn. Acad. Sci. (U.S.A.) 52: 454-461.

Hopkins, B.E., [illegible]gner, H. Jr., and Smith, J.W. (1976) Sodium and potassium activated adenosine triphosphatase of the nasal salt gland of the duck (*Anas platyrhynchos*). J. Biol. Chem. 251: 4365-4371.

Houslay, M.D., [illegible]n, G.B., Bridsall, N.J.M. and Metcalfe, J.C. (1975) Lipid phase transitions control β-hydroxybutyrate dehydrogenase activity in reconstituted-lipid protein complexes. FEBS Lett. 51: 146-151.

Huang, T.S., Chen, C.P., Weffler, V. and Raftery, A. (1961) A stable reagent for the Liebermann-Burchard reaction. Application to rapid serum cholesterol determination. Anal. Chem. 33: 1405-1407.

Inagaki, C., Lindenmayer, G.E. and Schwartz, A. (1974) Effects of sodium and potassium on binding of ouabain to the transport adenosine triphosphatase. J. Biol. Chem. 249: 5135-5140.

Israel, Y. and Titus, E. (1967) A comparison of microsomal Na^+, K^+-ATPase with K^+ acetylphosphatase. Biochim. Biophys. Acta 139: 450-459.

Jacobson, K. and Papahadjopoulos, D. (1975) Phase transitions and phase separations in phospholipid membranes induced by changes in temperature, pH and concentration of bivalent cations. Biochemistry 14: 152-161.

Jarnefelt, J. (1972) Lipid requirements of functional membrane structure as indicated by the reversible inactivation of (Na^+ + K^+)-ATPase. Biochim. Biophys. Acta 266: 91-96.

Jean, D.H., Albers, R.W. and Koval, G. (1975) Sodium-potassium activated adenosine triphosphatase of *Electrophorus* electric organ. X. Immunochemical properties of the lubrosolubilized enzyme and its constituent polypeptides. J. Biol. Chem. 250: 1035-1040.

Jean, D.H. and Albers, R.W. (1977) Molecular organization of subunits of electroplax, (sodium + potassium) activated adenosine triphosphatase. J. Biol. Chem. 252: 2450-2451.

Jørgensen, P.L. (1972) Purification of (Na^+ + K^+)-ATPase from the outer medulla of mammalian kidney. In: Role of Membranes in Secretory Processes. Eds., L. Bolis, R.D. Keynes and W. Wilbrandt. pp. 247-262 Amsterdam; North Holland.

Jørgensen, P.L. (1974a) Purification and characterization of (Na^+ + K^+)-ATPase. III. Purification from outer medulla of mammalian kidney after selective removal of membrane component by sodium dodecylsulphate. Biochim. Biophys. Acta 356: 36-52.

Jørgensen, P.L. (1974b) Purification and characterization of (Na^+ + K^+)-ATPase. IV. Estimation of the purity of the molecular weight and polypeptide content per enzyme unit in preparations from the outer medulla of rabbit kidney. Biochim. Biophys. Acta 356: 53-67.

Jørgensen, P.L. (1975a) Isolation and characterization of the components of the sodium pump. Q. Rev. Biophys. 7: 239-274.

Jørgensen, P.L. (1975b) Purification and characterization of (Na^+ + K^+)-ATPase. V. Conformational changes in the enzyme. Transitions between the Na^+ form and the K^+ form studied with tryptic digestion as a tool. Biochim. Biophys. Acta 401: 399-415.

Jørgensen, P.L. (1977) Purification and characterization of Na,K-ATPase. VI. Differential tryptic modification of catalytic functions of the purified enzyme in presence of NaCl and KCl. Biochim. Biophys. Acta 466: 97-108.

Jørgensen, P.L. and Skou, J.C. (1971) The influence of detergents on the activity of ($Na^+ + K^+$)-ATPase in preparations from the outer medulla of rabbit kidney. Biochim. Biophys. Acta 233: 366-380.

Jørgensen, P.L., Skou, J.C. and Solomonson, L.P. (1971) Purification and characterization of ($Na^+ + K^+$)-ATPase. II. Preparation by zonal centrifugation of highly active ($Na^+ + K^+$)-ATPase from the outer medulla of rabbit kidney. Biochim. Biophys. Acta 233: 381-394.

Jost, P.C., Griffith, O.H., Capaldi, R.A. and Vanderkooi, G. (1973) Evidence for boundary lipid in membranes. Proc. Natl. Acad. Sci. (U.S.A.) 70: 480-484.

Judah, J.D., Ahmed, K. and McLean, A.E.M.,(1962) Ion transport and phosphoproteins of human red cells. Biochim. Biophys. Acta 65: 472-480.

Kahlenberg, A., Galsworthy, P.R. and Hokin, L.E. (1967) Na,K-ATPase: acyl phosphate "intermediate" shown to be glutamyl phosphate. Science 157: 434-436.

Kahlenberg, A., Galsworthy, P.R., and Hokin, L.E. (1968) Studies on the characterization of the sodium-potassium transport adenosine triphosphatase. II. Characterization of the acyl phosphate intermediate as an L-glutamyl-gamma-phosphate residue. Arch. Biochem. Biophys. 126: 331-324.

Kawai, K., Nakao, M., Nakao, T. and Fujita, M. (1973) Purification and some properties of Na^+,K^+ -transport ATPase preparations at various purification steps from pig brain. J. Biochem., Tokyo 73: 979-991.

Kepner, G.R. and Macey, R.I. (1968) Membrane enzyme systems. Molecular size determinations by radiation inactivation. Biochim. Biophys. Acta 163: 188-203.

Kimelberg, H.K. (1975) Alterations in phospholipid dependent ($Na^+ + K^+$)-ATPase activity due to lipid fluidity. Effects of cholesterol and Mg^{++}. Biochim. Biophys. Acta 413: 143-156.

Kimelberg, H.K. (1976) Protein liposomes interactions and their relevance to the structure and function of cell membranes. Mol. Cell. Biochem. 10: 171-190.

Kimelberg, H.K. (1977) In: Dynamic Aspects of Cell Surface Organization. Eds., G. Poste and G. Nicolson. North-Holland - New York. pp. 205-294.

Kimelberg, H.K. and Papahadjopoulos, D. (1971) Interactions of basic proteins with phospholipid membranes: Binding and changes in the sodium permeability of phosphatidylserine vesicles. J. Biol. Chem. 246: 1142-1148.

Kimelberg, H.K. and Papahadjopoulos, D. (1972) Phospholipid requirements for ($Na^+ + K^+$)-ATPase activity: Head-group specificity and fatty acid fluidity. Biochim. Biophys. Acta 282: 277-292.

Kimelberg, H.K. and Papahadjopoulos, D. (1974) Effects of phospholipid acyl chain fluidity, phase transitions and cholesterol on ($Na^+ + K^+$)-stimulated adenosine triphosphatase. J. Biol. Chem. 249: 1071-1080.

Koyal, D., Rao, S.N. and Askari, A. (1971) Studies on the partial reactions catalyzed by the ($Na^+ + K^+$)-activated ATPase. I. Effects of simple anions and nucleoside triphosphates on the alkali-cation specificity of the p-nitrophenylphosphatase. Biochim. Biophys. Acta 225: 11-19.

Ku, D., Akera, T., Pew, C.L. and Brody, T.M. (1974) Cardiac glycosides: correlation among Na,K,-ATPase, sodium pump and contractility in the guinea pig heart. Naunyn-Schmeideberg's Arch. Pharmacol. 285: 185-200.

Kudoh, F., Nakamura, S., Yamaguchi, M. and Tonomura, Y. (1979) Binding of ouabain to Na^+,K^+-dependent ATPase during the ATPase reaction. J. Biochem. 86: 1023-1028.

Kuo, A.L. and Wade, C.G. (1979) Lipid lateral diffusion by pulsed nuclear magnetic resonance. Biochemistry 18: 2300-2308.

Kyte, J. (1971a) Purification of the sodium and potassium dependent adenosine triphosphatase from canine renal medulla. J. Biol. Chem. 246: 4157-4165.

Kyte, J. (1971b) Phosphorylation of a purified Na,K-ATPase. Biochem. Biophys. Res. Commun. 43: 1259-1265.

Kyte, J. (1972a) Titration of the cardiac glycoside binding site of the Na,K-ATPase. J. Biol. Chem. 247: 7634-7641.

Kyte, J. (1972b) Properties of the two polypeptides of sodium and potassium dependent adenosine triphosphatase. J. Biol. Chem. 247: 7642-7649.

Kyte, J. (1975) Structural studies of sodium and potassium ions activated adenosine triphosphatase. J. Biol. Chem. 250: 7443-7449.

Ladbrooke, B.D. and Chapman, D. (1969) Thermal analysis of lipids, proteins and biological membranes. Chem. Phys. Lipids 3: 304-367.

Lane, L.K., Anner, B.M., Wallick, E.T., Ray, M.V. and Schwartz, A. (1978) Effects of phospholipase. A treatment of the partial reactions of ouabain binding to a purified sodium and potassium activated adenosine triphosphatase. Biochem. Pharmacol. 27: 225-231.

Lane, L.K., Copenhaver, J.H. Jr., Lindenmayer, G.E. and Schwartz, A. (1973) Purification and characterization of ^3H ouabain binding to the transport adenosine triphosphatase from outer medulla of canine kidney. J. Biol. Chem. 248: 7197-7200.

Laris, P.C. and Letchworth, P.E. (1962) Cation influence on inorganic phosphate production in human erythrocytes. J. Cell. Comp. Physiol. 60: 229-234.

Lee, A.G., Bridsall, N.J., Metcalfe, J.C. Toon, P.A. and Warren, G.B. (1974) Clusters in lipid bilayers and the interpretation of thermal effects in biological membranes. Biochemistry 13: 3699-3705.

Lee, K.S. and Klaus, W. (1971) The subcellular basis for the mechanism of inotropic action of cardiac glycosides. Pharmacol. Rev. 23: 193-261.

Lelievre, L., Zachowski, A., Charlemagne, D., Laget, P. and Paraf, A. (1979) Inhibition of $(Na^+ + K^+)$-ATPase by ouabain. Involvement of calcium and membrane proteins. Biochim. Biophys. Acta 557: 399-408.

Linden, C.D. and Fox, C.F. (1973) A comparison of characteristic temperatures for transport in two unsaturated fatty acid auxotrophs of *Escherichia coli*. J. Supramol. Struc. 1: 535-544.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

Mahler, H.R. and Cordes, E.H. (1966) In: Biological Chemistry, Chapter of Metabolism of Carbohydrates, Harper-Row, New York. p. 434.

Mandel, F., Wallick, E.T. and Schwartz, A. (1977) Mg-stimulated ouabain binding to purified $(Na^+ + K^+)$-ATPase from lamb kidney. Fed. Proc. 36: (3): 274 (Abstr.).

Mandersloot, J.G., Roelofsen, B. and De Gier, J. (1978) Phosphatidylinositol as the endogenous activator of the $(Na^+ + K^+)$-ATPase in microsomes of rabbit kidney. Biochim. Biophys. Acta 508: 478-485.

Matsui, H. and Schwartz, A. (1966) Purification and properties of a highly active ouabain-sensitive $Na^+ + K^+$-dependent adenosine triphosphatase from cardiac tissue. Biochim. Biophys. Acta 128: 380-390.

McMurchie, E.J., Raison, J.K. and Cairneross, K.D. (1973) Temperature-induced phase changes in membranes of heart: A contrast between the thermal response of poikilotherms and homeotherms. Comp. Biochem. Physiol. 44B: 1017-1026.

Morero, R.D., Bloj, B., Farias, R.N. and Trucco, R.E. (1972) The allosteric transitions from membrane-bound enzymes: Behaviour of erythrocyte acetylcholinesterase from fat deficient rats. Biochim. Biophys. Acta 282: 157-165.

Nagai, K., Izumi, F. and Yoshida, H. (1966) Studies on potassium dependent phosphatase: its distribution and properties. J. Biochem. (Tokyo) 39: 295-303.

Nagai, K. and Yoshida, H. (1966) Biphasic effects of nucleotides on potassium dependent phosphatase. Biochim. Biophys. Acta 128: 410-412.

Neufeld, A.H. and Levy, H.M. (1970) The steady state level of phosphorylated intermediate in relation to the two sodium dependent adenosine triphosphatase of calf brain microsomes. J. Biol. Chem. 245: 4962-4967.

Nicholson, G.L. (1976) Transmembrane control of the receptors on normal and tumor cells. I. Cytoplasmic influence over cell surface components. Biochim. Biophys. Acta 457: 57-108.

Nishigaki, I., Chen, F.T. and Hokin, L.E. (1974) Studies on the characterization of the sodium-potassium transport adenosine triphosphatase. XV. Direct chemical characterization of the acyl phosphate in the enzyme as an aspartyl-β-phosphate residue. J. Biol. Chem. 249: 4911-4916.

Nogouchi, T. and Freed, S. (1971) Dissociation of lipid components and reconstitution at -75°C of Mg^{++} dependent, Na^+ and K^+ stimulated, adenosine triphosphatase in rat brain. Nature (New Biol.) 230: 148-150.

Ohnishi, T. and Kawamura, H. (1964) Roles des phosphatides dane l'adenosine triphosphatase sensitive a l'ouabaine localisee dane les membranes d'erythrocyte. J. Biochem. (Tokyo) 56: 377-378.

Opit, L.J. and Charnock, J.S. (1965) A molecular model for a sodium pump. Nature 208: 471-474.

Palatini, P., Dabbeni-Sala, F. and Bruni, A. (1972) Reactivation of a phospholipid-depleted sodium, potassium-stimulated ATPase. Biochim. Biophys. Acta 288: 413-422.

Palatini, P., Dabbeni-Sala, F., Pitotti, A., Bruni, A. and Mandersloot, J.C. (1977) Activation of (Na^+ + K^+)-dependent ATPase by lipid vesicles of negative phospholipids. Biochim. Biophys. Acta 466: 1-9.

Papahadjopoulos, D. (1976) The role of cholesterol as a membrane component: Effects on lipid-protein interactions. In: Lipids (vol 1) Eds., R. Paoletti, G. Porcellati and G. Jacini. Raven Press: New York.

Papahadjopoulos, D., Cowden, M. and Kimelberg, H. (1973) Role of cholesterol in membranes: Effects on phospholipid-protein interactions, membrane permeability and enzyme activity. Biochim. Biophys. Acta 330: 8-26.

Papahadjopoulos, D., Poste, G., Schaeffer, B.E. and Vail, W.J. (1974) Membrane fusion and molecular segregation in phospholipid vesicles. Biochim. Biophys. Acta 352: 10-28.

Perrone, J.R., Hackney, J.F., Dixon, J.F. and Hokin, L.E. (1975) Molecular properties of purified sodium + potassium activated adenosine triphosphatase and their subunits from the rectal gland of *Squalus acanthias* and the electric organ of *Electrophorus electricus*. J. Biol. Chem. 250: 4178-4184.

Peterson, G.L. (1977) A simplification of the protein assay method of Lowry *et al*. which is more generally applicable. Anal. Biochem. 83: 346-356.

Phillips, M.C. and Hauser, H. (1972) The inter- and intra- molecular mixing of hydrocarbon chains in lecithin/water system. Chem. Phys. Lipids 8: 127-133.

Phillips, M.C., Williams, R.M. and Chapman, D. (1969) On the nature of hydrocarbon chain motions in lipid liquid crystals. Chem. Phys. Lipids 3: 234-244.

Pink, D.A. and Chapman, D. (1979) Protein-lipid interactions in bilayer membranes: A lattice model. Proc. Natl. Acad. Sci. (U.S.A.) 76: 1542-1546.

Pitotti, A., Dabbeni-Sala, F. and Bruni, A. (1980) Phospholipid-dependent assembly of mitochondrial ATPase complex. Biochim. Biophys. Acta 600: 79-90.

Poo, M. and Cone, R.A. (1974) Lateral diffusion of rhodopsin in the photoreceptor membrane. Nature 247: 438-441.

Post, R.L. and Kume, S. (1973) Evidence for an aspartyl phosphate residue at the active site of sodium and potassium ion transport adenosine triphosphatase. J. Biol. Chem. 248: 6993-7000.

Post, R.L., Merritt, C.R., Kinsolving, C.R. and Albright, C.D. (1960) Membrane ATPase as participant in the active transport of Na^+ and K^+ in the human erythrocyte. J. Biol. Chem. 235: 1796-1802.

Priestland, R.N. and Whittam, R. (1972) The temperature dependence of activation by phosphatidylserine of the sodium pump adenosine triphosphatase. J. Physiol. (Lond.) 220: 353-361.

Racker, E. (1977) Perspectives and limitations of resolutions - Reconstitution experiments. J. Supramol. Struct. 6: 215-228.

Racker, E., Knowles, A.F. and Eytan, E. (1975) Resolution and reconstitution of ion-transport systems. Ann. [redacted]. Acad. Sci. 264: 17-33.

Rega, A.F., Garrahan, P.J. and Pouchan, M.I. (1970) Potassium activated phosphatase from human red blood cells. The asymmetrical effects of K^+, Na^+, Mg^{++} and adenosine triphosphate. J. Membrane Biol. 3: 14-25.

Rendi, R. and Uhr, M.L. (1964) Sodium, potassium-requiring adenosinetriphosphatase activity. I. Purification and properties. Biochim. Biophys. Acta 89: 520-531.

Repke, K.H.R. (1963) Metabolism of Cardiac Glycosides. In: New Aspects of Cardiac Glycosides (Proceedings of the first International pharmacological conference, Stockholm) vol. 3, pp. 47-73. Ed., G. Wilbrant, London. Permagon Press.

Rhee, H.M. and Hokin, L.E. (1975) Inhibition of the purified sodium-potassium activated adenosine triphosphatase from the rectal gland of *Squalus acanthias* by antibody against the glycoprotein sub-unit. Biochem. Biophys. Res. Commun. 63: 1139-1145.

Rhee, H.M. and Hokin, L.E. (1979) Inhibition of ouabain binding to $(Na^+ + K^+)$-ATPase by antibody against the catalytic subunit but not by antibody against the glycoprotein subunit. Biochim. Biophys. Acta 558: 108-112.

Rice, D.M., Meadow, M.D., Scheinman, A.O., Goni, F.M., Gómez-Fernandez, J.C., Moscarello, M.A. Chapman, D. and Oldfield, E. (1979) Protein-lipid interactions. A nuclear magnetic resonance study of sarcoplasmic reticulum Ca^{++}, Mg^{++}-ATPase, lipophilin and proteolipid apoprotein-lecithin systems and a comparison with the effects of cholesterol. Biochemistry 18: 5893-5903.

Richardson, T. and Tappel, A.L. (1962) Swelling of fish mitochondria. J. Cell Biol. 13: 43-54.

Robinson, J.D. (1973) Variable affinity of the $(Na^+ + K^+)$-dependent adenosine triphosphatase for potassium. Studies using beryllium inactivation. Arch. Biochem. Biophys. 156: 232-243.

Roelofsen, B. and Van Deenen, L.L.M. (1973) Lipid requirement of membrane bound ATPase. Studies on human erythrocyte ghosts. Eur. J. Biochem. 40: 245-257.

Roelofson, B., Zwaal, R.F.A. and Van Deenen, L.L.M. (1971) Lipoprotein integrity and enzymatic activity of the erythrocyte membrane. Adv. Exp. Med. Biol. 14: 209-228.

Ruoho, A. and Kyte, J. (1974) Photoaffinity labelling of the ouabain binding site on $(Na^+ + K^+)$-adenosine triphosphatase. Proc. Natl. Acad. Sci. (U.S.A.) 71: 2352-2356.

Sachs, S., Rose, J.D. and Hirschowitz, B.I. (1967) Acetyl phosphatase in brain microsomes: a partial reaction of ($Na^+ + K^+$)-ATPase. Arch. Biochem. Biophys. 119: 277-281.

Sandermann, H. (1978) Regulation of membrane enzymes by lipids. Biochim. Biophys. Acta 515: 209-237.

Schatzmann, H.J. (1953) Herzglycoside als Hemmstoffe fur den aktives kalium and natrium transport durch die erythrocytenmembran. Helv. Physiol. Pharmacol. Acta 11: 346-354.

Schoner, W., Paul, S.H. and Patzelt-Wenezler, R. (1977) Myocardial failure. Ed. G. Ricker, A. Weber and J. Goodwin. pp. 104-119, Berlin : Springer-Verlag.

Schoner, W., Von Ilberg, C., Kramer, R. and Seruber, W. (1967) On the mechanism of Na^+- and K^+-stimulated hydrolysis of adenosine triphosphate. Eur. J. Biochem. 1: 334-343.

Schwartz, A. (1976) Is the cell membrane Na^+, K^+-ATPase enzyme system the pharmacological receptor for digitalis. Circ. Res. 39: 2-7.

Schwartz, A., Allen, J.C. and Harigaya, S. (1969) Possible involvement of cardiac Na^+, K^+-ATPase in the mechanism of action of cardiac glycosides. J. Pharmacol. Exp. Ther. 168: 31-41.

Schwartz, A., Allen, J.C., Van Winkle, W.B. and Munson, R. (1974) Further studies on the correlation between the p-inotropic action of ouabain and interaction with the Na^+, K^+-adenosine triphosphatase: isolated perfused rabbit and cat hearts. J. Pharmacol. Exp. Ther. 191: 119-127.

Schwartz, A., Lindenmayer, G.E. and Allen, J.C. (1975) The sodium-potassium adenosine triphosphatase: Pharmacological, physiological and biochemical aspects. Pharmacol. Rev. 27: 3-134.

Seelig, J. and Waespe-Sarcevic, N. (1978) Molecular order in Cis and Trans unsaturated phospholipid bilayers. Biochemistry 17: 3310-3315.

Sen, A.K., Tobin, T. and Post, R.L. (1969) A cycle for ouabain inhibition of sodium and potassium dependent adenosine triphosphatase. J. Biol. Chem. 244: 6596-6604.

Shattil, S.J., Anaya-Garlindo, R., Bennett, J., Colman, R.W. and Cooper, R.A. (1975) Platelet hypersensitivity induced by cholesterol incorporation. J. Clin. Invest. 55: 636-643.

Shimshick, E.J. and McConnell, H.M. (1973) Lateral phase separation in phospholipid membrane. Biochemistry 12: 2351-2360.

Shinitzky, M. and Inbar, M. (1976) Microviscosity parameters and protein mobility in biological membranes. Biochim. Biophys. Acta 433: 133-149.

Shinitzky, M. and Rivnay, B. (1977) Degree of exposure of membrane proteins determined by fluorescence quenching. Biochemistry 16: 982-986.

Siegel, G.J. and Goodwin, B. (1972) Sodium-potassium activated adenosine triphosphatase - potassium regulation of enzyme phosphorylation. Sodium-stimulated, potassium-inhibited uridine triphosphate hydrolysis. J. Biol. Chem. 247: 3630-3637.

Sinensky, M., Minneman, K.P. and Molinoff, P.B. (1979a) Increased membrane acyl chain ordering activates adenylate cyclase. J. Biol. Chem. 254: 9135-9141.

Sininsky, M., Pinkerton, F., Sutherland, E. and Simon, F.R. (1979b) Rate limitation of ($Na^+ + K^+$)-stimulated adenosinetriphosphatase by membrane acyl chain ordering. Proc. Natl. Acad. Sci. (U.S.A.) 76: 4893-4897.

Singer, S.J. (1974) The molecular organization of membranes. Ann. Rev. Biochem. 43: 805-833.

Singer, S.J. and Nicolson, G.L. (1972) The fluid mosaic model of the structure of cell membrane. Science 175: 720-731.

Skou, J.C. (1957) The influence of some cations on an adenosine triphosphatase from peripheral nerves. Biochim. Biophys: Acta 23: 394-401.

Skou, J.C. (1960) Further investigations on a ($Mg^{++} + Na^+$) activated adenosine triphosphatase, possibly related the the active, linked transport of Na^+ and K^+ across the nerve membrane. Biochim. Biophys. Acta 42: 6-23.

Skou, J.C. (1965) Enzymatic basis for active transport of Na^+ and K^+ across cell membrane. Physiol. Rev. 45: 596-617.

Skou, J.C. (1975) The ($Na^+ + K^+$) activated enzyme system and its relationship to transport of sodium and potassium. Q. Rev. Biophys. 7: 401-434.

Skou, J.C. and Esmann, M. (1979) Preparation of membrane-bound and of solubilized ($Na^+ + K^+$)-ATPase from rectal glands of *Squalus acanthias*: The effect of preparative procedures on purity, specific activity and molar activity. Biochim. Biophys. Acta 567: 436-444.

Skulskii, I.A., Mannineu, V. and Jarnefelt, J. (1973) Interaction of thallous ions with the cation transport mechanism in erythrocytes. Biochim. Biophys. Acta 298: 702-709.

Steim, J.M., Tourtellotte, M.E., Reinert, J.C., McElhaney, R.N. and Rader, R.L. (1969) Calorimetric evidence for the liquid-crystalline state of lipids in a biomembrane. Proc. Natl. Acad. Sci. (U.S.A.) 63: 104-109.

Stier, A. and Sackmann, E. (1973) Spin labels as enzyme substrates. Heterogeneous lipid distribution in liver microsomal membranes. Biochim. Biophys. Acta 311: 400-408.

Swanson, P.D. (1966) Temperature dependence of sodium ion activation of the cerebral microsomal adenosine triphosphatase. J. Neurochem. 13: 229-236.

Swanson, P.D., Bradford, H.F. and McIlwain, H. (1964) Stimulation and solubilization of the Na,K-ATPase of cerebral microsomes by surface active agents. Biochem. J. 92: 235-247.

Tanaka, R. (1969) Comparison of lipid effects on K^+-Mg^{++} activated p-nitrophenylphosphatase and Na^+-K^+-Mg^{++} activated adenosine triphosphatase of membrane. J. Neurochem. 16: 1301-1307.

Tanaka, R. and Abood, L.G. (1964) Phospholipid requirement of Na^+, K^+-activated adenosine triphosphatase from rat brain. Arch. Biochem. Biophys. 108: 47-5..

Tanaka, R. and Sakamoto, T. (1969) Molecular structure in phospholipid essential to activate $Na^+ + K^+ + Mg^{++}$-dependent ATPase and $K^+ + Mg^{++}$-dependent phosphatase of bovine cerebral cortex. Biochim. Biophys. Acta 193: 384-393.

Tanaka, R., Sakamoto, T. and Sakamoto, Y. (1971) Mechanism of lipid activation of Na,K-Mg-activated adenosine triphosphatase and K,Mg-activated phosphatase of bovine cerebral cortex. J. Memb. Biol. 4: 42-51.

Tanaka, R. and Strickland, K.P. (1965) Role of phospholipid in the activation of Na^+, K^+-activated adenosine triphosphatase of beef brain. Arch. Biochem. Biophys. 111: 583-592.

Tanaka, R. and Teruya, A. (1973) Lipid dependence of activity-temperature relationship of $(Na^+ + K^+)$-activated ATPase. Biochim. Biophys. Acta 323: 584-591.

Taniguchi, K. and Iida, S. (1971) The binding of ouabain to Na^+-K^+-dependent ATPase treated with phospholipase. Biochim. Biophys. Acta 233: 831-833.

Taniguchi, K. and Iida, S. (1972) The effect of phospholipids on the apparent activation energy of $(Na^+ + K^+)$-ATPase. Biochim. Biophys. Acta 274: 536-541.

Taniguchi, K. and Iida, S. (1973) The role of phospholipids in the binding of ouabain to sodium and potassium dependent adenosine triphosphatase. Mol. Pharmacol. 9: 350-359.

Taniguchi, K. and Tonomura, Y. (1971) Inactivation of $(Na^+ + K^+)$ dependent ATPase by phospholipase treatment and its reactivation by phospholipids. J. Biochem. 69: 543-557.

Tashima, Y. and Hasegawa, M. (1975) The mode of activation by potassium of potassium-dependent and ouabain sensitive phosphatase activity. Arch. Biochem. Biophys. 171: 568-574.

Ting-Beall, H.P., Clark, D.A., Sueler, C.H. and Wells, W.W. (1973) Studies on the interaction of chick brain microsomal Na^+,K^+-ATPase with copper. Biochim. Biophys. Acta 291: 229-236.

Titus, E.O. and Hart, W.M. Jr. (1974) The use of sulfhydryl reagents to identify proteins undergoing ligand-dependent conformational changes associated with the function of $(Na^+ + K^+)$-ATPase. Ann. N.Y. Acad. Sci. 242: 246-253.

Tobin, T., Akera, T., Baskin, S.I. and Brody, J.M. (1973) Calcium ions and sodium-and-potassium-dependent adenosine triphosphatase: its mechanism of inhibition and identification of the E_1-P intermediate. Mol. Pharmacol. 9: 336-349.

Tobin, T., Baskin, S.I. and Akera, T. (1972) Nucleotide specificity of the Na^+ stimulated phosphorylation and ^3H-ouabain binding reactions of Na^+. Mol. Pharmacol. 8: 256-263.

Tobin, T. and Sen, A.K. (1970) Stability and ligand sensitivity of ^3H-ouabain binding to Na^+, K^+-ATPase. Biochim. Biophys. Acta 198: 120-131.

Toda, G. (1968) The effects of cations on the inhibition of sodium and potassium activated ATPase by beryllium. J. Biochem. (Tokyo) 64: 457-464.

Towle, D.W. and Copenhaver, J.H. Jr. (1970) Partial purification of a soluble Na^+, K^+-dependent ATPase from rabbit kidney. Biochim. Biophys. Acta 203: 124-132.

Trauble, H. and Eibl, H. (1974) Electrostatic effects on lipid phase transitions: Membrane structure and ionic environment. Proc. Natl. Acad. Sci.(U.S.A.) 71: 214-219.

Trauble, H. and Overath, P. (1973) The structure of *Escherichia coli* membranes studied by fluorescence measurements of lipid phase transitions. Biochim. Biophys. Acta 307: 491-512.

Uesugi, S., Dulak, N.C., Dixon, J.F., Hexum, T.D., Dahl, J.L., Perdue, J.F. and Hokin, L.E. (1971) Studies on the characterization of the sodium-potassium transport adenosine triphosphatase. VI. Large scale partial purification and properties of a lubrol solubilized bovine brain enzyme. J. Biol. Chem. 246: 531-543.

Van Alstyne, E., Bartschat, D.K., Poc, S. and Lindenmayer, G.E. (1978) Cardiac membrane vesicles enriched in ouabain sites. Fed. Proc. 37: 779 (Abstr.).

Verkleij, A.J., De Kruyff, B., Ververgaert, P.H.J.Th., Tocanne, J.F. and Van Deenen, L.L.M. (1974) The influence of pH, Ca^{2+} and protein on the thermotropic behaviour of the negatively charged phospholipid, phosphatidylglycerol. Biochim. Biophys. Acta 339: 432-437.

Ververgaert, P.H.J.Th., Verkleij, A.J., Elbers, P.F. and Van Deenen, L.L.M. (1973) Analysis of the crystallization process in lecithin liposomes: A freeze-etch study. Biochim. Biophys. Acta 311: 320-329.

Wacker, H., Muller, F. and Semenza, G. (1976) Incorporation of hydrophobic aminopeptidase from hog kidney into egg lecithin liposomes: Number and orientation of aminopeptidase molecules in the lecithin vesicles. FEBS Lett. 68: 145-152.

Walker, A.J. and Wheeler, K.P. (1975) Polar head-group and acyl side-chain requirements for phospholipid dependent ($Na^+ + K^+$)-ATPase. Biochim. Biophys. Acta 394: 135-144.

Wallick, E.T., Lane, L.K. and Schwartz, A. (1979) Biochemical mechanism of the sodium-pump. Ann. Rev. Physiol. 41: 397-411.

Wallick, E.T. and Schwartz, A. (1974) Thermodynamics of the rate of binding of ouabain to the sodium, potassium adenosine triphosphatase. J. Biol. Chem. 249: 5141-5147.

Wallick, E.T., Schwartz, A., Michael, L.H. and Huggins, R.A. (1977) Ouabain binding to heart muscle and inotropy. Fed. Proc. 36: 972 (Abstr.).

Warren, G.B., Housaly, M.D., Metcalfe, J.C. and Bridsall, N.J.M. (1975) Cholesterol is excluded from the phospholipid annulus surrounding an active calcium transport protein. Nature 255: 684-687.

Warren, G.B., Toon, P.A., Bridsall, N.J.M., Lee, A.G. and Metcalfe, J.C. (1974) Reversible lipid transition of the activity of pure adenosine triphosphatase lipid complexes. Biochemistry 13: 5501-5507.

Weber, K. and Osborn, M. (1969) The reliability of molecular weight determination by dodecylsulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244: 4406-4412.

Wheeler, K.P. (1975) Role of phospholipid in the intermediate steps of the sodium-plus-potassium ion-dependent triphosphatase reaction. Biochem. J. 146: 729-738.

Wheeler, K.P. and Walker, A.J. (1975) Differential effects of lipid depletion on membrane sodium-plus-potassium ion-dependent adenosine triphosphatase and potassium ion dependent phosphate. Biochem. J. 146: 723-727.

Wheeler, K.P., Walker, A.J. and Barker, D.M. (1975) Lipid requirement of the membrane sodium-plus-potassium ion dependent adenosine triphosphatase system. Biochem. J. 146: 713-722.

Wheeler, K.P. and Whittam, R. (1970a) The involvement of phosphatidylserine in adenosine triphosphatase activity of the sodium pump. J. Physiol. (Lond.) 207: 303-328.

Wheeler, K.P. and Whittam, R. (1970b) ATPase activity of the sodium pump needs phosphatidylserine. Nature 225: 449-450.

Whittam, R. (1962) The asymmetrical stimulation of a membrane adenosine triphosphatase in relation to active transport. Biochem. J. 84: 110-118.

Whittam, R., Hallam, C. and Wattam, D.G. (1976) Observations on ouabain binding and membrane phosphorylation by the sodium pump. Proc. R. Soc. Lond. B. 193: 217-234.

Whittam, R. and Wheeler, K.P. (1970) Transport across cell membranes. A. Rev. Physiol. 32: 21-60.

Wiley, J.S. and Cooper, R.A. (1975) Inhibition of cation cotransport by cholesterol enrichment of human red cell membranes. Biochim. Biophys. Acta 413: 425-431.

Yoshida, H., Izumi, F. and Nagai, K. (1966) Carbamylphosphate, a preferential substrate of K^+-dependent phosphatase. Biochim. Biophys. Acta 120: 183-186.

Yoshida, H., Nagai, K., Ohashi, T. and Nakagawa, Y. (1969) K^+-dependent phosphatase activity observed in the presence of both adenosine triphosphatase and Na^+. Biochim. Biophys. Acta 71: 178-185.

Zachowski, A., Lelievre, L., Aubry, J., Charlemagne, D. and Paraf, A. (1977) Roles of proteins from inner face of plasma membranes in susceptibility of (Na^+ + K^+)-ATPase stimulated Mg^{++}-adenosinetriphosphatase to ouabain. Proc. Natl. Acad. Sci. (U.S.A.) 74: 633-637.