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The Sodium-Potassium Adenosine Triphosphatase: Pharmacological, Physiological and Biochemical Aspects*

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I. Introduction

The cell membrane, by virtue of its complex structure and enzymatic characteristics, is not only able to control the passage of substrates and ions into and out of the cell but also contains important immunological, hormonal, and pharmacological receptor sites. Reports published in 1973 indicate that there have been extensive efforts to localize receptors in cell membranes that are specific for insulin (131), acetylcholine (336), and glucagon (528). Newer techniques for tissue fractionation, enzyme purification, and chromatography have aided considerably in these studies.

It is possible that, in terms of evolution, one of the earliest functions of the cell membrane was the maintenance of appropriate intracellular concentrations of sodium and potassium. Therefore, it is not unlikely that one of the first enzymatic functions of the cell membrane was some type of pump mechanism. Schatzmann (556) in studying sodium and potassium fluxes in the red blood cell, a relatively simple membrane system that had been used by many investigators for studying transport, found that adenosine triphosphate (ATP) was required for transport (in agreement with the data of Straub and Gardos) and that a cardiac glycoside poisoned the cation pump. Skou (609) fractionated a crab nerve homogenate into a microsomal component which exhibited ATPase activity that was stimulated by the addition of sodium plus potassium in the presence of magnesium. The cationic-stimulated component of this ATPase was inhibited by the same cardiac glycosides used earlier by Schatzmann (610). Thus, one of the most exciting chapters in physiology, pharmacology, and biochemistry began. In the 15 years since this discovery, over 3000 papers have been published on various aspects of the Na^+,K^+-ATPase enzyme system. The documentation of literature references in this review is not meant to be complete, but it is, we hope, representative. A large number of reviews dealing with the Na^+,K^+-ATPase have been published and the reader is referred to these for information and concepts which are not included in the present review (71a, 116a, 132a, 151a, 226a, 229a, 284, 325a, 337a, 389, 430a, 493, 499, 550a, 577, 615, 616, 617a, 708a).

II. Current Concepts of Cell Membrane Structure

The main components of membranes are lipids and proteins which constitute about 40 and 50%, respectively, of the membranes by weight (86). Although carbohydrate is usually present, it probably represents less than 10% of the mass of the membrane (86). Originally, the cell membrane was thought to be arranged in a bimolecular leaflet (236), and such a model was eventually suggested chiefly on the basis of experiments with myelin (137). This model proposed that a protein layer was spread on both surfaces of a lipid bilayer in some type of globular configuration (529). In the ensuing years this somewhat simplified model has been extensively revised. At the present time there is little continued support for the "unit membrane concept," although most individuals have been reluctant to adopt a specific generalized membrane model [see reviews by Bretscher (86) and Hendler (262)].

The erythrocyte membrane has been studied in great detail chiefly because it is easy to obtain in pure form, is relatively simple, and is representative of membranes in general in terms of structure and function (86). Several proteins from the erythrocyte membrane have been identified, and studies are being carried out to determine how each of the proteins are located with respect to the lipid matrix. Two of the major components observed on gels seem to be located on the external surface of the membrane. One is a glycoprotein, and the other has been called component a. Component a has a molecular weight of about 100,000 and represents the only major protein exposed on the surface of the red blood cell. Bretscher's current suggestion is that component a may not only be a structural protein but may also have some specific enzymatic function and, more specifically, may be an "anion channel."

The major glycoprotein of the human erythrocyte membrane has been isolated and purified and its molecular anatomy has been partially characterized, chiefly through the intriguing work of Marchesi *et al.* (421, 424). This protein, called glycophorin, is a single polypeptide chain which carries many blood group antigens and lectin receptors. The polypeptide chain has three distinct segments or sites that have unique chemical properties and appear to be located in different regions of the membrane. All of the carbohydrates appear to be covalently linked to the N-terminal half of the polypeptide chain and are exposed to the external environment. The C-terminal third of the polypeptide appears to be located internal to the lipid matrix and may extend into the cytoplasm of the cell. The portion which connects the two segments appears to be composed of nonpolar and hydrophobic amino acids which "span" the fatty acid region of the membrane. The hydrophobic center segments of the polypeptide chain appear to be linked to 85 Å globular structures located within the lipid region of the membrane. The hydrophobic segments of the glycoprotein and the intramembraneous particle together "represent a new structural unit of the red cell membrane which may have important functional properties" (421). One of these functions may be related to the large number of negative charges this molecule carries and which could serve to keep erythrocytes apart from one another. Another possible function is that the negative charges may in some way bind important cations such as calcium. Moreover, it is possible that the glycoprotein is involved in the action of digitalis.

A general picture of the erythrocyte membrane includes a lipid bilayer which has an asymmetrical composition in its phospholipid and glycolipid components. A major protein and glycoprotein are located in a fixed orientation across the membrane, and many more proteins are associated with the inner surface of the bilayer. The erythrocyte membrane also has compositional asymmetry within its hydrophobic phase. Phosphatidylcholine, sphingomyelin, and glycolipids all contain very few polyunsaturated fatty acid residues. By contrast, phosphatidylethanolamine and phosphatidylserine are rich in these components, particularly arachidonic acids. It is possible that these polyunsaturated residues in the inner half of the bilayer provide a less ordered phase which may, therefore, be a better solvent for accommodating proteins than the outer half of the bilayer. The membrane presumably would always have a polarity, even in the absence of any associated protein. It is obvious, as indicated above, that some proteins extend across the entire bilayer. The carbohydrate may prevent the protein from diffusing out of the membrane into the cytoplasm and may act as a sort of "lock" on the protein. It is possible that lateral motion or rotation of lipids and proteins occur in the plane of the bilayer. According to Brewer and Passwater

(86a), the P=O radical, which is a component of all membranes and characterizes surfaces containing phospholipids, constitutes a physical explanation for membrane transport. When excited, the double bonds induce a powerful negative intrinsic field strength, which attracts internal potassium. In turn, the space change induced by the ion layer excites all surfaces. The "excited bands" return to the ground state in 10^{-4} seconds. The excess of cations within the membrane may be forced out largely in the direction of the plasma. Sodium ions tend to carry polar molecules with which they become associated while near the inner wall.

A spectrophotometric scan of a sodium dodecyl sulfate (SDS)-polyacrylamide gel after electrophoresis of the solubilized membrane reveals numerous polypeptide chains. As indicated above, only a few of these components have been associated with a biological activity: a) The red cell membrane contains a Na^+,K^+-ATPase and a calcium-ATPase. The Na^+,K^+,ATPase appears to be associated, as will be discussed later, with a polypeptide chain of about 100,000 daltons. The calcium ATPase, in a water extract of erythrocyte ghosts, was observed in 1970 by Wolf (709) and seems to be associated with a calcium efflux mechanism. It is possible that the calcium-stimulated ATPase may be present in the 200,000 dalton class of polypeptides, although the basic unit of the calcium ATPase may also be 100,000 daltons. The Na^+,K^+-ATPase and calcium ATPase may be comprised of at least one chemically identical phosphorylated component (aspartyl residue). b) An acetylcholinesterase with a reactive serine at the active site has been found to be associated with a polypeptide chain of 90,000 daltons. c) A number of blood group antigens, present on the carbohydrate-containing polypeptide chains, represent less than 15% of the total membrane protein. The four highest molecular weight polypeptides are released from the intact ghost by extraction with water and apparently, contain calcium ATPase activity. These components appear fibrinous in the electron microscope and were originally described by Marchesi and Steers (423), who called them spectrin. It is possible that spectrin may lie on the inner surface of the red blood cell (*i.e.*, that surface facing the cytoplasm) and it is suggested that this material may be a contractile protein and, as such, contributes to the mechanical properties of the intact red cell membrane. Studies of the binding between spectrin and glycophorin and pure phospholipids should prove to be important in the elucidation of the molecular organization of the red blood cell membrane (526).

Several types of receptor substances have been associated with the cell membrane and several of these have been studied extensively. One, the nicotinic acetylcholine binding site, appears to involve a polypeptide with a molecular weight of approximately 42,000 daltons (336). A second receptor substance, namely that for insulin, has been isolated from liver and fat cells by Cuatrecasas (131). This receptor also appears to be located exclusively on the surface of the cell and binds insulin. A third receptor substance which has been studied extensively by Rodbell (528) involves that for glucagon. For example, in rat liver plasma membranes, a regulatory component that specifically binds glucagon has been discovered along with a catalytic component which converts ATP to cyclic adenosine monophosphate (AMP). Hormonal activation is dependent on guanosine 5′-triphosphate (GTP) or ATP which binds reversibly to allosteric sites on the enzyme system.

The subject of this review, the Na^+,K^+-ATPase, represents another "receptor"-type protein that has functional activity and is associated with the cell membrane. Moreover, a component of this important enzyme system appears to be a receptor for the digitalis glycosides. Recent attempts at localization have been only partially successful, but it is thought that

both the magnesium ATPase and the sodium plus potassium component are located along the inner surface of the red cell ghost membranes (598). The use of ^{3}H-ouabain and radioautography at the ultrastructural level has been employed in attempts to specifically localized the digitalis site.

The Na^+,K^+-ATPase appears to be associated not only with the energy-linked movements of sodium and potassium but also with the control of calcium fluxes and may, indeed, be structurally related to the other receptor regions discussed above. Recent evidence, for example, suggests that there is an association between this enzyme system and at least one other important enzyme, adenylyl cyclase. Finally, there is the possibility that this enzymatic system represents an important site of action for digitalis glycosides. Whether the interaction between Na^+,K^+-ATPase and cardiac glycosides is directly or indirectly associated with a pharamcological action is an intriguing question and is discussed below.

III. Localization of the Na^+,K^+-ATPase

Since the erythrocyte membrane is the only membrane in this type of cell, there is no question that the Na^+,K^+-ATPase associated with the red cell is specifically located on the cell membrane. In most other tissues, however, localization of the Na^+,K^+-ATPase has proved difficult, and many studies have been carried out in attempts to localize the enzymatic system. A few of these studies are reviewed here since they show not only some of the difficulties involved but also suggest newer approaches to the problem.

A rather simple way to localize the Na^+,K^+-ATPase in, for example, the heart, would be to isolate well defined membrane fractions from various parts of the cardiac cell. This has not been successful, however, because of the difficulty in obtaining "clean" fractions. Such an achievement would be of considerable importance since the enzyme is believed to be localized in the sarcolemma. A number of procedures have been described which are claimed to yield sarcolemmal preparations of "high purity" and "good yield" (346, 637) but, in our judgment, these newer procedures offer no unique advantage.

Schultz and Wollenberger (571), with a histochemical technique involving lead precipitation, demonstrated at least two enzymes in different parts of the cell membrane in the myocardial cell which hydrolyzed ATP: One, a Na^+,K^+-ATPase that is inhibited by ouabain, was localized at the plasma membrane of the sarcolemma but not at the basal membrane. The second enzyme system that appears to involve calcium is not inhibited by ouabain, does not require sodium and potassium, and appears to be localized at the membranes of the intercalated disc at the nexus. Unfortunately, the lead procedure (the Wachstein-Meisel technique) tends to be relatively nonspecific and is difficult to reproduce. Even in the careful study of Marchesi and Palade (422), the ATPase activity was not dependent on sodium and potassium nor was it sensitive to ouabain. Many attempts have been made to modify the Wachstein-Meisel technique, but none has met with much success.

In 1972, Ernst (167, 168) examined the various procedures and, in a careful piece of work, devised a technique which involves use of paraformaldehyde-fixed tissue from the avian salt gland. These sections were incubated in a medium which permitted measurement of *p*-nitrophenyl phosphate activity by precipitating hydrolyzed phosphate with $SrCl_2$. After incubation at room temperature, the sections were treated with lead nitrate to convert the strontium phosphate precipitate to lead phosphate precipitates for visualization in the electron microscope. The reaction product was localized on the cytoplasmic side of the lateral and basal plasma membranes of the secretory cells. Little, if any, reaction product was associ-

ated with the apical surfaces of the secretory cell or with the endothelial surfaces of capillaries. Deposition of the reaction product appeared to be dependent upon magnesium and potassium and was sensitive to ouabain. Ernst (167, 168) reported that the absence of any detectable cytochemical localization of the transport enzyme on the luminal surface suggests that the Na^+,K-ATPase is oriented to move sodium into the cell. In this system, he used strontium as a "pure noncompetitive" inhibitor of the enzyme, causing 50% inhibition at low concentrations. It has little effect, however, on potassium affinity. This procedure appears to be a promising one for use in such tissues as the heart.

The ouabain-sensitive pump in frog choroid plexus is thought to reside on the apical surface of the epithelium (the cerebrospinal fluid side) and ^3H-ouabain should bind, therefore, only to the brush border membrane. This has been difficult to demonstrate, however, since it has not been possible to separate the basolateral membrane from the brush border by biochemical technology, and there are serious questions about the cytochemical studies carried out to date. The use of autoradiographic localization of ^3H-ouabain, introduced by Stirling (639), shows promise, and in the study by Quinton *et al.* (513), ^3H-ouabain was found to bind to the apical surface which was associated with the Na^+,K^+-ATPase. Therefore, it is attractive to suggest that sodium enters the epithelial cell from the blood and is subsequently "pumped" across the brush border into the cerebrospinal fluid by a ouabain-sensitive pump. These experiments, however, do not remove the possibility that a pump does exist in the basolateral membranes, as has been suggested for the avian salt gland (168). It may be that this area is absent or inaccessible in the choroid plexus.

Another approach was taken by Rostgaard and Møller (550) which involves isolation of "well defined" microsomal fractions from ox kidney cortex; these were analyzed by electron microscopy before and after freezing and deoxycholate activation in sections of pellets and in negatively stained preparations. Their results suggest that Na^+,K^+-ATPase active sites are located on the inner aspect of the cell membrane and that the magnesium-dependent ATPase active sites are located on the outer aspect of the cell membrane. The effect of so-called activation of Na^+,K^+-ATPase by freezing or by detergent treatment is to "open closed artifactitious microsomal vesicles, thus exposing more Na^+,K^+-ATPase active sites to the substrate" (550).

In 1974, Kyte (371a) prepared a ferritin-labeled antibody raised against a lipid-free purified catalytic component of the Na^+,K^+-ATPase. The antibody bound only to the inner surface of the vesicle. It is clear that a distinct localization has not been achieved unequivocally by these methods, and other cellular areas such as the sarcoplasmic reticulum of muscle cannot be categorically excluded (177) as lacking the enzyme.

IV. Attempts to Purify Na^+,K^+-ATPase from Heart and Other Tissues

Many attempts have been made to delineate the mechanism of the sodium pump (see below), but the results have been complicated by the impurity of the preparations employed in most of these studies. Purification of the Na^+,K^+-ATPase has proved to be difficult since the system is intimately associated with the membrane structure. Some success in purification has been achieved for a variety of tissues through the use of surface-active agents to solubilize the Na^+,K^+-ATPase. In recent years, a breakthrough in purification was obtained by use of these surface-active agents on microsomal fractions derived from tissues rich in sodium pump activity such as the outer medulla of kidney and the rectal gland of the dogfish shark.

The use of a detergent such as deoxycholate for preparation of Na^+,K^+-ATPase from cardiac and other tissues was first suggested by Skou (611). At about the same time, the effects of numerous detergents, both anionic and nonanionic, were examined in order to isolate Na^+,K^+-ATPase from brain (575). There are significant problems associated with the use of detergents in the isolation and purification of any membrane-associated, multisite (multiple subunits) enzymatic system. For example, some detergents such as digitonin and deoxycholate can produce extensive activation of ATPase activity. This may be due to chelation of heavy metals released during membrane disruption, a direct stimulatory effect on one or more of the partial reactions, or an "exposure" of latent enzymatic sites in the preparation by the removal of specific protein and/or lipid components [*e.g.*, exposure to detergents could lead to opening of vesicular structures, which would result in a greater accessibility of substrate and activators (320)]. For example, Winter (708) in 1972 showed that digitonin completely inhibited the Na^+,K^+-ATPase when it was used at higher concentrations (84, 575). In spite of the fact that the inhibition was complete, "complete retention of the sodium-dependent adenosine diphosphate (ADP)-ATP exchange activity and partial retention of the potassium-phosphatase activity was accomplished" (708). Somogyi *et al.* (629), found that Triton-X-100, deoxycholate, digitonin, and sonication increased, to some extent, the total ATPase activity up to a certain concentration limit. They showed, however, that only Triton was suitable for solubilization of the ATPase activities without any enzyme loss. Furthermore, various ions, as well as ethylenediaminetetraacetate (EDTA) influence the detergent action on the ATPase. Järnefelt (308) and Landon and Norris (375) originally suggested that disruptive agents may produce an interconversion between the magnesium-dependent, ouabain-insensitive and the Na^+,K^+-activated, ouabain-sensitive components. Somogyi *et al.* (629) suggested that detergent treatment activates the enzyme by causing a change in enzyme conformation and that interconversion problably does not occur. Jorgensen and Skou (320) concluded that deoxycholate, as indicated above, exposes latent enzymatic sites during the preparation, but suggested that the molecular activity of Na^+,K^+-ATPase is "not changed by treatment with detergent." They also concluded that detergent treatment does not involve an interconversion: "Detergents increase the activity of Na^+,K^+-ATPase several-fold without noticeable changes in the activity of Mg^{++}-ATPase, ouabain-insensitive component" (320). Cortas and Walser (130) suggested that low concentrations of deoxycholate may "preserve" a protein phospholipid interrelationship essential for the response to ouabain. When deoxycholate was omitted during preparation of the enzyme from a toad bladder, incomplete inhibition by ouabain was observed (*i.e.*, there was a sodium-potassium-dependent activity that did not seem to be inhibited by ouabain). The addition of phosphatidylserine modified the results in a dose dependent manner in both deoxycholate-treated preparations.

Banerjee *et al.* (54), with guinea pig kidney as the enzyme source, compared Lubrol with Triton-X-100 and found that Triton with added ligands was superior in that it extracted a soluble enzyme of higher specific activity and ouabain sensitivity than Lubrol. The concentration of Triton needed for maximum solubilization was 0.1 of that used by previous investigators [Hokin and his colleagues; (442)]. Moreover, their soluble enzyme had a 4-fold greater specific activity than that reported by the Hokin group. They also pointed out the interesting fact that high concentrations of Lubrol interfere with protein determinations, which, of course, might lead to erroneously high specific

activities unless adequate care is taken to correct for interference. This is possibly true for all activator agents employed. Chan (109), with radiolabeled sodium dodecyl sulfate, found that this detergent bound to membrane fragments and heating at 15°C did not dissociate the dodecyl sulfate-membrane complex. This may suggest that reversible alterations of membrane ATPase, characteristic of detergent treatment, are not simply due to the binding and dissociation of the detergent molecules but rather are probably due to detergent-induced "conformational changes." It is of interest that the activation of ATPase by low concentrations of sodium dodecyl sulfate, which produced a 2-fold increase in membrane ATPase activity by the addition of sodium or potassium, was not affected by ouabain. Hokin *et al.* (285) have criticized the reporting of "high specific activities" when the assays are carried out in the presence of detergents. In view of these results, it would appear important to exert caution in evaluating purification data. If specific activity is increased, a number of partial reactions catalyzed by the Na^+,K^+-ATPase should be concomitantly increased. These include the sodium-dependent phosphorylation, a sodium-dependent ATP-ADP exchange reaction and, what we feel to be of considerable importance, the rate and maximum amount of ouabain bound under a variety of ligand conditions (see below for further discussion).

A number of attempts have been made to solubilize and then to purify the Na^+,K^+-ATPase, with both mammalian and nonmammalian tissues. The first attempt at the former was carried out by Schwartz *et al.* (575), with guinea pig cerebral cortex microsomes. Lubrol (0.1%), a nonionic detergent consisting of a condensate of cetyl alcohol and a polyoxyethylene, yielded the highest activity in the solubilized state, although very little sodium activation was noted. A 3-hour exposure of a fragmented membrane preparation to digitonin (0.5%) in a sucrose medium yielded a soluble preparation which was stimulated by sodium. Deoxycholate (0.1%) proved to be the best detergent for the preparation of a workable microsomal Na^+,K^+-ATPase, but the specific activity (henceforth expressed as μmoles of inorganic phosphate per mg of protein per hour) was only about 50.

Hokin and his collaborators (285, 326, 328, 442, 675, 676) have placed major emphasis on solubilization and purification and have published an extensive series of such experiments. In 1967, they showed that a 1.2% Lubrol WX treatment of guinea pig cortical microsomes, suspended in 2 ml (15 minutes at 0°C) and then centrifuged, yielded a "soluble" preparation with specific activity of only 5 to 10 (442). With a sodium iodide-treated beef brain microsomal preparation, they found that Lubrol (three parts to one part protein) extraction yielded a specific activity of about 40. In this preparation, they found an apparent molecular weight of about 670,000, with the use of gel chromatography [*i.e.*, 6% agarose (442)]. By using a similar procedure, they then tried to purify the solubilized preparation. The enzyme was separated from some of the non-ATPase protein and from the detergent by various procedures and was purified to a specific activity of "10 to 15 times that present in the microsomes," yielding a final enzyme "solution" that was water-clear and "fairly free" of unbound Lubrol. The specific activity ranged from 15 for the microsomes to 130 for a carboxymethylcellulose-chromatographed Lubrol-extracted pellet (326). These methods were later improved through the use of isoelectric precipitation, zonal centrifugation, and a new ammonium sulfate fractionation procedure (675). The latter rendered the ATPase insoluble. The partially purified enzyme was examined by polyacrylamide gel electrophoresis after solubilization in a sodium dodecyl sulfate mercaptoethanol medium. One prominent band

was found with a molecular weight of 94,000; this was identified as the "phosphorylated subunit." The cholesterol content fell with purification. A large-scale procedure was reported which required one week and yielded 30 to 50 mg of enzyme for each 1.2 kg of bovine brain cortex. From the amount of the phosphorylated enzyme (see below for details about phosphorylating conditions), the investigators judged that their enzyme was approximately half-pure. The ammonium sulfate enzyme exhibited a high specific activity, ranging from 450 to 750. This required high amounts of Lubrol (about 20 mg/ml of microsomal protein), and probably explains the difficulty encountered in reproducing the results. Shirachi *et al.* (594) modified the Hokin procedure by lowering the concentration of Lubrol to 0.4% (w/v) and isolated a microsomal fraction by the procedure of Schwartz *et al.* (575). The final concentration of Lubrol after appropriate dilution was 0.2%, which was found to be optimal for the extraction of the Na^+,K^+-ATPase. With rat and guinea pig tissue, they found a specific activity of about 71 (Lubrol extract) for rat brain microsomes and about 21 for guinea pig heart. It is of interest that this is comparable to the Lubrol extract stage reported by Hokin's group [see their table I on page 535 (675)] of about 64 for beef brain cortex. In addition, the enzyme prepared by Shirachi *et al.* (595) did not require ATP or sodium for protection, and they did not subject their enzyme to zonal centrifugation or ammonium sulfate precipitation. Their procedure, however, is reproducible (unpublished experiments from our laboratory) and has been applied to liver, kidney, heart, and brain (595).

The kidney, in many ways, appears to be a good tissue source from which to obtain a highly purified Na^+,K^+-ATPase. Towle and Copenhaver (672) described the partial purification and properties of a soluble Na^+,K^+-ATPase derived from rabbit kidney cortex (with frozen kidneys obtained from Pel-Freeze) and treated with 0.33% deoxycholate followed by ammonium sulfate fractionation and an interesting glycerol stabilization step. Their enzyme preparation was completely inhibited by ouabain and yielded a specific activity of 300, which is very high for the kidney cortex.

We and others have found that the medullary area of the kidney yields preparations with considerably higher activity than that of the cortex. Since sodium is transported against a very steep electrochemical gradient in the thick ascending limbs of Henle, it is logical to expect a very high concentration of Na^+,K^+-ATPase in this area. Accordingly, Jørgensen and Skou (319), by using the outer medulla of rabbit kidney and deoxycholate (DOC) with sucrose density-gradient centrifugation, reported an Na^+,K^+-ATPase specific activity of 881 with an estimated purity of 31 to 61%. By using zonal centrifugation and a similar deoxycholate-treated preparation, the same laboratory (321) later obtained a Na^+,K^+-ATPase preparation with specific activity of 900 to 1200 and a yield of 1.5 to 2.5 mg of protein per g of tissue. They also reported that with rate zonal centrifugation the specific activity increased to 1500 with an estimated purity of about 49%. These investigators concluded that they were dealing with plasma membrane fragments with a high density of enzyme sites per unit of membrane area. Purity was estimated by using a molecular weight of about 250,000 and a molecular activity of 12,850 min^{-1}. Hokin *et al.* (285) have criticized this study, indicating that the specific activity may be due to the activating effect of detergent present in the assay medium rather than to a definitive purification. Furthermore, a number of investigators, including ourselves, have found difficulty in reproducing this procedure of Jørgensen and Skou (321). Similar criticisms, however, can be levied against every procedure designed to

purify the Na^+,K^+-ATPase, since all require numerous tedious steps that involve a relatively long period of time. It is thus not surprising that it is difficult to reproduce "recipes." The possibility of species differences further complicates reproduction of results.

As indicated above, Banerjee *et al.* (54) found that Triton-X-100 was superior to Lubrol for solubilization, and they obtained a specific activity from 140 to 200 for the enzyme from guinea pig kidney. It is of importance that the insoluble, crude Na^+,K^+-ATPase preparations exhibit almost identical properties to the considerably purified and soluble enzyme (352).

A major effort in the isolation, purification, and characterization of Na^+,K^+-ATPase was published in a series of papers by Kyte (368–371). With canine renal medulla and a method slightly modified from the Jørgensen-Skou procedure and from the original Skou procedure (612), an enzyme was obtained which had a specific activity of 350 to about 1000. The enzyme in the supernatant, which had been solubilized with deoxycholate, had a specific activity of about 360. Kyte characterized the proteins in the purified Na^+,K^+-ATPase preparation by means of Sepharose 4B gel filtration, polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate, and ultracentrifugation with a Spinco model E ultracentrifuge. The major finding was that there were only two polypeptides in the Na^+,K^+-ATPase preparation, one with a molecular weight of about 84,000 and the other, 57,000. The smaller molecular weight protein appeared to be a sialoglycoprotein and the larger one possibly a "catalytic" subunit, since it was phosphorylated by ATP in the native state. Kyte successfully cross-linked the two polypeptides to one another, with dimethyl suberimidate, to yield a new component with an apparent molecular weight higher than either of the two proteins. This suggests that the two proteins reside in close proximity when in the intact (native) Na^+,K^+-ATPase lipoprotein complex. Also, Kyte concluded that one large polypeptide could be cross-linked with one small polypeptide. He suggested that the two polypeptides are present in the purified Na^+,K^+-ATPase preparation in a mass ratio for large to small chain of 1.7:1. This mass ratio probably corresponds to a molar ratio of one large chain to two small chains. The large polypeptide chain is soluble in simple aqueous solvents in the absence of detergents and lipids, although it no longer possesses any enzymatic activity. No successful attempt (reported, at least) at reconstitution has been made at the time of this writing (December 1973, but see the end of this section for a review of some attempts at reconstitution). A combination of both purified polypeptides after exposure to sodium dodecyl sulfate without added lipid would probably fail to yield activity.

By using this preparation and a "derivatized" strophanthidin with an affinity for the enzyme at least two orders of magnitude higher than the parent compound (the tight and rapid binding of this inhibitor made it an ideal reagent for a kinetic titration of the cardiac glycoside binding sites of Na^+,K^+-ATPase), Kyte (370) found 1 mole of glycoside binding site present per mole of the higher molecular weight polypeptide chain (*i.e.*, the component that can be specifically phosphorylated). Kyte concluded that the preparation of soluble enzyme contains a uniform collection of enzyme molecules that binds 1 mole of cardiac glycoside per mole of large polypeptide chain and only phosphorylates under optimal conditions to a level of 0.4 mole per mole. However, determinion of glycoside binding sites was by a kinetic titration procedure and not by a direct binding one. The former estimates an upper limit of the concentration of specific sites, while the latter probably estimates a lower limit, or perhaps, if carried out

under "appropriate" ligand conditions, a true estimation. As Kyte (370) pointed out, the maximum binding of ^{3}H-ouabain is 94 ± 6 pmol per μmole of ATP hydrolyzed per minute. However, this figure may be fortuitous, since cardiac glycosides bind to the dephosphoenzyme as well as to the phosphoenzyme (see later discussion). Furthermore, deoxycholate can alter the turnover number of the enzyme. At maximum phosphorylation, Kyte found only 0.36 moles of phosphate attached to the polypeptide per mole of cardiac glycoside binding sites (370). Albers *et al.* (18) also found that the number of cardiac glycoside sites was about twice the number of phosphorylation sites, although we have found a 1:1 relationship with a crude heart enzyme. It is possible that the proteins form a dimer so that the existence of two sites per functional Na^+,K^+-ATPase molecule creates the *functional* existence of only one site (638). Half-of-the-site reactivity has been shown for a number of enzymes and enzyme systems. We wonder, however, whether it is appropriate, as Kyte and others have done, to compare the stoichiometry of binding either by the kinetic procedure or by the direct binding method to the formation of phosphorylated intermediate (see above).

In 1973, Hart and Titus (254, 255) described an interesting preparative technique with rabbit brain homogenized in a deoxycholate buffer, followed by suspension and separation by flotation in a linear gradient of potassium iodide, and then harvesting a lipoid pellet by centrifugation in potassium iodide. After treatment with sodium dodecyl sulfate, disc gel electrophoresis revealed seven bands, the most prominent of which had a mobility corresponding to a molecular weight of about 98,000. Sulfhydryl groups play an important role in the Na^+,K^+-ATPase. Their reactivity toward alkylating agents such as N-ethylmaleimide is a function of ligand-controlled conformational states, according to Hart and Titus (254, 255), and such reactivity, therefore, provides interesting means for identification of a protein component(s) of an enzyme. These authors recognize the highly complicated and allosteric nature of the enzyme system and the fact that N-ethylmaleimide has been shown to inhibit the potassium-dependent dephosphorylation of the enzyme as well as the Na^+,K^+-ATPase activity and the potassium-stimulated, *p*-nitrophenylphosphatase activity. But this reagent stimulates the ADP-ATP exchange reaction [this phenomenon formed part of the basis for the elucidation of the sodium-dependent, ADP-ATP exchange reaction by Albers and his group (171–173)]. In low concentrations, N-ethylmaleimide appears to inhibit selectively the potassium-dependent dephosphorylation of the enzyme, while leaving the sodium-dependent phosphorylation step intact (58). Sodium and ATP appear to protect the enzyme against the N-ethylmaleimide inhibition of the dephosphorylation step. Accordingly, in parallel incubations, Hart and Titus (254, 255) treated the enzyme protein with ^{3}H- or ^{14}C-N-ethylmaleimide in the absence and presence, respectively, of protecting ligands, after which the enzyme was solubilized with sodium dodecyl sulfate. The two labeled forms of the enzyme lipoproteins were solubilized and mixed together, and that portion of the mixture containing a significantly reduced proportion of ^{3}H to ^{14}C label was marked as containing SH-groups that were specifically sensitive to (*i.e.*, protected by) ligand-induced stabilization of enzyme conformation. A single protein band was identified as conformationally sensitive by this procedure and corresponded in molecular weight to the one that supposedly is phosphorylated by ATP through a magnesium- and sodium-dependent reaction (Kyte's heavy polypeptide†; see also refs. 127 and 675).

The glycoprotein (lighter polypeptide) is of interest, although very little work has been done with respect to the purified Na^+,K^+-ATPase. However, a major glyco-

protein of the human erythrocyte membrane has been isolated and characterized in detail (582, 583, and see section II). This glycoprotein seems to be very similar to the one reported by Kyte. Both hydrophilic regions and domains are shown, thereby indicating an amphipathic molecular structure. This would make this component ideal for interaction with membranes, ions and possibly inhibitors. It should be pointed out that two polypeptide components have also been isolated now from bovine and human erythrocytes (106, 705) and from heart (701). The latter was apparently (according to the abstract) identified by an antibody reaction yielding 3.2×10^4 and 9.6×10^4 molecular weight protein components. Finally, in the latest of a series of papers from Hokin's laboratory (285), an enzyme was purified from rectal gland of *Squalus acanthias,* the spiny dogfish shark. It is of interest that this species was selected; it has been shown by Bricker and his colleagues (82) that erythrocyte membranes isolated from this dogfish yielded a Na^+,K^+-ATPase with an activity considerably higher than that observed for the same enzyme in red blood cells of all other species studied previously. The animal is unique in the elasmobranch class in that the extracellular fluid is almost isosmotic with the ocean, and the sodium concentration of the extracellular fluid is about 250 mEq/l. Despite this, the sodium concentration in the red blood cell is maintained at about 10 to 20 mEq/l and, therefore, sodium ions must be extruded by a very active enzymatic process. This is also undoubtedly true of glandular material such as the rectal gland.

Accordingly, Hokin and his colleagues (285, 517) used a procedure similar to one they had used previously, namely, a Lubrol extraction requiring a 16% solution with a final concentration of about 1.6%, followed by zonal centrifugation and ammonium sulfate fractionation. The membrane preparation yielded a specific activity of about 400. Solubilization produced a 2-fold activation; purification by zonal centrifugation and a "novel" ammonium sulfate fractionation yielded an enzyme preparation with a specific activity of 1500. The overall yield of enzyme at the membrane stage was 70%. A mince of 10 rectal glands weighing about 10 to 15 g of fresh weight yielded about 20 to 30 mg of purified, very stable enzyme. Polyacrylamide gel electrophoresis carried out in the presence of sodium dodecyl sulfate yielded two polypeptides—a "catalytic" subunit with a molecular weight of 97,000 (72% of total protein) and a glycoprotein with a molecular weight of 55,000 (19% of total protein)—and a "protein" which migrated with a tracking dye (8.5% of total protein). This seems to be a good tissue to use, since the catalytic subunit and the glycoprotein can be isolated in milligram quantities and, after gel chromatography in the presence of sodium dodecyl sulfate, 90% of the protein can be recovered. The protein composition appears to be about 66% for the catalytic subunit, 28% for the glycoprotein, and 5% for the remainder. Both proteins are found in the form of vesicles which bear a resemblance to cytochrome oxidase. About 4000 pmol of acylphosphate per mg of protein were formed from ATP in the presence of magnesium and sodium, which is two to three times more than the highest previously reported. According to these investigators, this attests to the high purity of the enzyme preparation. No ouabain binding was reported; the turnover number of the preparation was about 6300 min^{-1}, which is in the range found for most Na^+,K^+-ATPase preparations. It is not known from these studies or from previous studies whether or not the glycoprotein is a subunit of the Na^+,K^+-ATPase. If both polypeptides are required then the enzyme is about 90 to 95% pure; if only the catalytic subunit is associated with the enzyme, the preparation reported by Hokin and his colleagues is 66 to 72% pure.

Considerable interest has been paid to

the purification of the Na^+,K^+-ATPase from the heart since the sodium pump is thought to be a critical participant in maintaining the conditions required for proper cardiac function. In addition, the cardiac glycoside receptor associated with the pump may be involved in the cardiotonic effect of digitalis. Because the contractile fibers entrap fragmented membranes in homogenates of heart muscle to complicate differential centrifugation techniques, a great deal of difficulty has been encountered in the studies designed to achieve purification. Also it is possible that the density of sodium pump sites per milligram of cardiac membranes may be lower (at least as isolated) than that from other tissues such as kidney or brain. The presence of an extensive organelle and membraneous reticular network in muscle also complicates purification procedures.

Procedures for isolating *relatively* high activity and stable cardiac Na^+,K^+-ATPase preparations originated in the laboratory of Skou (611). He was the first to discover that the addition of a heat-stable, acid-precipitable factor derived from the supernatant of a cell-free heart suspension, when added back to a "microsomal" preparation, markedly inhibited the ouabain-insensitive component. Studies in our laboratory showed that the factor was probably a basic protein, cardiac histone (576).

A number of investigators have isolated Na^+,K^+-ATPase from cardiac tissue (14, 35, 37, 107, 215, 216, 340, 346, 432, 506, 521, 572, 594, 612, 637, 654). The most recent preparation employed for studies that have been published involves a combined detergent salt treatment after initial suspension and homogenization with a Polytron device (432); this is followed by a sequential series of centrifugations leading to a final membrane preparation with a reasonably reproducible specific activity. The latter ranges from 20 to 70 μmoles of inorganic phosphate per mg of protein per hour depending upon the species, rapidity of removal of the organ and other factors. It is estimated that the purity is in the range of 5 to 10%. At this time no demonstrable enzymatic activity connected with anything but Na^+,K^+-ATPase has been detected. With appropriate treatments, such as aging, sulphydryl protective agents, and various modifications of detergent and/or salt treatment, it is possible to obtain a "working" preparation that is low in ouabain-insensitive activity. A detailed "recipe" for isolation of the Na^+,K^+-ATPase from heart is available (581).

Although no one particular procedure appears to be superior, a number of methods were either modified or devised for specific purposes. As an example, the procedure by Stam *et al.* (637) yielded a preparation purportedly rich in sarcolemma; therefore, the authors felt that the Na^+,K^+-ATPase was a "marker" for the myocardial cell membrane. Unfortunately, the preparation was very low in activity (about 2.9 μmoles of inorganic phosphate per mg of protein per hour was ouabain-sensitive) and since it was not pure, it was difficult to exclude other cellular sites. The procedure used by Akera *et al.* (14) was designed to improve the existing methods so that a workable preparation could be obtained from a comparatively small amount of cardiac tissue. The procedure employed by Repke (521), who was the first to study in depth the cardiac Na^+,K^+-ATPase system, was designed for extensive comparative studies. The methodology described by Gibson and Harris (215, 216) was used to isolate the enzyme from human myocardial tissue.

Lane *et al.* (377) in this laboratory reported in 1973 a relatively rapid procedure for the purification of the Na^+,K^+-ATPase from the outer medulla of canine kidney. The purification technique requires the following sequential treatment of microsomes obtained from the tissue: a) exposure to sodium iodide for 30 minutes (note: in ref. 377 the time used was 10

minutes; however, 30 minutes ensures the efficacy of this step); b) extraction with deoxycholate; c) precipitation with glycerol; d) extraction with a medium containing deoxycholate plus cholate; and e) fractionation with ammonium sulfate followed by dialysis. The final Na^+,K^+-ATPase was similar to that found by Kyte (369) with the same tissue source and by Hokin *et al.* (285) for the rectal gland of the dogfish shark. The Na^+,K^+-ATPase activity was up to 1550, which is higher than that obtained by Kyte (369), and the preparation bound between 3000 and 4000 pmol of ouabain per mg of protein. The yield was 16 to 18 mg of protein per 100 g of starting tissue. The preparation contained two proteins of 89,000 and 56,000 daltons; the former was phosphorylated by ATP, while the latter was a glycoprotein.

It was reported in 1973 that application of some aspects of this purification sequence (377) to heart muscle led to a significant increase in purification of the cardiac Na^+,K^+-ATPase (486). The final specific activity was 160 to 170, and the preparation bound approximately 400 pmol of ouabain per mg of protein. Recently a new procedure (486a) has yielded preparations from cardiac muscle with specific activities of 400 to 500. Such preparation bound up to 70 pmol of ouabain per mg of protein and contained only 5 proteins of which the major component was the 95,000 dalton polypeptide. The results suggests, however, that the heart enzyme, "notorious" with respect to difficulty of purification, may be identical, or nearly identical, to those in mammalian kidney and in the nonmammalian rectal gland of the dogfish shark.

In summary, fragmented preparations which exhibit Na^+,K^+-ATPase activity typically include a variety of lipids and many different proteins, but recent purification studies have reduced the possible constituents of the Na^+,K^+-ATPase to two proteins, lipids, and perhaps small polypeptides. The characteristics of the two major proteins, derived from three different laboratories and two enzyme sources (*i.e.*, outer medulla of canine kidney and the rectal gland of the dogfish shark), are presented in table 1. Hokin *et al.* (285) observed a third protein peak at the position of the tracking dye upon gel electrophoresis or chromatography for the dogfish shark Na^+,K^+-ATPase. Since reconstitution of these proteins into an active complex has not been obtained to date, it is not known whether both of the proteins are required for activity, although the higher molecular weight entity, which can be phosphorylated (see below), is presumably part of the enzyme. An amino acid analysis by Kyte (371) showed that both proteins contain a significant percentage of hydrophobic amino acids, which is consistent with their intimate relationship to a membranous environment. Caution should be applied in determining hydrophobicity since the conclusions are based upon the selection of specific amino acids. We should point out that small polypeptides, undetected to date, may also be present in our Na^+,K^+-ATPase preparations.

Jørgensen (317) in 1974 reported purification of Na^+,K^+-ATPase from the outer medulla of kidneys from rabbits, sheep, pigs, and dogs. His procedure employs deoxycholate and low sodium dodecyl sulfate (in the presence of ATP) treatments of a microsomal fraction in conjunction with rate-zonal and isopycnic-zonal centrifugations. His final enzyme preparation has a 96,000 dalton polypeptide which can be phosphorylated by ATP and a 57,000 dalton glycoprotein. The preparation exhibited specific activities of 1500 to 2200 units, binds up to 4000 pmol of ATP or ouabain per mg of protein and can be phosphorylated up to a maximum of about 7500 pmol per mg of protein.

Nakao and his colleagues (341, 458–461) reported a procedure that yields Na^+,K^+-ATPase preparations with specific activities of up to 7000 units. Their procedure, with pig brain as a tissue source, involved

TABLE 1

Characterization of proteins from purified Na^+, K^+-ATPase preparations

Characteristic	References		
	Kyte (370, 371)	Hokin *et al.* (285)	Lane *et al.* (377)
Molecular weight of phosphorylated protein (A) [a]	84,000–139,000	97,000	89,000–96,000
Molecular weight of glycoprotein (B) [a]	57,000–35,000	55,000	56,000–49,000
Molar ratio of A:B	1:2 [b]	2:1	1:1
Maximum ligand bound [c] (pmoles/mg protein)	5720–4000 [d]	4080	3000–4000
Minimum molecular weight [e] of Na^+, K^+-ATPase	175,000–250,000 [d]	245,000	250,000
Maximum turnover number [f] (min^{-1})	1100–2900 [d]	6300	6500

[a] Where two numbers are present, the first was obtained by polyacrylamide gel electrophoresis and the second by gel chromatography; both separation procedures were carried out in the presence of sodium dodecyl sulfate.

[b] Based on molecular weights of 139,000 for A and 35,000 for B; with molecular weights of 84,000 (A) and 57,000 (B), the molar ratio is approximately 1.0:0.9.

[c] Titration of glycoside, N-(4'-Amino-n-butyl)-3-aminoacetylstrophanthidin), effects on Na^+,K^+-ATPase activity (370); phosphorylation of enzyme in presence of [γ^{32}P]ATP, magnesium and sodium (285); ^{3}H-ouabain binding to the preparation in the presence of magnesium plus inorganic phosphate or plus ATP and sodium (377).

[d] First numbers were determined in the presence of deoxycholate which depressed the turnover number and increased the apparent number of glycoside interaction sites.

[e] The following relationships allow an estimate of the purity of these membrane preparations with respect to the Na^+,K^+-ATPase:

1) moles of ligand bound/g protein = 1 mole ligand bound/x g protein

2) [(molecular weight estimate (g)/x g)] × 100 = estimate of percent purity

"Ligand" refers to a solute that reacts with the membrane preparation in a manner directly proportional to Na^+,K^+-ATPase activity, *e.g.*, phosphorylation from [γ^{32}P]ATP (503) or binding of ^{3}H-ouabain (580).

[f] Calculation for maximum turnover number = $\frac{\mu\text{moles ATP hydrolyzed min}^{-1}\text{ mg}^{-1}\text{ protein}}{\mu\text{moles enzyme mg}^{-1}\text{ protein}}$

treatment of a microsomal fraction with deoxycholate and sodium iodide. Subsequently, the preparation was solubilized by Lubrol. Passage of the soluble fraction through an aminoethyl cellulose column yielded three peaks of varying activities and labilities. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate showed that all of the peaks were composed of one prominent polypeptide with a molecular weight of about 100,000 and faint and variable smaller bands. One of the peaks could be phosphorylated to a maximum of about 7000 pmole per mg of protein. These results may suggest that polypeptides other than the one which can be phosphorylated are contaminants or, at the most, modifiers of Na^+,K^+-ATPase activity. On the other hand, some caution must be invoked with respect to this procedure, because the resulting enzyme preparation was extremely labile, and the yield was very low.

These highly purified Na^+,K^+-ATPase preparations can incorporate between 4000 and 7500 pmol per mg of protein of phosphate from ATP and can bind up to 4000 pmol of ATP or ouabain per mg of protein (table 1). (The rationale for the use of these probes is discussed below.) If there is one reactive site for each probe on a functional molecule of the enzyme, 4000 pmol per mg of protein equates to a functional molecular weight of 250,000 g of protein per mole of enzyme. This calculated value is in the range of the molecular weights determined by Kepner and

Macey (342) who used radiation *in vacuo* inactivation techniques. They found molecular weights of 190,000 for the enzyme in guinea pig kidney microsomes and 250,000 for the enzyme in erythrocyte ghosts. Although it is possible to postulate various ratios of the large protein to the glycoprotein (*i.e.*, if, in fact, the latter is a component and not a contaminant) in order to arrive at these molecular weights, it should be pointed out that the purification procedures may well yield a product that is a mixture of native and denatured proteins. Nonetheless, the fact that similar preparations have been obtained by four different laboratories does suggest that there may be two of the "catalytic" proteins per functional molecule of the Na^+,K^+-ATPase.

The Na^+,K^+-ATPase is known to require lipids for catalytic activity. There is considerable controversy about the specificity of lipids which are required (28, 95, 126, 143, 163, 182, 200, 258, 263, 300, 337, 382, 417, 546, 548, 557, 636, 641, 645, 646, 648, 655, 682, 694). There is reason to believe that phosphatidylserine may be a requirement or at least is more effective than other lipids in stimulating catalysis (174, 232, 286, 348, 471, 478, 644, 647, 649, 651–653, 693). Reports published in 1971 and 1972 also suggest that cholesterol is required (311, 468). The reader is referred to a recent critical review of this subject in the paper by Roelofsen and Van Deenen (547).

The role of the lipids, besides forming a matrix for the enzyme, appears to involve modulation of the conformation of the Na,K^+-ATPase. In this regard, Arrhenius plots of Na^+,K^+-ATPase activity reveal a break at about 20°C (113–116, 241, 242, 503, 510, 579, 621, 642, 651, 652). Grisham and Barnett (242) showed that this temperature corresponded to a transition in the state of the lipids extracted from a Na^+,K^+-ATPase preparation. The transition in the state of the extracted lipids reflected a conversion from a more ordered state (below 20°C) to a less ordered state (above 20°C). Charnock *et al.* (113) reported that phospholipase C treatment of a Na^+,K^+-ATPase preparation altered the specific activity but did not change the nature of the response to temperature. Phospholipase A treatment, however, converted the Arrhenius plot to a linear form. Addition of phosphatidylserine to the phospholipase A-treated preparation restored the response to temperature to that of the control preparation.

Barnett (62) concluded that the change in the state of the lipids altered a rate-limiting step in the reaction sequence for ATP hydrolysis. Further analysis revealed that the following "partial reactions" were not altered by the lipid transition: a) phosphorylation of the Na^+,K^+-ATPase by ATP in the presence of magnesium and sodium; b) potassium-stimulated *p*-nitrophenylphosphatase; and c) rates of ouabain binding supported by ATP, magnesium, and sodium. Studies carried out in this laboratory (686) showed that the rate of ouabain binding was not affected by the transition of the lipids, whether the reaction was carried out in the presence of ATP, magnesium plus sodium, ATP plus magnesium without sodium, or magnesium plus inorganic phosphate. Barnett (62) concluded that the partial reaction, namely the conversion of a potassium-sensitive form of the Na^+,K^+-ATPase to a sodium-sensitive form (see below), is affected by the physical state of the lipids. It is of interest that Zetterqvist and Mårdh (729), by employing rapid-quenching techniques, concluded that the latter partial reaction is the rate-limiting step in the catalyzed hydrolysis of ATP.

Barnett (62), in a further interpretation of his data, suggested that at 37°C the lipids are a mosaic mixture of fluid and ordered (or highly oriented) structures. Molecules of Na^+,K^+-ATPase in the highly ordered regions would be less active than those molecules in the fluid regions. Thus, the partial replacement of

lipids by detergents, such as Lubrol, Triton, and deoxycholate, may increase or decrease the number of Na^+,K^+-ATPase molecules in fluid regions or may cause a generalized increase or decrease in the fluidity *per se* of all hydrophobic regions. This action may explain, at least partially, detergent "activation" and "inactivation" (see above). It is clear from these considerations that multiple combinations in the membranous environment of various phospholipids and neutral lipids may be consistent with Na^+,K^+-ATPase activity, albeit at different turnover rates. Barnett (62) also suggests that aging may alter the fluidity of membranes.

It is also interesting to speculate how the lipid content of a particular subcellular membrane may regulate the expression *in vivo* of the Na^+,K^+-ATPase. The biosynthesis of this system is poorly understood. It is possible that the sodium pump is synthesized in such a manner that it is exclusively limited to the cell membrane, its presumed location (see section III). Conversely, the system may be localized in multiple subcellular membranes, but the lipid content of these membranes may vary, so that Na^+,K^+-ATPase activity is manifested in the cell membrane but not in other membranes. Similar considerations may apply to the expression of Na^+,K^+-ATPase in isolated membrane fractions. It seems possible that upon tissue homogenization and during long isolation procedures (particularly when collecting the membrane fragments in pellets), some exchange of lipid moieties may occur among fragments of different subcellular membranes. Also, membranes *in vivo* are not generally vesicular but *assume* vesicular shapes after the homogenization and isolation treatment. This means that the ends of membrane fragments must "weld" together to form the vesicle, presumably through hydrophobic interactions. It seems reasonable to suppose that such "welding" may yield a vesicle formed by fragments derived from different subcellular membranes. These considerations may form a possible basis to explain why membrane preparations can be obtained with high adenylate cyclase activity, presumably another cell membrane system, and low Na^+,K^+-ATPase activity (643) and *vice versa*. Such changes during isolation also offer an explanation for "sarcoplasmic reticulum" preparations containing Na^+,K^+-ATPase and exhibiting changes in calcium binding upon exposure to cardiac glycosides (165). The alternate explanation for this preparation is that the Na^+,K^+-ATPase is a component *in vivo* of the sarcoplasmic reticulum.

Reconstitution of the Na^+,K^+-ATPase must be achieved for two reasons. The first is *to define the minimal requirements for Na^+,K^+-ATPase activity*. This can only be attained by isolating all of the components of the system and then selectively recombining the components until a glycoside-sensitive Na^+,K^+-ATPase activity is established. Such experiments could reveal whether both the high molecular weight and the glycoprotein are required for Na^+,K^+-ATPase function, whether very small polypeptides(*e.g.*, proteolipids) are also required, and the extent to which specific lipids are required for catalysis and glycoside binding. Eventually, another recombination approach could also prove valuable. These experiments would attempt to incorporate other membrane systems (*e.g.*, adenylate cyclase, the calcium-binding proteins of the sarcoplasmic reticulum, *etc.*) into the hydrophobic membrane matrix which contains the Na^+,K^+-ATPase. This approach should yield insight into how the Na^+,K^+ATPase may be regulated in the tissue.

The second reason why reconstitution of the enzyme is of importance is to *determine whether the requirements for Na^+,K^+-ATPase activity and for sodium pump function are identical or different and to demonstrate whether this system can transport cations uphill through an ATP-dependent and cardiac glycoside-sen-*

sitive reaction. The latter is necessitated by the Ling hypothesis, *viz.,* the gradients of sodium and potassium across the membrane are not due to a pump *per se* but result from anionic binding sites in the intracellular space which have higher affinity for potassium than sodium (400–409). Reconstitution for these purposes would involve incorporation of a Na^+,K^+-ATPase into an artificial membrane system such that the system can pump sodium and potassium against electrochemical and chemical gradients, respectively (234). Jain and his colleagues (305) reported the results of experiments that suggested incorporation of a rat brain cortical Na^+,K^+-ATPase preparation into an artificial membrane formed from oxidized cholesterol and alkanes. Upon addition of the preparation to the medium bathing one side of the membrane, they found ATP-induced changes in the electrical characteristics of the membrane when ATP was added to the same side as the enzyme. Ouabain added to the medium bathing the opposite surface inhibited these changes. These experiments were carried out in the presence of magnesium, sodium, and potassium. The authors concluded: "These observations provide circumstantial evidence for the reconstitution of the active cation pump across the artificial bilayer" (305). The authors pointed out, however, that the changes did not correlate in a quantitative sense with the specific activity of the Na^+,K^+-ATPase preparation. Furthermore, no evidence of reconstitution was observed with Na^+,K^+-ATPase preparations derived from a variety of other tissues and species. The experiments reported by these investigators have not yet been repeated.

In 1974 Hokin (283) reported the incorporation of his highly purified Na^+,K^+-ATPase preparation into lipid vesicles suspended in a medium containing radiolabeled sodium. He found that addition of ATP to the suspension medium induced a movement of sodium into the vesicle through a process inhibited by ouabain when the latter was inside but not outside the vesicle. The nucleotide specificity for influx paralleled the specificity requirements for Na^+,K^+-ATPase activity. Furthermore, Hokin stated that when the sodium concentrations were equivalent inside and outside the vesicle, the ATP-dependent influx caused sodium to accumulate inside. Thus, these experiments suggested that the Na^+,K^+-ATPase and the sodium pump are equivalent in a macromolecular sense and that the system has the ability to pump uphill. Goldin and Tong (personal communication from Dr. G. Guidotti and ref. 230b) have succeeded in reconstituting the kidney Na^+,K^+-ATPase into lipid vesicles. They were able to induce uptake of sodium through a process which required external ATP and which was inhibited by internally trapped cardiac glycosides. They were able to induce a 3-fold higher concentration of sodium (60 mM) in the vesicles than that initially present (20 mM). The transport of sodium was accompanied by the transport of chloride in an amount required to maintain bulk electrical neutrality.

Electron micrographs from typical areas of fixed pellets from each successive step in the purification of the canine medullary Na^+,K^+-ATPase by the procedure of Lane *et al.* (377) are shown in figure 1 a–d. Both crude microsomal (fig. 1a) and NaI-treated fractions (fig. 1b) appear heterogeneous, being comprised of large, empty vesicles, as well as smaller dense vesicles. In contrast, the glycerol precipitated fraction (fig. 1c) is composed almost exclusively of large, roughly concentric whorls of membranes that exhibit the typical trilaminar profiles of many biological membranes. The ammonium sulfate fractions are composed of small flocculant particles with no discernible substructure (fig. 1d). Freeze-etch studies (figs. 2, 3) of these fractions reveal membranes of crude microsomal preparations whose surfaces

FIG. 1. Electron micrographs of Na^+,K^+-ATPase preparations at various stages of purification by the method of Lane *et al.* (377). Fixed in 1.5% glutaraldehyde in 0.05 M phosphate buffer and postfixed in osmium tetroxide (×37,000). a, microsomal fraction from canine kidney outer medulla (specific activity of Na^+,K^+-ATPase = 59 μmol P_i/mg/hour). b, NaI-treated microsomal fraction (specific activity = 170); c, glycerol-precipitated fraction after solubilization with deoxycholate (specific activity = 1290); and d, enzyme fraction after deoxycholate-cholate solubilization of fraction C and ammonium sulfate precipitation followed by dialysis (specific activity = 1537). These micrographs were taken by Dr. Margaret A. Goldstein, Departments of Cell Biophysics and Medicine, Baylor College of Medicine.

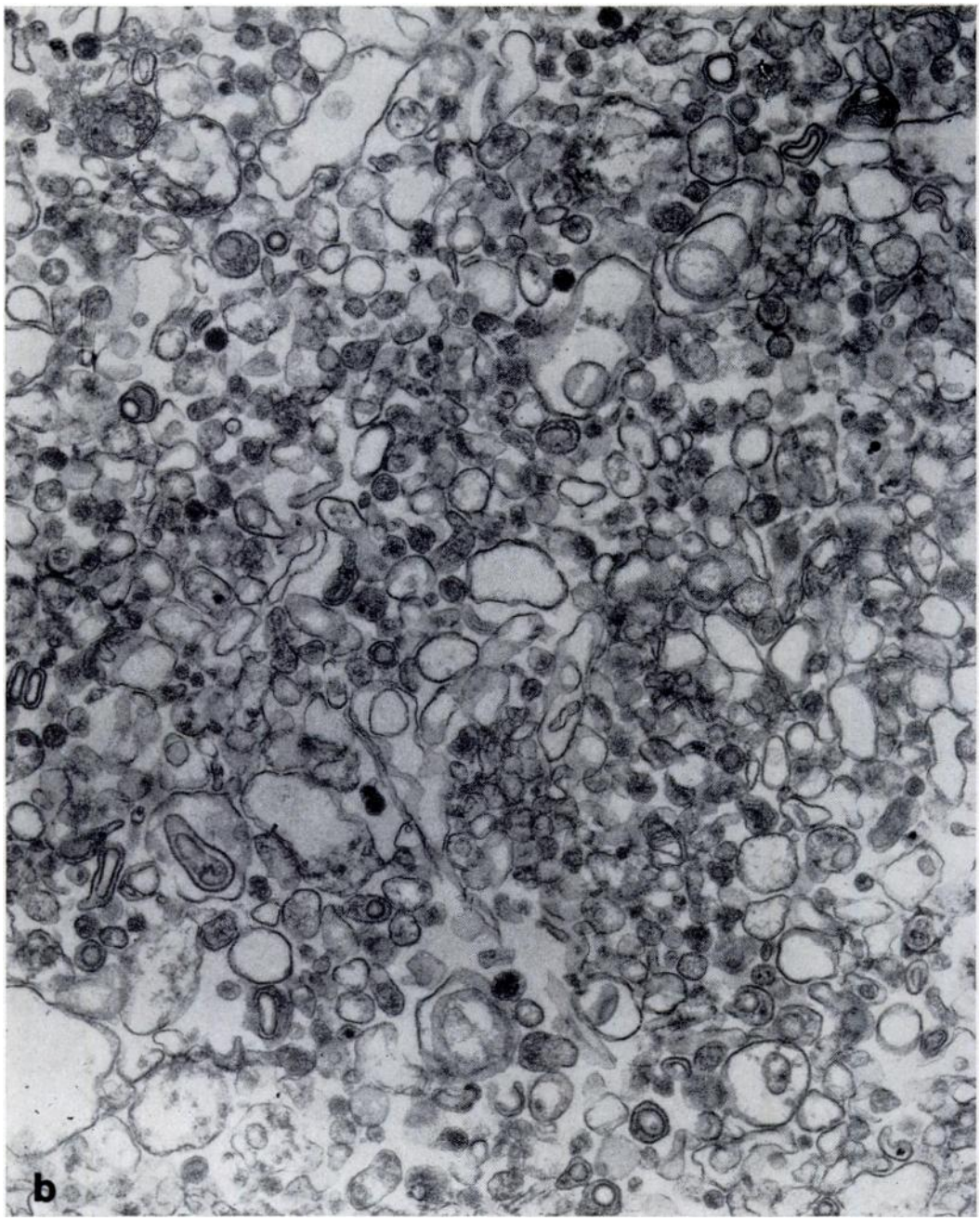

FIG. 1b.

exhibit protein particles of varying size and distribution. The "purer" ammonium sulfate fraction (fig. 3) appears as rough vesicles with densely packed particles of the same size embedded in the membrane.

V. Studies on the Molecular Nature of the Sodium Pump

Most mammalian cells require an intracellular environment containing low sodium and high potassium, even though

FIG. 1c.

these cells are typically bathed by a medium containing high sodium and low potassium. The cell membrane is characterized by a low but finite permeability to sodium, a higher permeability to potassium, and a transmembrane potential with the inside of the cell being electrically negative relative to the outside. The sodium

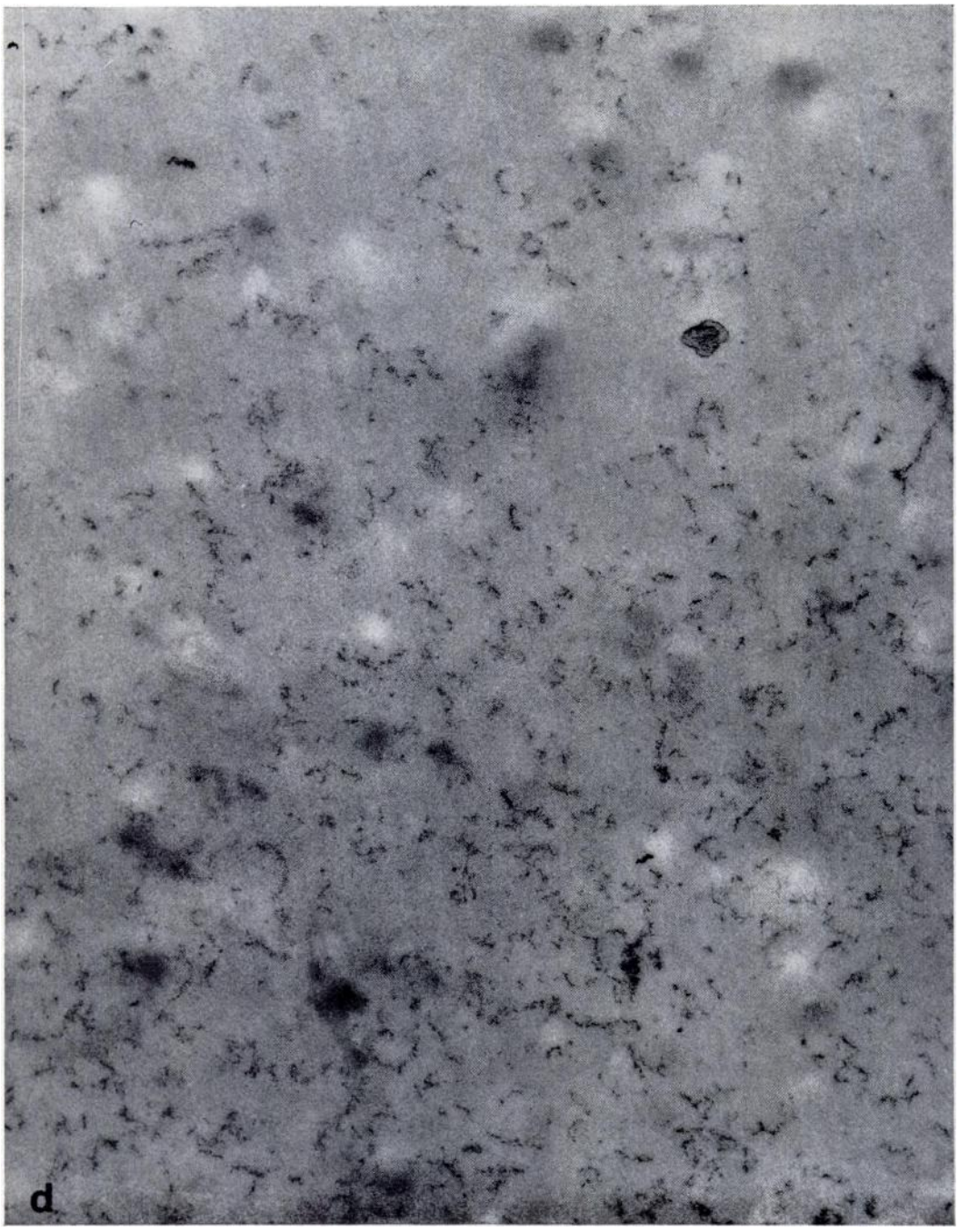

FIG 1d.

pump is thought to play a key role in the maintenance of these transmembrane cation and potential gradients. The net effect of the forward reaction of the pump is to move sodium ions out of the cell against an electrochemical gradient and potassium ions into the cell down an electrical, but against a chemical, gradient with energy derived from the hydrolysis of ATP (fig. 4). The stoichiometry of this reaction is believed to be three sodium ions pumped out per two potassium ions

pumped in per molecule of ATP hydrolyzed (199, 205, 584, 697). Thus, the pump may be electrogenic, since one net positive charge is moved out of the cell. [The contribution of this electrogenicity to the resting membrane potential varies from tissue to tissue (343, 577)]. The end result of the forward reaction is that at least part of the energy released by the hydrolysis of ATP is transduced into an increase in the potential energies of the transported cations. An understanding of how the sodium pump works will require a solution to the fundamental problem of how the energy available in ATP is converted into osmotic and electrical work.

The manifestation of the sodium pump in fragmented membrane preparations is a Na^+,K^+-ATPase activity discovered in 1957 by Skou (609). This enzyme hydrolyzes ATP in the presence of sodium and potassium through a mechanism inhibited by cardiac glycosides, which are specific inhibitors of the sodium pump in intact transporting systems (219, 556). While fluxes of sodium and potassium cannot be measured in fragmented membrane preparations that have Na^+,K^+-ATPase activity, the similarities of these preparations compared to those from intact transporting systems strongly suggest that the Na^+,K^+-ATPase activity is the catalytic expression of the complete and coupled enzyme transport complex (501, 614). [The definitive evidence for this conclusion would be the incorporation of Na^+,K^+-ATPase into an artificial membrane; the system should then catalyze the uphill movements of sodium and potassium (see the previous section)]. We will use the terms "Na^+,K^+-ATPase" and "sodium pump" to denote the system in fragmented membrane preparations and intact transporting systems, respectively, but the terms, as used, will apply to the same system.

A. Orientation within the Membrane

Early studies of the nature of the sodium pump revealed that it was asymmetrically oriented within the membrane. Intact transporting systems were used, in which the intracellular and extracellular salt concentrations could be varied and maintained independently. [The preparations most effectively used to date are the resealed erythrocyte ghost (272, 280, 695) and the internally perfused giant squid axon (104).] The forward direction of the reaction, for example, requires the presence of internal sodium and ATP and external potassium and the pump is inhibited specifically by cardiac glycosides added to the external medium. When the drug is placed in the intracellular space, there is no effect. These characteristics suggest that the nucleotide and glycoside react with sites located on the internal and external surfaces (*i.e.*, with respect to the cell) of the membrane, respectively (46, 49, 104, 105, 148, 220, 276, 357, 385, 558, 584, 695, 696). Sodium interacts with sites located on the internal surface of the membrane to activate the enzymatic reaction. These sites have a relatively high affinity for sodium and a low affinity for potassium and will subsequently be referred to as the "sodium activation sites." Potassium reacts with sites located on the external surface of the membrane (the "potassium activation sites"), which exhibit a relatively low affinity for sodium but a high affinity for potassium. Potassium interaction with the sodium activation sites or sodium with potassium activation sites prevents or retards the transport-hydrolysis reaction (110). A variety of monovalent cations (excluding sodium) are effective substitutes for potassium at the potassium activation sites, but no monovalent cation can substitute for sodium at the sodium activation sites (46, 220, 385, 496, 500, 614, 695, 696).

The physical pathways used by sodium and potassium in their movement through the membrane are unknown with respect to transport mediated by the pump, although several possibilities have been suggested (159, 307): a) penetration

FIG. 2. Visualization of canine kidney Na^+,K^+-ATPase fractions by freeze etch electron microscopy. Fractions representing various steps in the purification of canine kidney Na^+,K^+-ATPase (377) were prepared for freeze etching by conventional techniques (658b). Freeze fraction and etching were carried out in a Balzers 360 M apparatus and replicas examined in a Jeolco 100 B electron microscope. Representative field of crude canine microsomal preparation is presented. Vesicles of various sizes made up of smooth lipid regions (L) with intercalated protein particles (P) are seen. Convex fracture faces (large double arrows)

through pores lined with fixed negative charges or carbonyl groups; b) penetration *via* a complex with a membrane-localized carrier; or c) penetration by initial "tight" binding to the membrane (pump), with subsequent distortion in the system such that the bound cations are exposed to the opposite surface, and affinities of the pump decrease (or the cations are shifted to lower affinity sites) to encourage dissociation of the cations. Since the movements of sodium and potassium occur through the membrane, it is reasonable to suppose that the pump completely penetrates the structure, with opposite surfaces of the pump exposed to the intracellular and extracellular spaces. It is assumed that the pump *per se* is a protein(s); therefore, complete penetration is consistent with modern concepts of membrane structure (86, 358, 391, 412, 604, 677, 683, and Introduction). On the other hand, the alternate possibility of an orientation more consistent with the structure proposed by the unit membrane theory (137, 138, 262, 529) has not been eliminated.

B. General Considerations of Mechanism

The sodium pump must have high affinity sites on its internal surface in order to bind sodium out of an intracellular milieu containing low sodium and high potassium. Also, the affinity of these sites must be low for potassium, and there should be three sites on a pump unit that bind sodium in order to account for the stoichiometry of the forward reaction. After movement of sodium through the membrane, these ions are exposed to the extracellular space but, at some point, must remain attached to the pump. Since the extracellular space contains high sodium, however, the binding forces must be less than that originally present at the internal surface, so that the ions dissociate into the external space to complete the efflux reaction. The same requirements must be met for potassium influx, with the exceptions that the movement is in the opposite direction and that there should be two binding sites for this cationic species. There are a number of ways in which the cationic affinities may be altered (577): a) these changes may reflect a shift of the cation to progressively weaker binding sites as it traverses the membrane; b) only one physical site may be involved per bound cation, but its affinity may be altered as it is oriented from the internal to the external surface (sodium) or *vice versa* (potassium); or c) the site may vary in accessibility so that the unloaded site exists predominantly at one surface while the loaded site exists at the opposite surface.

Shaw (592) in 1954 proposed a simple model to explain the coupled uphill transport of sodium and potassium. He suggested that a carrier, with alternating high affinities for the two cationic species could explain the reaction. One state of the carrier would have high affinity for sodium and low affinity for potassium, and a second state would have high affinity for potassium and low affinity for sodium. Each state of the carrier would also have an inside and an outside orientation with respect to the membrane surfaces. Finally, transformations between these states would occur at the external and internal surfaces and would be associated with chemical changes in the state of the pump. There is good evidence that such a mechanism applies to the pump. This evidence will be discussed below, but there is other evidence which appears to support another type of mechanism, one of simultaneous sodium and potassium interaction.

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represent the inner portion (facing interior of vesicle) of the bimolecular lipid layer with associated proteins Concave faces (small double arrows) represent the outer half of bimolecular layer. It is apparent that the membrane protein particles differ in size from 80 to 150 Å and in spatial distribution. (× 40,000.) These micrographs were taken by Dr. W. Barry Van Winkle, Department of Cell Biophysics, Baylor College of Medicine.

FIG. 3. Fraction of 29 to 50% $(NH_4)_2SO_4$ cut of canine kidney preparation. Well formed vesicles are less numerous than in figure 2. Circular outlines of vesicles (large arrows) as well as random sheets predominate. Protein particles are of mainly one size, ~ 110 Å, and densely cover most surfaces (small arrows) as contrasted to some large particle-free regions in figure 2. (× 32,000.) (Mottled background in both figures from glycerol – H_2O medium). These micrographs were taken by Dr. W. Barry Van Winkle, Department of Cell Biophysics, Baylor College of Medicine.

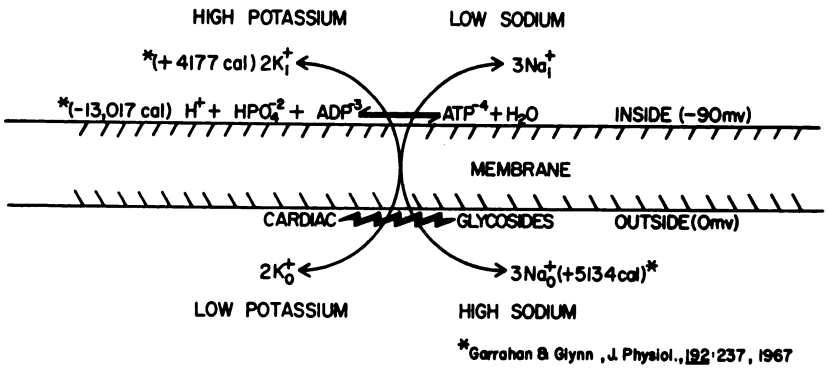

FIG. 4. Membrane orientation, stoichiometry and suggested energetics of the forward reaction of the sodium pump.

A sequential mechanism for the sodium pump, such as described above, requires that sodium interact with one state of the system followed by potassium interaction with a second state. Most models of this type require that there be an interdependence of the apparent affinities of the sodium and potassium activation sites (52). In other words, the degree of sodium interaction with its activation sites modulates the apparent affinities of the potassium activation sites and *vice versa*. Actually, the two species of sites may be physically independent in an autosteric or allosteric sense. In many sequential mechanisms, however, sodium and potassium would modulate the steady-state levels of the pump "conformations" that have high affinities for sodium and potassium. In this case, the response of pump activity to varying sodium or potassium concentrations would reflect the dissociation constant of a site for a particular cation and changes in the steady-state level of that site. Opposed to this postulation, the two species of interaction sites could be independent if they existed simultaneously. This would imply that sodium and potassium react in a random manner with their respective activation sites to form a specific complex with a particular part of the system.

C. Nature of the Sodium and Potassium Activation Sites

The Na^+,K^+-ATPase from a variety of sources and the sodium pump in erythrocytes respond in a sigmoidal manner to increasing concentrations of sodium (196, 240, 500, 534, 538, 609, 632). This deviation from a rectangular hyperbolic response is a function of the potassium concentration in the assay medium, *viz.*, the higher the potassium concentration the greater the deviation. As the sodium concentration becomes higher, a second deviation from the rectangular hyperbolic response is seen in fragmented membrane preparations in which high sodium inhibits activity. The degree of inhibition is inversely related to the potassium concentration. The antagonism between low sodium and high potassium is generally thought to reflect competition between these ligands for the sodium activation sites, whereas the antagonism between high sodium and low potassium is thought to reflect competition for the potassium activation sites. The response of a Na^+,K^+-ATPase activity to increasing concentrations of potassium is also sigmoidal, particularly in the presence of high sodium. The antagonism seen at low potassium is believed to reflect competition between sodium and potassium for the potassium activation sites (196, 203, 240, 273, 281, 500, 534, 538, 555, 609, 632). Similar to the case for sodium activation, higher potassium in the presence of low sodium causes inhibition of activity; this appears to reflect competition by the ligands for the sodium activation sites.

The sigmoidal nature of the sodium and potassium activation curves could be a consequence of three factors. First, the stoichiometry of the pump requires multiple sodium (three) and potassium (two) interactions per pump molecule per turnover cycle (see above). This requirement *per se* means that the activation responses must be sigmoidal, since it is generally predicted by the following relationship, where the multiple interaction sites are equivalent and independent.

$$\frac{v}{V_m} = \frac{1}{\left(1 + \frac{k_s}{s}\right)^n}$$

n = number of interaction sites which must be occupied by the substrate (or here, the activator ligand). The same type of response is seen when multiple nonequivalent sites must be occupied by the same ligand species (49, 203, 281, 398, 509, 555). The second factor which may modulate the sigmoidal nature is the interrelationship or interdependence between the multiple sites for the same ligand. The first sodium interaction with the pump, for example, may increase or decrease the affinity of the second and/or third site on the pump for sodium. A similar relationship may exist between the potassium activation sites (534, 538, 632, 668). [See the interesting dialog between Robinson and Garrahan (200, 535) with respect to interdependence *versus* independence of the sodium or potassium activation sites.] A third influence is the possible interdependence between the sodium and potassium activation sites. Thus, in the sequential model for sodium and potassium interaction, the degree of sodium binding to its sites could modulate the steady-state level of pump "conformers" that can interact with potassium and *vice versa* (see above and ref. 52).

We have shown that the activation of a beef brain Na^+,K^+-ATPase by potassium is consistent with potassium interaction at two-nonequivalent but independent sites with high affinity for potassium (398). These sites had low affinity for sodium. The data for sodium activation were consistent with a three-equivalent site model, where the sites were independent and exhibited high affinity for sodium and low affinity for potassium. Furthermore, in this model, the sodium activation sites and the potassium activation sites were independent. This is consistent with the *simultaneous* existence of the sodium and potassium activation sites as opposed to their sequential existence (but see below), and the requirements of the model for sodium and potassium activation of the Na^+,K^+-ATPase is in accord with the stoichiometry proposed for the sodium pump. In an earlier study, however, Ahmed *et al.* (3) found that a two-equivalent site model explained their data for sodium activation of a rat brain Na^+,K^+-ATPase preparation. Their study was carried out over a range of low sodium and potassium concentrations. We could not eliminate the possibility (on statistical grounds) that a two-equivalent site model also applies to our data for sodium activation. In the latter case, however, the fit of a two-equivalent site model at low potassium (0.5mM) was better than at higher potassium.

With respect to potassium activation of the sodium pump in intact transporting systems, it is generally agreed that the pump responds in a sigmoidal and saturable manner to increasing concentrations of external potassium in erythrocyte (196, 203, 273, 281, 555) and squid axon (49) preparations. External sodium raises the amount of external potassium required to activate the pump by one-half of maximum. The erythrocyte pump, for example, has been reported to be activated by a similar amount by 0.14 to 0.4 mM potassium in the absence of external sodium and by 0.9 to 2.1 mM in the presence of 130 to 160 mM external sodium (203, 500, 509, 696). In squid axon, the pump is activated by 0.61 mM external potassium in the absence of sodium and 5.5 mM potas-

sium in the presence of 160 mM external sodium (49). The inflexion for a break or deviation from a rectangular hyperbolic response occurs at very low potassium concentrations. Sjodin and Beaugé (607) and Sjodin (605) observed that sodium efflux in muscle cells responded in a sigmoidal manner to external potassium in the presence of 120 mM external sodium. In the absence of external sodium, however, the response was a rectangular hyperbola. In erythrocyte ghost preparations, Garrahan and Glynn (203) found that even in the presence of very low concentrations of potassium, the response was sigmoidal. This is consistent with the existence of multiple sites for potassium. These authors (203) also suggested that the sites were nonequivalent (*i.e.*, they exhibited different affinities for potassium), since the inflexion of the curve describing the response of the pump to external potassium occurred at a much lower potassium concentration (*i.e.*, about 15 μM) than that required to active the pump by one-half of the maximum. [It is interesting that the two-nonequivalent site model derived for a Na^+,K^+-ATPase preparation predicts an inflexion between 10 and 20 μM potassium in the absence of sodium (398).]

Rectangular hyperbolic responses to potassium were observed for crab nerve and mammalian nonmyelinated nerve preparations (50, 514). However, Baker *et al.* (49) have pointed out that this response may reflect higher potassium in the mesaxon and periaxon spaces, and, thus, around the external surface of the pump, than the actual potassium concentration in the medium bathing the preparation. In squid axon, sodium efflux responds in a sigmoidal manner to increasing concentrations of external potassium (49).

The pump in erythrocyte and squid axon preparations responds to external potassium (either sodium efflux or potassium influx) in a manner described kinetically by models that have two-equivalent potassium activation sites (49, 196, 281, 555; but see ref. 203). In this regard, two-equivalent site models may mimic two-nonequivalent site models and *vice versa* if the affinity of the nonequivalent sites are not substantially different from each other (398).

The response of the sodium pump to increasing concentrations of internal sodium has been found to be a saturable function of internal sodium in erythrocyte preparations and for the isolated muscle of the barnacle preparation (87, 196, 500). Hoffman (274) and Garay and Garrahan (196) presented evidence that internal potassium competed with internal sodium for the sodium activation sites of the erythrocyte pump. In striated muscle, sodium efflux was found to be proportional to a cubed or higher function of internal sodium (250, 344, 345, 450). This is consistent with the requirement that multiple internal sites per pump molecule must be occupied by sodium in order for the pump to function. On the other hand, sodium efflux in squid axons appears to be a linear and nonsaturable function of internal sodium (48, 88, 271, 606). Thomas (658) reported in 1972 that sodium efflux in the snail neuron was a linear function of internal sodium, although a threshold of about 1 mM internal sodium was required. A linear response to increasing internal sodium is seemingly inconsistent with the requirement that multiple sites exist per pump molecule, and the lack of saturation is also disturbing. There are several explanations for these observations: a) The affinity of the sodium activation sites for sodium is much less in these preparations than in mammalian preparations; this could account for the apparent lack of saturability. b) A compartment could exist around the internal sites such that the concentration of sodium in the compartment differs from that measured for (or perfused into) the intracellular space. c) The tacit assumption that interaction of

cations with the internal sites is limited only by the rate of diffusion of the cations to the sites may be wrong; this assumption implies that cation interaction with the pump is not rate limiting, therefore, saturability occurs. Conversely, if the state of bound sodium converts to a different state much more rapidly than the initial sodium interaction, saturability might not be observed. d) There may be differences in the coupling of sodium efflux to potassium influx. Mullins and Brinley (449) found that potassium influx was a nonlinear function of internal sodium concentrations whereas sodium efflux was a linear function. They suggested that these differences may reflect a flexibility in the coupling between sodium and potassium transmembrane movements as catalyzed by the sodium pump.

In 1973, Garay and Garrahan (196) presented a kinetic description for sodium activation of sodium efflux in intact erythrocyte preparations. They found that sodium efflux responded in a sigmoidal and saturable manner to increasing concentrations of internal sodium. Their model for sodium activation required three sodium activation sites per pump. The apparent affinity of these sites for internal sodium was modulated by internal potassium in a competitive manner; this affinity was about 50 times higher for sodium than for potassium. Also, their data were explained by a composite model for sodium (three-equivalent and independent sites) and potassium (two-equivalent and independent sites) activation, wherein the two species of sites existed simultaneously.

There is little information beyond the general characteristics described above about the physical nature of the sodium and potassium activation sites. Kepner and Macey (342) noted that a protein of 250,000 molecular weight with a density of 1.3 would correspond to a spherical particle with a diameter of about 85 Å. The radii of dehydrated sodium and potassium are about 0.95 to 1.33 Å, respectively, so that with a pump molecule, it may be feasible to assume an independence between ion interactions. Of course, the hydrated cations are larger, and it is not known whether the hydration sphere is stripped from the cations as they interact with the system. Also, it is unclear as to the nature of the forces that allow cation interaction. One possibility is electrostatic interaction. A second possibility is that an ionophoric-like site or component (508, 707) exists within the pump and substitutes for the hydration sphere of the cation. To a large extent the affinity would be determined by the size of the sphere of the site into which the dehydrated cation enters. Such a site is attractive in that ionophores do exhibit a substantial ability to discriminate between different cation species (*e.g.*, valinomycin has an affinity for potassium which is three orders of magnitude greater than its affinity for sodium). In this regard, in 1974 Shamoo and Albers (587) reported the isolation of a substance from *Electrophorus electricus* microsomes (with high Na^+,K^+-ATPase activity) that could be incorporated into an artificial lipid bilayer in the presence of sodium. When the enzyme was incorporated, the bilayer then exhibited a marked increase in sodium conductance. Shamoo (586) stated in 1974 that the sodium-dependent ionophoric activity may be associated with the smaller polypeptide (*i.e.*, the glycoprotein) of a highly purified Na^+,K^+-ATPase preparation. It will be necessary for these interesting findings to be reproduced before definitive conclusions are made.

Some evidence was presented in 1974 that a size-restrictive determinant may be operative in the affinities exhibited by the potassium activation sites of a beef brain Na^+,K^+-ATPase preparation (398). A site with high affinity for potassium and low affinity for sodium was found to modulate the interaction of a cardiac glycoside with the system (396). Since these relative affinities were consistent with those of a potassium activation site, a model incorporating

the dissociation constants for potassium and sodium was applied to potassium activation of Na^+,K^+-ATPase activity. As described above, we found that this site and a second site were required to explain the data. A comparison between the sites of the dissociation constants for sodium and potassium (*i.e.*, 0.213 mM for potassium and 13.7 mM for sodium for the site involved in modulation of glycoside binding; 0.0091 mM for potassium and 74.1 mM for sodium for the second site) showed that the affinity of the second site for potassium was greater than the affinity of the first site but that the affinity of the second site for sodium was less than that for the first site. This difference could be explained by the second site being slightly larger than the first, since dehydrated potassium is slightly larger than dehydrated sodium. This argument assumes that a major determinant of the affinity is the *size* of the site, and it also assumes that the dehydrated cation binds to the site as opposed to the hydrated cation. Such a difference in affinities appears hard to explain if electrostatic interactions were the major determinant. Further suggestive evidence for size restriction is that the affinity of the first site decreases slightly for sodium as the temperature is lowered, while the affinity of this site for potassium markedly increases with a drop in temperature (686). A much stronger argument for size restriction is evident if the sodium activation sites are converted to the potassium activation sites during the enzyme (pump) turnover cycle, as proposed originally by Shaw (592). In this case, the dissociation constant for sodium (1 mM or less) and potassium (10 mM or greater) for the sodium activation state would be converted to values of 15 mM or greater for sodium and 0.5 mM or less for potassium for the potassium activation state. Such affinity changes in opposite directions for two similar cationic species do not appear possible if all that is being altered is the electronic distribution around a negatively charged, fully exposed residue.

We have previously pointed out that several factors extraneous to the system *per se* may modulate the function of the pump *in situ* (577). One of these is water structure. It is now generally agreed that the water close to macromolecular structures is more highly ordered than the water some distance away. Structured water may alter ionic mobilities of sodium and potassium and may greatly increase the mobility of protons through "tunneling" effects. Exactly how structured water would modulate pump function is unclear, but it appears to be almost certain that the pump does not operate on the basis of ions dissolved in "normal" water.

A second modulating factor is pH. Although it is well recognized that macromolecular conformation is a function of pH, this factor may be important for a second reason, *viz.*, a proton is a product of ATP hydrolysis. Lindenmayer *et al.* (398) and Mitchell (444) have proposed that water (*i.e.*, a reactant with ATP for hydrolysis) may participate in the reaction by physically entering the pump from the external surface and that the proton, along with ADP and inorganic phosphate, leaves the membrane at the internal surface. Such a case may have mechanistic advantages beyond the obvious requirements that ATP hydrolysis remain electrically neutral at the inside surface and that the proton not leave the cell (*i.e.*, in order to restrict the electrogenicity of the reaction to one net positive charge pumped out per cycle). Thus, the structure of water as a reactant, the mobility of protons, and the external pH may modulate the turnover rate of the sodium pump. It is interesting that Albers and Koval (20) and workers in this laboratory (398) suggested that a molecule of water entering the reaction may originate from the hydration sphere of potassium. In this case, structured water, by modulating the mobility of potassium, could regulate the rate at which both potassium and water

enter the reaction. A further consideration of proton concentrations and mobilities is the possibility that a proton crosses the membrane from outside to inside such that the forward reaction of the pump is electrically neutral (*i.e.*, three sodium ions pumped out per two potassium ions and one proton pumped in) as opposed to being electrogenic, at least in some tissues. Finally, Fujita *et al.* (193) observed a potassium-stimulated ATPase activity over a pH range of 5 to 6 as opposed to the the Na^+,K^+-ATPase activity observed at a higher pH. This reaction was insensitive to cardiac glycosides. However, we have not been able to confirm this observation.

A third factor that may modulate the function of the sodium pump is macromolecular complexes (*e.g.*, mucopolysaccharides) adjacent to either or both surfaces of the pump. These complexes typically possess negatively charged residues that may bind monovalent or divalent (or higher?) cations and/or protons. In this regard, it is of interest that a protein closely associated with or a part of the Na^+,K^+-ATPase is a glycoprotein (see above). Marchesi and his colleagues (421, 424, 582, 583) analyzed the structure of a glycoprotein, "glycophorin," from erythrocyte membranes and suggested that its negative charges were located in the external milieu. Thus, the glycoprotein associated with the Na^+,K^+-ATPase may have a considerable regulatory role in pump function, even though it may not be required *per se* for catalytic activity.

Divalent cations, particularly magnesium and calcium, also modulate the pump and its chemical analog, Na^+,K^+-ATPase. Magnesium is a cofactor for the forward reaction but may have additional regulatory roles. For example, it appears to compete with sodium for the sodium activation sites and may increase the affinity of the potassium activation sites for potassium (397). Calcium appears to have multiple effects. In high concentrations, calcium competes with magnesium for sites involved in the role of magnesium as a cofactor. A second effect involves a possible competition of calcium with sodium for the sodium activation sites (310, 322, 324, 325, 397, 488, 490, 564, 566, 663, 664, 703). A recent analysis carried out in this laboratory suggested that the affinity of these sites for calcium may be about 3 times greater than the affinity for sodium and about 30 times greater than the affinity for potassium (397). Calcium may substitute for sodium during phosphorylation of the Na^+,K^+-ATPase by ATP (564, 566, 664). A third effect of calcium is that it seems to decrease the amount of potassium required to activate the Na^+,K^+-ATPase by half-maximum. This can be explained by one of two possible mechanisms. First, calcium may act in an allosteric or autosteric manner to increase the affinity of the potassium activation sites for potassium. It is of interest in this respect, that the system's reactivities to potassium and to another divalent cation, beryllium, appear to be interrelated (532, 670). Alternatively, calcium may partially substitute for potassium at a potassium activation site (397), in spite of the report that the phosphoenzyme formed in the presence of calcium may be more stable than that formed in the presence of magnesium (664). Interestingly, Blum and Hoffman (80) found a calcium-induced increase in potassium permeability of erythrocyte membranes. This increase appeared to be mediated by the sodium pump in that the effect was inhibited by cardiac glycosides (also, see 361). The extent to which calcium may actually modify the activity of the sodium pump would depend on the affinity of these sites for calcium (*versus* the affinities for potassium and sodium) and the concentrations of calcium around the external and internal surfaces of the pump. In this respect, both heart and brain are thought to have intracellular free calcium concentrations of 1μM or less (47). Therefore, calcium interaction with the sodium activation sites

in situ may be negligible, at least in these two tissues. Conversely, the interstitial free calcium may range around 2.5 mM, so calcium modulation of potassium interaction (or substitution for potassium, in part) could have physiological significance (397).

D. Reactive States of the Sodium Pump and Na^+,K^+-ATPase

There are two general types of mechanisms by which the sodium pump can operate: 1) Internal ATP (or more likely the magnesium·ATP complex, see below), internal sodium, and external potassium react in a random manner to form a complex with the pump that contains all three sets of reactants at one time. This complex decomposes to yield the products of the reaction, *i.e.*, external sodium, internal potassium, and internal ADP and inorganic phosphate. 2) The overall reaction is composed of a series of partial or elementary reactions that connect different conformers or "states" of the pump. These "states" are characterized by their ability to interact with a particular set of reactants and/or to yield a particular set of products. It is not possible to eliminate either type of mechanism from consideration at the present time. The second mechanism does predict the existence of different states of the pump which could potentially be detected by experiment. In its favor, multiple states have been detected by the use of a variety of probes.

Fragmented membrane preparations with relatively high Na^+,K^+-ATPase activities are phosphorylated by [γ-^{32} P] ATP when sodium and magnesium are present in the reaction medium (38, 41, 42, 75–77, 119, 120, 127, 172, 214, 260, 290, 333, 426, 455, 456, 466, 492, 493, 501–503, 520, 544, 567, 568, 620, 705). The amount of phosphate incorporated into the enzyme system under a constant set of reaction conditions correlates with the level of Na^+,K^+-ATPase activity, *i.e.*, the higher the activity the greater the level of phosphorylation (503). The phosphomembrane complex is acid stable and not extractable with solvents used to remove phospholipids. Thus, the phosphate from ATP is presumed to be attached to a protein in the membrane through a covalent bond. Nagano *et al.* (455) and Hokin *et al.* (290) found that the phosphoprotein complex was labile to alkaline treatment, stable between pH 1–3 and was split by hydroxylamine or acetylphosphatase. The complex was also susceptible to methanolysis or ethanolysis (44, 45, 260). While there are problems in the interpretation of some of these results, particularly those for hydroxylamine (40, 45, 117, 123, 124, 172, 544, 577), the conclusion derived from these studies was that the phosphate was linked to a carboxyl group on the protein through an "acyl" covalent linkage. Studies reported in 1967 and 1968 suggested that the phosphate was attached to the *gamma*-carboxyl of a glutamyl residue (327, 328), but later (1973, 1974) reports indicate clearly that the phosphate is attached to the *beta*-carboxyl of an aspartyl residue (283, 464a, 497).

Schwartz *et al.* (577) pointed out in 1972 that the use of acid to denature the membranes may favor formation of an enzyme acyl phosphate from the inorganic phosphate which could be electrostatically linked to the membrane system. Alternatively, the low pH may induce an acid-catalyzed migration of phosphate (21). Alexander and Rodnight (21) in 1974 showed that when the phosphorylating reaction is terminated by sodium dodecyl sulfate at neutral pH, the pH profile of stability and the sensitivity to hydroxylamine of the bound phosphate is similar to that obtained after acid denaturation.

Transphosphorylation (*i.e.*, from ATP to the membrane protein) requires sodium and magnesium and no other monovalent cation substitutes for sodium (503; but see ref. 620 with respect to one report of a lithium effect), which is significant since no other monovalent cation can apparently substitute for sodium at the sodium activation sites of the intact sodium pump

or on Na^+,K^+-ATPase with respect to activating the system. If potassium is also included in the reaction medium, the phosphoprotein level decreases. This suggests that potassium either prevents the formation of the complex or stimulates its breakdown. Formation was found to be quite rapid at low temperatures and the level of the complex reached a steady-state level within a matter of seconds. Addition of unlabeled ATP causes the level of the phosphoprotein to decrease, which is consistent with the conclusion that the phosphoprotein is a labile compound. Addition of potassium with unlabeled ATP increases the rate of breakdown by at least 5-fold over that seen only with unlabeled ATP. This suggests that potassium stimulates the hydrolysis of the acylphosphate. Monovalent cations which substitute for potassium with respect to activation of Na^+,K^+-ATPase or the sodium pump also stimulate the hydrolysis of the acylphosphate. In 1969, Post *et al.* (499) reported other data that further support the conclusion that the predominant action of potassium may be to stimulate the rate of hydrolysis of the acylphosphate.

Albers *et al.* (170–173, 599) have presented evidence consistent with the tenet that there are multiple forms of the phosphoprotein. They found that the microsomes isolated from the electric organ of the electric eel *(Electrophorus electricus)*, had a particularly high Na^+,K^+-ATPase activity and that this preparation catalyzed an ATP-ADP exchange reaction under specific conditions: a) in the presence of sodium and low magnesium; and b) in the presence of sodium, magnesium, and the sulfhydryl reagent, N-ethylmaleimide. Subsequently, the association of this reaction with other Na^+,K^+-ATPase preparations was confirmed (57, 633–635). Further investigation by these workers (171–173, 599) showed that N-ethylmaleimide, oligomycin, arsenite or 2,3-dimercaptoethanol inhibited the potassium-stimulated breakdown of the phosphoprotein, which suggests that potassium activates the reaction by interacting with only one of the two forms of the phosphoprotein. Post *et al.* (499) confirmed this interpretation by showing that potassium but not ADP decreased the level of the acylphosphate in "native" microsomes. Conversely, ADP but not potassium caused the disappearance of the phosphoprotein in N-ethylmaleimide-treated microsomes. These two forms of the acylphosphate have been designated as E_1-P, reactive to ADP, and E_2-P, reactive to potassium. Since there is evidence that the two forms of the enzyme are chemically similar (*i.e.*, the phosphate is probably linked to the same amino acid residue), the difference in reactivity probably reflects subtle but important different conformations of the enzyme.

Banerjee and Wong (57) reported that potassium in a guinea pig kidney preparation had two effects on the ADP-ATP exchange reaction: a) inhibition at low magnesium and sodium which was overcome by increasing sodium; and b) stimulation in the presence of 32 mM sodium and 0.4 mM magnesium which was abolished by N-ethylmaleimide. They proposed that the stimulatory effect of potassium may be due to a slowing of the conversion of E_1-P to E_2-P (see the scheme below).

The characteristics of the two forms of the phosphoprotein fit into a scheme for the turnover cycle of the Na^+,K^+-ATPase. Post *et al.* (503) and Kanazawa *et al.* (332) reported that the rates of formation and breakdown of the phosphoprotein were consistent with the turnover number (*i.e.*, the catalytic rate) of the enzyme. In other words, the phosphoprotein has the characteristics required of an intermediate state in the sequence of ATP hydrolysis, as catalyzed by the Na^+,K^+-ATPase. The following scheme was suggested by Siegel and Albers (599) to account for this sequence.

$$\text{(a)}\quad \text{ATP} + E_1 \overset{Mg^{2+},\,Na^+}{\rightleftharpoons} E_1\text{-P} + \text{ADP}$$

$$\text{(b)}\quad E_1\text{-P} \overset{Mg^{2+}}{\rightleftharpoons} E_2\text{-P}$$

(c) $E_2P + H_2O \xrightleftharpoons{K^+} E_2 + P_i$

(d) $E_2 \rightleftharpoons E_1$

In 1968, Albers *et al.* (18) applied this reaction sequence to sodium and potassium translocation catalyzed by the sodium pump.

A second way which has been used to identify reactive states of the Na^+,K^+-ATPase is nucleotide binding. General theories for macromolecular-catalyzed reactions require that an early step in the reaction is the combination of the substrate with the enzyme, and a number of studies in the last several years have been directed toward detection of an ATP or ADP complex with the Na^+,K^+-ATPase. In 1971, Jensen and Nørby (312) and Nørby and Jensen (469) and Hegyvary and Post (259) reported evidence of ATP binding to the system, which was obtained by a flow dialysis technique. Their assays required low temperatures (0–2°C) and the presence of an agent to chelate endogenous magnesium to minimize hydrolysis of ATP. Scatchard plots of the binding data were rectilinear, with the possible exception of binding at high concentrations of ATP (259), and yielded dissociation constants of 0.12 to 0.22 μM. Thus, the binding of ATP to the membrane preparations responded to increasing free ATP in a saturable manner described by adsorption to a single species of independent (*i.e.*, non-cooperative) sites. Several experiments suggested that the binding was related to Na^+,K^+-ATPase instead of other proteins of the preparations: a) amounts of nucleotide bound correlated with Na^+,K^+-ATPase activities but not with ouabain-insensitive ATPase activities; b) pretreatment with ouabain prevented binding; c) the structural requirements for nucleotide binding appeared to be consistent with the requirements for hydrolysis of ATP as catalyzed by the Na^+,K^+-ATPase (259); d) the maximum number of ATP binding sites essentially equaled the capacity of the preparation to be phosphorylated by ATP (259); and e) the molecular activity of the enzyme based on the number of ATP binding sites was similar to that estimated from other parameters (*e.g.*, phosphoprotein formation, cardiac glycoside binding) (259).

ATP binding was independent, or almost independent, of sodium when potassium was absent, although high concentrations of sodium may have had a slight stimulating effect (259). On the other hand, potassium markedly decreased the amount of nucleotide bound, and this effect of potassium was antagonized by sodium (259, 312, 469).

In 1974, Nørby and Jensen (470) found that sodium modulates the apparent affinity of Na^+,K^+-ATPase for ATP. Addition of up to 12 mM sodium increased the affinity by a factor of two. However, the authors concluded that this effect of sodium was due to contamination of their preparation by 0.5 to 0.6 mM potassium. Sodium had little effect on the affinity for ATP when a washed preparation, which reduced endogenous potassium to 70 μM, was employed. Other monovalent cations, except lithium, which are effective substitutes for potassium also reduce ATP binding, and a titration of the response of ATP binding to increasing concentrations of these cations suggested that the latter acted through multiple and perhaps cooperative sites (259). Two mechanisms were proposed to account for the antagonism between potassium and ATP. Nørby and Jensen (469) plotted apparent dissociation constants for ATP against potassium concentrations and found that the constants approached a maximum value with increasing potassium. This result led these authors to conclude that a complex type of competition exists between ATP and potassium such that the presence of one of the ligands on the enzyme altered the affinity for the second ligand (also see ref. 480). Data reported by Nørby and Jensen are consistent with the existence of an enzyme state

in which ATP and potassium are simultaneously bound.

Reaction	Dissociation Constant
(a) $E + ATP \rightleftharpoons E\text{-}ATP$	0.12 μM for ATP
(b) $E + K^+ \rightleftharpoons K^+\text{-}E$	87 μM for potassium
(c) $K^+\text{-}E + ATP \rightleftharpoons K^+\text{-}E\text{-}ATP$	0.69 μM for ATP
(d) $E\text{-}ATP + K^+ \rightleftharpoons K^+\text{-}E\text{-}ATP$	500 μM for potassium

(Numerical values for the constants were derived from the best fit of the above relationships to data by Nørby and Jensen.)

Post *et al.* (495) suggested a slightly different mechanism. Potassium reacts with the enzyme to accelerate dephosphorylation (as described above) after which it remains bound to the dephospho-form of the enzyme. ATP, by binding to an activating site, displaces potassium, which allows the enzyme to enter a state susceptible to phosphorylation, *i.e.*, E_1. Siegel and Goodwin (600), employing uridine triphosphate (UTP) as a substrate (in an attempt to study the effects of potassium when phosphorylation is rate-limiting), reached a similar conclusion, *viz.*, potassium can inhibit by stabilizing a conformer that has reduced affinity for sodium and nucleotides.

The concentration of magnesium in these studies was reduced to 1×10^{-9} M or less (469) by chelating agents. Thus, ATP binding appears to be independent of magnesium; ATP hydrolysis, however, requires magnesium and, in fact, the activity of Na^+,K^+-ATPase is at a maximum when the magnesium to ATP ratio is one or two and the two ligands are present in millimolar concentrations. Under these conditions, more than half of the ATP is complexed with magnesium. Also, an analysis of catalytic rates at various magnesium and ATP concentrations and ratios led Hexum *et al.* (264) to the conclusion that the magnesium·ATP complex is the true substrate for the enzyme, that free ATP is a competitive antagonist of this complex, and that free magnesium inhibits in a noncompetitive manner.

Thus, one may question whether the above reactions, with respect to the magnesium-*independent* binding of the ATP, have any relevance to the physiological turnover cycle of the enzyme at higher temperatures. Several studies suggest that they do. Robinson (534), for example, showed that the dissociation constant (or K_m) for Na^+,K^+-ATPase activity for ATP increases with increasing potassium in the assay medium when magnesium is present at concentrations equal to ATP. Also, the low dissociation constant for ATP with respect to binding in the absence of magnesium (*i.e.*, 0.12 to 0.22 μM) is similar to the K_m (*i.e.*, 0.5μM) reported by Post *et al.* (503) for phosphorylation of the enzyme by ATP when the ratio of magnesium to ATP was maintained at one.

Kaniike *et al.* (334) found that ADP also binds to membrane preparations containing Na^+,K^+-ATPase activity, with a dissociation constant of 0.34 μM in the presence of 5 mM sodium, in the absence of potassium and a chelating agent, and at 0°C. Scatchard plots of ADP binding at various ADP concentrations were rectilinear, signifying that binding occurred through a single species of sites on the enzyme which were independent in an allosteric or autosteric sense. Potassium raised the dissociation constant for ADP, and this effect was overcome by sodium through a competitive type of antagonism.

Tonomura and Fukushima (671), on the basis of studies carried out with a rapid mixing device, suggested that a series of nucleotide enzyme complexes exist in the sequence of ATP hydrolysis in a manner similar to proposed schemes for myosin ATPase:

$$ATP + E \rightleftharpoons E_1ATP \rightleftharpoons E_2ATP \rightleftharpoons E^{ADP}_{\sim P}$$
$$\rightleftharpoons E \sim P + ADP$$
$$\rightarrow E + P_i + ADP$$

They concluded that potassium had at least two effects on this sequence. Potassium shifts $E^{ADP}_{\sim P}$ back to E_2ATP but stimulates the hydrolysis of $E\sim P$ to free en-

zyme and inorganic phosphate. Low concentrations of sodium favored formation of E_2ATP from E_1ATP, but this conversion was inhibited by high sodium. These results, therefore, suggest that low sodium should favor nucleotide interaction with the Na^+,K^+-ATPase. In 1974 we (393) presented evidence consistent with this concept (see the discussion of ouabain binding studies presented below).

In 1974, Skou (619) reported experiments in which the effects of sodium, potassium, ATP, and the magnesium·ATP complex were examined on the activity of Na^+,K^+-ATPase remaining after exposure to N-ethylmaleimide. He concluded that potassium acted at a "sodium-activation site" to reduce the affinity of the enzyme for ATP. Conversely, ATP *or* the magnesium·ATP complex increased the affinity of the "sodium-activation sites" for sodium.

Brodsky and his colleagues (90, 588–591) have carried out a series of studies of nucleotide interaction with a Na^+,K^+ATPase preparation derived from mucosal epithelial cells of turtle bladder. They found a magnesium-dependent interaction of ATP with this preparation which was stable during acid treatment of the microsomes. In the presence of magnesium, sodium, and potassium, ouabain inhibited part of the nucleotide binding, which suggested that this part of the binding is related to the Na^+,K^+-ATPase. Further studies (90) suggested that ATP may be covalently attached to the protein through a phosphoryl-acyl bond, an adenosinyl-protein bond and perhaps a phosphoramido bond. It is interesting that the amount of acid-stable, covalently linked ATP was only 5 to 10% of the amount of acylphosphate (*i.e.*, E-P; see above) formed in the presence of ATP, magnesium plus sodium. In 1974, Brodsky (89) reported similar results for a Na^+,K^+-ATPase preparation derived from the eel electric organ. It should be pointed out that Post *et al.* (503), Fahn *et al.* (172), and Rodnight *et al.* (544) could not detect the existence of an acid-stable, ATP-enzyme complex.

In 1967, Garrahan and Glynn (206) showed that the sodium pump in resealed erythrocyte ghosts could catalyze the synthesis of ATP through a ouabain-sensitive mechanism. This was observed when the internal medium of the ghosts contained a high concentration of potassium and a very low concentration of sodium. In addition to these concentrations, ATP, ADP and inorganic phosphate were added to give a low ratio of $[ATP]/[ADP]\cdot[P_i]$. The synthesis of ATP was also stimulated by an external medium containing a high concentration of sodium and no potassium. These authors suggested that the synthesis of ATP under these conditions represents reversal of the forward reaction such that potassium efflux and sodium influx were coupled to ATP synthesis. Glynn and Lew (228) subsequently showed that potassium efflux is associated with ATP synthesis with a ratio of ouabain-sensitive potassium efflux to ouabain-sensitive ATP synthesis of two to three. Lant *et al.* (383) showed an interdependence of the ouabain-sensitive components of sodium influx and potassium efflux. Thus, these studies provide evidence that the incorporation of inorganic phosphate into ATP, as catalyzed by the sodium pump, is coupled to the downhill movements of sodium and potassium through a reversal of the pump. In a study reported in 1968, Lant and Whittam (384) showed that synthesis of ATP required downhill movements of both sodium and potassium. The downhill movement of only one of the cations did not catalyze ATP formation (also see ref. 142).

The pump can be made to catalyze a one for one exchange of internal sodium for external sodium in erythrocytes (202–204, 681). This exchange reaction requires external sodium and does not occur in the presence of physiological concentrations of potassium. The reaction requires the presence but probably not substantial hydrol-

ysis of internal ATP (225, 226) and also requires that internal potassium be present or that the concentration of inorganic phosphate be greater than that of ATP. Oligomycin, which stimulates the ADP-ATP exchange reaction (254), inhibits the sodium-sodium exchange reaction (278). Since the sodium-sodium exchange is modulated by the concentration of ADP, these studies suggest that sodium-sodium exchange *per se* is composed of at least two partial reactions: a) formation of E_1-P; and b) conversion of E_1-P to a different state, presumably E_2-P. A sodium-sodium exchange has also been found in squid axon (49, 146, 147).

A ouabain-sensitive efflux of potassium is also catalyzed by erythrocyte ghosts when either external sodium or external potassium is present. The latter is thought to reflect an exchange of internal potassium for external potassium (229).

In 1968, we (394) reported that a fragmented membrane preparation of Na^+,K^+-ATPase could be phosphorylated by inorganic phosphate in the presence of magnesium and potassium but in the absence of cardiac glycosides. In subsequent studies carried out in our own laboratory, we failed to reproduce this observation. However, Post and his colleagues (504, 505, 652a) did repeat this experiment with washed membrane fragments to eliminate endogenous sodium which inhibits the reaction. In 1973, Dahms and Boyer (133) and Dahms *et al.* (134) reported studies which examined the ^{18}O exchange reactions catalyzed by Na^+,K^+-ATPase. They found that enzyme preparations derived from the *Electrophorus* electroplax catalyze a rapid exchange between water oxygen and inorganic phosphate. This exchange did not appear to require nucleotide and was inhibited by sodium, ouabain, N,N′-dicyclohexyl-carbodiimide or p-mercuribenzoate. Oligomycin, which may block the conversion of E_1-P to E_2-P, did not inhibit the potassium-activated inorganic phosphate-water exchange. These studies are consistent with a potassium-activated reversal of a terminal step in the hydrolysis of ATP as catalyzed by the Na^+,K^+-ATPase.

It is of interest that Kanazawa and Boyer (331) found that the calcium-activated ATPase of sarcoplasmic reticulum also catalyzed an inorganic phosphate-water exchange. This system, like the Na^+,K^+-ATPase, is phosphorylated by ATP, yielding an acyl phosphate (415, 428, 429, 717, 718). Bastide *et al.* (65) in 1973 reported that the region around the phosphorylated residue was similar for the calcium-ATPase and Na^+,K^+-ATPase and that the phosphate from ATP was covalently attached to an aspartyl residue (*i.e.*, β-carboxyl of the residue) in both systems. These two ATPases have other similarities. For example, both catalyze an ADP-ATP exchange reaction, both can be phosphorylated by inorganic phosphate when appropriate conditions are employed, and the overall reactions catalyzed by both systems can be reversed such that ATP is synthesized at the expense of ADP and inorganic phosphate (see above and references 416, 430, 487, 716). Further comparison of the calcium ATPase and Na^+,K^+-ATPase suggests that calcium acts like sodium, and magnesium acts like potassium. The differences in the cation specificities but similarities in the phosphorylation site suggest that these two ATPases use similar mechanisms to channel energy into the transport process and that the cationic specificity of transport is a function of separate macromolecular determinants.

Post and his colleagues (504, 505, 652a) have reported that *fragmented membrane Na^+,K^+-ATPase preparations* can also synthesize ATP under specific conditions. They incubated the enzyme preparation in the presence of magnesium and inorganic phosphate ($^{32}P_i$) at 0°C for a short period of time which may form some type of phosphoenzyme complex. The membranes were then exposed to a medium containing

ADP, *trans*-1,2-diaminocyclohexanetetraacetic acid (CDTA), low ATP, and high sodium and the reaction was stopped by acid treatment; after centrifugation, chromatographic separation of the components in the supernatant revealed radiolabeled ATP. The energetics of this synthesis are unclear. The membrane fragments may be in vesicular form but the vesicles may be permeable. If so, ionic gradients across the vesicles may not exist although this is not certain. It may be possible that the ionic media used in this experiment rendered the vesicles impermeable. This may not be important, however, because solubilized preparations also work (652a), thus suggesting that when the enzyme is in an appropriate configuration, sodium may produce a change in the molecular architecture so that "conformational energy" is used to esterify ADP. The second medium (see above) did have high sodium, and Post showed that the rate constant for conversion of the "low-energy" phosphoenzyme complex to ATP was a saturable function of the sodium concentration; saturation required *one molar* or higher sodium. An alternate possibility is that the reduction of free magnesium by CDTA favors dissociation of magnesium from the phosphoenzyme complex, leaving the latter in an unstable or "high-energy" form. Post and co-workers (504, 505, 652a) showed evidence consistent with the suggestion that the reaction was a function of the Na^+,K^+-ATPase. Heating at 100°C, exposure to ouabain or exposure to sodium and potassium before the initial mix blocked the reaction.

It is well known that N-ethylmaleimide inhibits Na^+,K^+-ATPase activity. With respect to partial reactions catalyzed by this system, this agent increases the ADP-ATP exchange but inhibits the potassium-dependent breakdown of E_2-P (172, 173). Banerjee and his colleagues (58, 59) and Hart and Titus (254) showed that sodium plus ATP protects against inhibition of Na^+,K^+-ATPase activity and potassium-dependent phosphatase activity (see below). These data suggest that the reactivity of sulfhydryl groups on the Na^+,K^+-ATPase is a function of ligand-controlled conformations of the system.

Additional evidence that the Na^+,K^+-ATPase can be placed in various conformational states was subsequently obtained by Hart and Titus (255, 661) who measured the reactivity of a Na^+,K^+-ATPase isolated from the outer medulla of rabbit kidney to N-ethylmaleimide. They found that the ability of this reagent to alkylate sulfhydryl groups on the Na^+,K^+-ATPase was dependent on the ligands in the assay medium. Sodium plus ATP (*i.e.*, consistent with the E_1 conformation; see above) protected sulfhydryl groups in a 98,000 dalton polypeptide. In the presence of magnesium, consistent with the E_2 form of the enzyme, potassium and ouabain protected against alkylation. Magnesium, ATP plus sodium, however, exposed sulfhydryl groups. This increased reactivity is consistent with the phosphorylated state, and ouabain and oligomycin further increased sulfhydryl reactivity of the phosphorylated enzyme. It is of interest that these workers could find no ligand-sensitive changes in sulfhydryl reactivity of proteins with molecular weights lower than the 98,000 polypeptide. This suggests that the 45,000 to 55,000 dalton glycoprotein present in highly purified Na^+,K^+-ATPase preparations may not undergo ligand-dependent conformational changes. Hart and Titus quantitated sulfhydryl reactivity by measuring radiolabeled N-ethylmaleimide binding. This study provides *direct* evidence that Na^+,K^+-ATPase exists in various states and supports previous suggestions that N-ethylmaleimide occludes the enzyme in specific states and that sodium, potassium, ATP, oligomycin, and ouabain react with specific sites on the system to modify its reactivity.

Hoffman (278) reported that the reactivity of the sulfhydryl groups differs on each side of the sodium pump in resealed erythrocyte ghost preparations. Ethacrynic

acid, N-ethylmaleimide and *p*-chloromercuriphenyl sulfonic acid all inhibit the pump. Ethacrynic acid reacts only at the external surface and does not inhibit glycoside interaction with the pump. N-Ethylmaleimide acts at both the external and internal surfaces and does inhibit glycoside interaction; *p*-chloromercuriphenyl sulfonic acid cannot penetrate the membrane. When the latter agent is present in the external medium it does not affect the binding of the glycoside, but it does prevent the glycoside reaction when incorporated into the internal medium of the ghost. Hoffman (278) concluded, therefore, that there must be two different classes of sulfhydryl groups associated with the sodium pump and that these are located on the opposite surfaces of the pump.

Ahmed and co-workers (1, 4) reported that deuterated water, when substituted for water, inhibited Na^+,K^+-ATPase. This inhibition was noncompetitive with ATP but was competitively antagonistic with sodium with respect to sodium activation of the enzyme. In addition, deuterated water inhibits sodium-dependent phosphorylation (from ATP) which provides additional evidence that the phosphorylated state may be an intermediate. Ahmed and his colleagues concluded that water at the sodium site modulates the conformation of the site and, as such, regulates phosphorylation of the enzyme. Deuterated water appears to be an uncompetitive antagonist of potassium with respect to activation of the Na^+,K^+-ATPase. It is interesting, however, that replacement of water by deuterated water stimulates the potassium phosphatase. In association with this stimulation, deuterated water decreases the apparent K_m for potassium. This decrease is similar to one induced by ATP plus sodium, and both effects are inhibited by oligomycin. It was suggested by these workers (1, 4) that deuterated water may shift the Na^+,K^+-ATPase conformation from one with low affinity to another with high affinity for potassium. It is well recognized that deuterated water as opposed to water can induce different macromolecular conformations because of solvent effects. The Na^+,K^+-ATPase-sodium pump, however, may well function through the dehydration-rehydration of the cations. Also, water is a reactant in the ATPase reaction *per se*, allowing direct splitting of ATP or hydrolysis of the acylphosphate. Further studies of this type may yield increased insight into the "partial reactions" of the system.

E. Cardiac Glycoside Interaction

It is well recognized that the sodium pump of intact transporting systems and the Na^+,K^+-ATPase activity of broken membrane preparations are inhibited by low concentrations of digitalis glycosides (fig. 5; refs. 6, 219, 247, 274, 275, 330, 414, 440, 488, 496, 556, 609, 610, 706). In general, only those members of this drug class with cardiotonic actions are effective in-

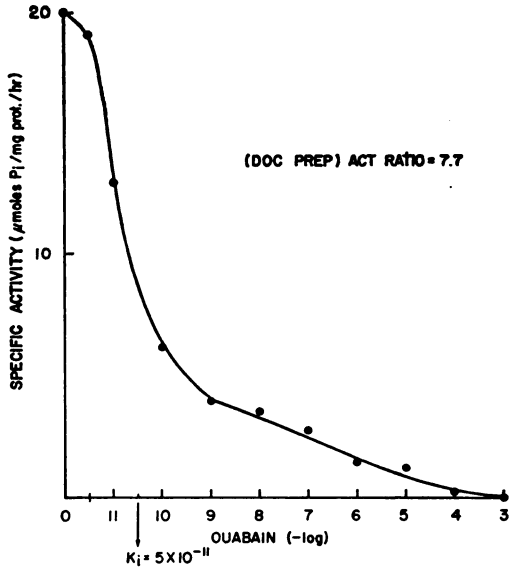

FIG. 5. Sensitivity of pig heart Na^+,K^+-ATPase to ouabain. Assays were carried out at 37°C for 10 minutes in the presence of ouabain as shown, and the concentrations of ATP, magnesium, sodium and potassium were optimal for Na^+,K^+-ATPase activity.

hibitors (489, 491, 524). This section reviews studies that have been concerned with the nature of the glycoside-Na^+,K^+-ATPase interaction and how such interaction leads to inhibition of this enzyme. Subsequent sections of this review will be concerned with the evidence that the pump may be associated with the pharmacological receptor for the drug and how interaction with the Na^+,K^+-ATPase may lead to alterations in physiological function. With respect to chemical agents that modify Na^+,K^+-ATPase activity, most experiments have dealt with the "molecular" action of cardiac glycosides. This does not imply that we have a complete insight into the mechanism, but enough information is available to allow discussion of its action within the framework of Na^+,K^+-ATPase function at the macromolecular level. An understanding of digitalis action on the Na^+,K^+-ATPase may reveal molecular characteristics of the pump.

In intact membrane preparations, cardiac glycosides inhibit the sodium pump only when they are present in the milieu bathing the preparation (*i.e.*, the extracellular space). This suggests that the receptor for the drug resides on the external surface of the membrane (103, 148, 276, 357, 695, 696).

The Na^+,K^+-ATPase must be closely associated with a receptor for cardiac glycosides since the most highly purified enzyme preparations, possessing only two major polypeptides, retain their sensitivity to the drug (285, 317, 371, 377). As stated above, it is unclear whether both of these polypeptides are required for Na^+,K^+-ATPase activity, but there are several experimental approaches that may clarify which of the polypeptides (or both?) react with cardiac glycosides. One approach is to synthesize a radiolabeled analog of the drug that binds through those forces required for the parent drug, but which, after binding, can be covalently attached to the receptor region by a chemical or physical intervention (*e.g.*, exposure to light). In 1974, Ruoho and Kyte (543a) synthesized an ethyl diazomalonyl derivative of cymarin and photoaffinity labeled the digitalis binding site on the Na^+,K^+-ATPase, which presumably was associated with the larger, "catalytic" component; the binding site was probably on the outside of the membrane (371a). Other analogs of potential use include the haloacetate derivatives of glycosides first synthesized by Hokin and his colleagues (289, 552), although these derivatives nonspecifically label many residues on the less pure enzyme preparations (282). A wide variety of other possibilities, however, can be envisioned. After covalent attachment the system could be solubilized with detergent and subjected to the common separation techniques (*e.g.*, polyacrylamide gel electrophoresis, gel chromatography, *etc.*). Localization of the label on one or the other of the polypeptides would constitute evidence for location of the receptor.

A second approach is to raise antibodies to the individual polypeptides and then to test their ability to alter catalytic activity of and ouabain binding to the intact Na^+,K^+-ATPase preparation. This type of approach should yield information about the location of the glycoside receptor and the possible interrelationships between the polypeptides with respect to Na^+,K^+-ATPase. Experiments reported from this laboratory in 1974 in collaboration with Dr. Vincent Butler, Jr. of the Columbia College of Physicians and Surgeons in New York (441) have enhanced the feasibility of this approach. Antibodies to the highly purified Na^+,K^+-ATPase preparation, derived from the outer medulla of canine kidney, were reacted with a ouabain-treated enzyme. This exposure resulted in the separation of the antibodies into two groups: a) those antibodies which when reacted with the ouabain-enzyme and eluted from the complex inhibited catalytic activity but not ouabain binding; and b) those antibodies which did not react with the ouabain-enzyme, only slightly

inhibited catalytic activity but did inhibit ouabain binding. This suggested that the antibodies raised against the intact enzyme preparation consisted of at least two and probably many species of antibodies directed against different antigenic determinants or conformations. These components may be select regions on the membrane system and may require specific orientations or higher-order structures of these regions. We will shortly review a large number of studies that suggest a direct and close relationship between catalytic activity and the ability of the receptor to bind cardiac glycosides. In other words, the reactivity of the Na^+,K^+-ATPase, in general, correlates with the reactivity of the receptor. The results with the antibodies reviewed here, however, suggest that part of the receptor is relatively independent of reactive or catalytic sites on the enzyme. An alternate interpretation is that the antibodies which inhibit ouabain interaction may bind around but not on the receptor region to physically limit access of drug to the receptor.

A third approach was initiated by Rivas *et al.* (527) who reported the extraction of a ouabain-hydrophobic protein complex from electroplax membrane by use of a chloroform-methanol solution. Application of this experiment to the preparations containing only two major polypeptides may permit some conclusion about the locus and the physical nature of the receptor for cardiac glycosides.

In 1967, Matsui and Schwartz (434) reported the first studies designed to measure quantitatively the interaction of cardiac glycosides with Na^+,K^+-ATPase. They examined ^{3}H-digoxin binding to fragmented membrane preparations, isolated from beef heart, that contained Na^+,K^+-ATPase activity. This and subsequent studies (18, 435, 580) strongly suggest that cardioactive glycosides bind only to receptor sites on membrane fragments associated with the Na^+,K^+-ATPase. The amount of drug bound to these preparations correlates with the Na^+,K^+-ATPase activity (*i.e.*, the higher the activity, the greater the binding). In 1974 studies which employed highly purified Na^+,K^+-ATPase preparations suggested that there is one nucleotide binding site and perhaps two phosphorylation sites (see above) for each glycoside receptor in the preparation (317). In studies reported in 1967 and 1968 (18, 434, 435, 580), it was found that inactive or less active cardiac glycosides (*i.e.*, with respect to their effects on the heart) and other steroids could not compete with digoxin, ouabain, or other cardiotonic glycosides for the receptor. On the other hand, cardiotonic glycosides could effectively compete (*e.g.*, ouabain *versus* digoxin).

Since the cardiac glycoside receptor appears to lie on the external surface of the cell membrane and the active site for ATP resides on the internal surface of this membrane, it is reasonable to conclude that inhibition by the drug is an allosteric event, in that alterations in conformation of the pump must be invoked to connect glycoside interaction on the external surface to inhibition of ATP hydrolysis at the internal surface. This consideration, in general, applies to the mechanism of Na^+,K^+-ATPase and the sodium pump. Sodium and potassium are moved through the membrane, and it is difficult to envision how such movements could take place without an alteration or, more likely, a series of alterations in the structure of the system catalyzing these transmembrane movements. Attempts have been made to detect such changes through the use of various spectroscopic techniques (395, 438, 453, 454, 723).

Originally, it ws found that digoxin did not bind when the assay medium contained only buffer (435). As outlined in a previous section, considerable evidence was accumulated in the early and middle 1960's that the Na^+,K^+-ATPase could be placed in certain reactive states. These data led to the hypothesis that the glycoside receptor

existed in multiple conformations but that only one or a few conformations were conducive to drug interaction. Furthermore, it was felt that the conformation appropriate for binding may coincide with a reactive state of the Na^+,K^+-ATPase. The first condition observed to promote digoxin interaction appeared to justify this hypothesis. A reaction medium containing ATP plus magnesium plus sodium (*i.e.*, a condition which leads to phosphorylation of the Na^+,K^+-ATPase; see above) promoted ^{3}H-digoxin binding through a reaction which approached completion in 3 minutes at 37°C (434,435). This originally suggested that the drug bound to the phosphorylated enzyme. Consistent with this conclusion, inclusion of potassium, which reduces the level of the phosphoenzyme complex, markedly inhibited digoxin or ouabain binding to the Na^+,K^+-ATPase preparation over the 3- to 5-minute assay periods employed in these experiments. Acetylphosphate, a substrate for the potassium-dependent phosphatase activity associated with Na^+,K^+-ATPase (see part VI of this review), was also found to support binding. Again, however, addition of potassium inhibited glycoside interaction. This led to the following scheme as a description of the turnover cycle of Na^+,K^+-ATPase and of the "site" in the sequence at which cardiac glycosides react.

$$\begin{array}{ccccc} & & \text{Acetylphosphate} & & \\ & & \downarrow & & \\ \text{E} + \text{ATP} & \xleftarrow{Mg^{++},\,Na^+} & \text{E} \sim \text{P} & \xrightarrow{K^+} & \text{E} + \text{P}_i \\ & & \downarrow \text{digitalis} & & \\ & & (\text{E} \sim \text{P})\ \text{digitalis} & & \end{array}$$

Thus, it was concluded that the digitalis-like glycosides inhibited the Na^+,K^+-ATPase and the sodium pump by binding to a phosphorylated state of the system.

Subsequent studies, however, reported other conditions favorable for binding but which did not appear to be consistent with the existence of a "high-energy" phosphoenzyme, *i.e.*, acylphosphate complex (table 2; refs. 18, 435, 580). These conditions included: a) ATP plus magnesium without sodium; b) magnesium plus inorganic phosphate, arsenate or acetate; and c) magnesium or manganese alone. Magnesium plus inorganic phosphate does, in fact, lead to phosphorylation of the Na^+,K^+-ATPase in the presence of a cardiotonic glycoside such as ouabain (this will be discussed below), but the other conditions listed are not conducive to phosphorylation unless, of course, the phosphate is derived from an endogenous source (*e.g.*, an endogenous organic

TABLE 2

Effect of sodium on specific ^{3}H-digoxin binding to cardiac Na^+,K^+-ATPase supported by various cations, nucleoside di- and triphosphates, and anions

Addition	^{3}H-Digoxin bound to protein	
	Without Na^+	100 mM with Na^+
	pmol/mg	
None	0	0
ATP	0	10.3
Mg^{++} + ATP[a]	14.5	37.3
ADP	0.5	0.7
Mg^{++} + ADP	4.8	11.1
Mg^{++} + CTP	14.3	26.1
(5 mM) Mg^{++}	11.5	0
(5 mM) Mn^{++}	31.7	16.3
(0.05 mM) Ca^{++}	2.0	0
(0.5 mM) Ca^{++}	2.7	0
(5 mM) Ca^{++}	4.0	0.5
(20 mM) Ca^{++}	9.0	2.9
(5 mM) Sr^{++}	5.3	16.1
(4 mM) Pi	0.7	0
Pi + Mg^{++}	42.8	1.4
Tris-arsenate (As)	0	0
As + Mg^{++}	38.8	1 9
Tris-SO_4^{2-}(SO_4)	5.4	0
SO_4 + Mg^{++}	5.6	0
Tris-acetate + Mg^{++}	13.9	0

The cations, except when otherwise indicated, were used in 5 mM concentration; Tris salts of the nucleosides and nucleotides, in 2 mM concentration; the anions in 2 to 4 mM concentration. The specific activity of the cardiac enzyme varied between 26 and 40 μmol P_i/mg protein/hour. [From Schwartz *et al.* (580).]

[a] The abbreviations used are: ATP, adenosine triphosphate; ADP, adenosine diphosphate; CTP, cytidine triphosphate.

phosphate?). Furthermore, potassium inhibits binding supported by any of these conditions, while sodium stimulates binding in the presence of magnesium plus ATP but inhibits binding promoted by the other conditions. It is difficult to envision anything in common about these binding conditions except for their observed ability to promote glycoside binding. Most of the promoters or inhibitors, however, are either reactants, products, or activators (*i.e.*, manganese serves as an analog of magnesium) of the forward reaction of Na^+,K^+-ATPase. As such, they must have sites of some specificity on the system so that they presumably act to modify the conformation of the Na^+,K^+-ATPase and the glycoside receptor.

Studies carried out in this laboratory in 1974 suggest that all of these conditions promote digitalis binding to the same species of receptor. For example, binding supported by a) ATP plus magnesium plus sodium, b) ATP plus magnesium without sodium, c) magnesium plus inorganic phosphate, or d) magnesium alone yield (in the presence of a high glycoside concentration and after a sufficient length of exposure of drug to the enzyme) the same level of glycoside binding to a highly purified Na^+,K^+-ATPase preparation (294).*

Second, when dissociation of a radiolabeled drug from the receptor was examined by "chasing" bound-labeled drug with free unlabeled drug in the presence of the conditions used to promote binding, the rate of dissociation was essentially the same (*i.e.*, $T_{1/2}$ was about 2.5 hours at 37°C, pH 7.4) regardless of the conditions used to promote binding (377, 565). This suggested that the nature of the drug-receptor complex, once formed, is independent of the conditions employed to promote the association of drug and receptor. (But there is a complication to this conclusion; see below.)

Third, the various conditions which promote digitalis interaction with the Na^+,K^+-ATPase are different, in that they allow glycoside binding to occur at different rates. This would suggest that binding promoted by the various ligand conditions proceeds through different pathways (perhaps through different conformations of the receptor). Plots in the Arrhenius form of the rate constants for ouabain binding at various temperatures, however, yield rectilinear responses. From the slopes, it was found that the energies of activation for the binding reactions supported by ATP plus magnesium plus sodium (21.2 ± 0.8 kcal per mole), by ATP plus magnesium without sodium (23.4 ± 0.8 kcal per mole), and by magnesium plus inorganic phosphate (22.8 ± 1.5 kcal per mole) were essentially the same (686). This again suggests that the receptor conformations induced by these three ligand conditions are either identical or very similar. It is possible, therefore, that there may be only one ligand-induced state of the Na^+,K^+-ATPase which coincides with the orientation of the receptor required for binding. It is further possible that this state is one in a series that constitutes the turnover cycle of the system (25). If so, the different rates of binding promoted by the various ligand conditions may reflect dif-

* It has been suggested that a Scatchard type of analysis is required to obtain an accurate estimate of the maximum number of ouabain binding sites. This is incorrect, as can be seen by considering the equilibrium $[D][R]/[DR] = K_d$. The dissociation constant, K_d, is the reciprocal of the ratio of the rate constants for the formation of the drug-receptor complex (k_f) and for the dissociation of the complex (k_b); k_b is about 7.7×10^{-5} sec^{-1} at 37° C (377); k_f ranges from 3×10^3 to 4.4×10^4 M^{-1} sec^{-1} (61, 396). Thus, in the presence of excess ouabain [*i.e.*, when free ouabain always remains constant even when all receptors are bound (for the example here, 10 μM)], the ratio [R]/[DR] would range from 0.0026 to 0.00018, which is too small to be detected experimentally. Thus, the estimate of maximum binding in the presence of excess ouabain should provide a more accurate index of values for maximum glycoside binding (*i.e.*, number of receptor sites) to a particular Na^+,K^+-ATPase preparation than that obtainable from extrapolation from Scatchard plots.

ferent steady-state levels of the particular reactive enzyme state compatible with glycoside binding. As discussed below, this conformer appears to be one in which the potassium activation sites, or at least one of these sites, exist at the external surface of the pump (*i.e.*, reside in a position to react with potassium).

The studies reviewed above support the conclusion that there is only one binding conformation of the glycoside receptor and that there is only one species of the glycoside-receptor complex, regardless of the conditions used to promote its formation. This conclusion is by no means shared by all investigators. A number of other studies have provided evidence for the existence of at least two glycoside-receptor complexes and for the existence of two different species of the digitalis receptor or two different binding conformations of one receptor. In fact, results from this laboratory reflect inconsistencies in the aforementioned "single conformer" hypothesis. If a fragmented membrane preparation is reacted with ouabain under specific ligand conditions and then is resuspended in a drug-free, buffered medium of low ionic strength, the rate at which the bound drug dissociates from the receptor is a function of the ligands used to promote binding (10, 23, 24). The complex formed in the presence of ATP, magnesium and sodium dissociates quite rapidly, whereas the complex formed in the presence of ATP plus magnesium without sodium, magnesium plus inorganic phosphate, or magnesium alone dissociates at slow and equivalent rates. Addition of potassium or higher concentrations of other monovalent cation salts reduces the dissociation rate of the complex formed in the presence of ATP, magnesium, and sodium down to those rates observed for the other complexes (10, 24). If the dissociation of the complex formed in the presence of magnesium and inorganic phosphate is carried out in the presence of ATP and sodium, the normally slow rate of dissociation is increased (578, 662). Thus, one type of complex can be converted into the other type. On the other hand and as stated above, *if the dissociation is carried out in the presence of the binding ligands, the rates are slow but equivalent for all of the binding conditions discussed here* (377). The difference in dissociation rates in a low ionic strength medium is not immediately explicable, but these data clearly suggest that ligands do react with the ouabain-receptor complex, probably in a freely reversible manner with free ligands in solution. The conversion from slow to rapid dissociation by ATP plus sodium may reflect a change in the affinity of the complex for magnesium, inorganic phosphate, and/or the stability of a phosphoenzyme complex formed from inorganic phosphate in the presence of magnesium and ouabain.

Hart and Titus (254, 255) provided further evidence that the glycoside-receptor complex formed in the presence of magnesium plus inorganic phosphate differs from the complex formed in the presence of ATP, magnesium, and sodium. In the former case, reaction with ouabain decreased the reactivity of sulfhydryl groups in a 98,000 dalton peptide to N-ethylmaleimide, while in the latter case ouabain binding increased the reactivity of these groups.

Taniguchi and Iida (650) reported that at pH 6.1, Scatchard plots of ouabain binding to an ox brain Na^+,K^+-ATPase preparation revealed evidence for two species of binding sites—one of high affinity for the drug with a dissociation constant of about 0.18 μM and a second of low affinity with a dissociation constant of about 20 μM. The amount of ouabain bound to these two species of sites appeared to be similar. While the data can be interpreted in a different manner (see above and refs. 252, 294), there is at least one report that is consistent with the possibility that there may be more than one binding site for digitals (376).

The "maximum amount" of ouabain bound to crude Na^+,K^+-ATPase preparations is higher when the reaction is promoted by magnesium and inorganic phosphate than when promoted by magnesium, sodium, and ATP (18, 580, 669). Sodium and potassium inhibit the glycoside interaction promoted by magnesium plus inorganic phosphate or magnesium or manganese alone, but in the presence of ATP plus magnesium, sodium stimulates while potassium inhibits binding (18, 580, 618, 669, 679, 698). Van Winkle *et al.* (679) reported differential effects of sodium and potassium on binding supported by magnesium plus inorganic phosphate; the effects depended on how near the reaction was to completion. These results may be interpreted in several ways: a) different binding conformations of one receptor species exist or more than one receptor is present; or b) sodium modulates the steady-state level of the binding conformation in different ways, depending on whether or not ATP is present. In 1973, Whittam and Chipperfield (698) reported differences in the pH profile for binding supported by combinations of ATP, magnesium, and sodium *versus* that supported by magnesium. This is consistent with the former explanation but could also be accounted for by the latter.

As stated above, ouabain reacts relatively rapidly with the Na^+,K^+-ATPase in the presence of a combination of ATP, magnesium, and sodium. This strongly suggests that the form of the enzyme designated as E_2-P coincides with a receptor conformation required for binding. It is interesting, therefore, that the rate of binding appears to be elevated by sodium interaction with two sets of sites. One site has an affinity for sodium in the range of that required for the formation of (E_2-P) from ATP [i.e., dissociation constant of about 1 mM for sodium (503)]. The second site has an affinity that is about 10 times lower than for the first site (*i.e.*, dissociation constant of about 14 mM). High sodium may not necessarily alter (E_2-P) but can act as an inhibitor of Na^+,K^+-ATPase activity, as a competitive antagonist to potassium, and also may inhibit the sodium-dependent ATPase activity ob-observed in the absence of potassium.*

These results suggest that the high affinity site for sodium is concerned with the formation of (E_2-P) and that the low affinity site is concerned with the stabilization of this complex. In terms of transmembrane movements, sodium interaction at the former site would reflect sodium interaction at the internal surface, whereas the latter site may be related to an interaction with the external surface, normally reflecting dissociation of sodium from the pump into the extracellular milieu. This suggests that potassium reacts with the (E_2-P)-sodium form of the enzyme and/or with a subsequent state of the enzyme in the turnover cycle to reduce the steady-state concentration of (E_2-P)-sodium. (The studies concerned with cation modulation of ouabain binding rates will be reviewed below.)

The complex (E_2-P) is thought to be an acylphosphate (see previous section); acylphosphates are, in general, unstable or "high energy." Energetically, this seems plausible, since this state derived from ATP which is also a "high-energy" compound. On the other hand, it seems unlikely that such a complex could be formed from inorganic phosphate, a "low-energy" compound, unless cation movements down electrochemical gradients were

* High sodium inhibited the sodium-ATPase activity of the highly purified preparation isolated from the outer medulla of kidney, by the procedure of Lane *et al.* (377, and unpublished observations). The results of other studies, however, are conflicting. Post *et al.* (495) reported that high sodium stimulated sodium-ATPase activity and the rate of breakdown of the phosphoenzyme complex (also see ref. 465). Kanazawa *et al.* (332) concluded that the rate of phosphoenzyme breakdown was insensitive to sodium, and Rodnight (543) reported that high sodium inhibited phosphorylation and ATP hydrolysis.

used as an energy source for the reaction. Such gradients, of course, do not exist in fragmented membrane preparations, and it was, therefore, surprising to find that ouabain in the presence of magnesium promoted the incorporation of inorganic phosphate into the enzyme (18, 299, 394, 395, 585). Originally, it was felt that the phosphoenzyme formed from inorganic phosphate was of a "low-energy" type, perhaps reflecting a partial reversal of the Na^+,K^+-ATPase reaction to a state towards the end of the turnover cycle [*i.e.*, after E_2-P (394)]. Subsequently, however, it was shown that the phosphoenzyme complexes formed from ATP in the presence of magnesium plus sodium, in one case, and from inorganic phosphate in the presence of magnesium plus ouabain, in the second case, were chromatographically identical after digestion of the radiolabeled phosphoenzyme (125, 602). There are several mechanisms by which an acylphosphate could be derived from inorganic phosphate [*e.g.*, see Siegel *et al.* (602)]. The most likely explanation is that ouabain binds to the enzyme in such a manner as to produce a conformation favorable for phosphorylation. If ouabain encourages rapid formation of an acylphosphate, the drug may bind prior to covalent attachment of the phosphate to the carboxyl group. This suggests that the acceleration in the rate of ouabain binding by inorganic phosphate (in the presence of magnesium) must be the result of some other reaction. A possible explanation may be that inorganic phosphate reacts with the enzyme through noncovalent forces and that this (E_2·P) complex is also favorable for ouabain interaction. The result of the latter would be to convert (E_2·P) to (E_2-P), presumably through a conformational alteration. This conversion may reflect a competition between (E_2·P) and the unsaturated lactone ring of the drug for a molecule of water in the part of the macromolecule in which water is limiting [fig. 6; Dr. Earl T. Wallick, personal communication (399)].

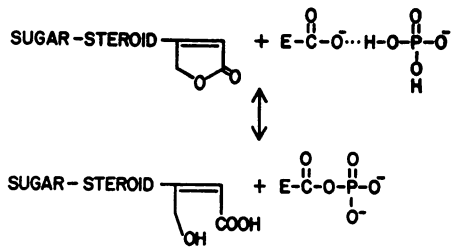

FIG. 6. Possible competition between acylphosphate and unsaturated lactone ring of cardiac glycoside for a molecule of water in an essentially dehydrated (intramembraneous) environment (suggested by Dr. Earl T. Wallick, Department of Cell Biophysics, Baylor College of Medicine).

It is emphasized that we have no data to support this concept. Repke and his colleagues (419) have carried out extensive studies on the structural nature of digitalis glycoride binding to Na^+,K^+-ATPase and have emphasized the importance of hydrogen bonding in the drug-enzyme complex formation.

The Na^+,K^+-ATPase in fragmented membrane preparations will catalyze ATP hydrolysis when sodium, potassium, and magnesium are present in the assay medium. If a low concentration of a cardiotonic glycoside is used in the assay medium, inhibition of the catalytic rate occurs and increases with time (18, 574). At some point, total inhibition is approached if the free glycoside concentration is not too low. A parallel result is observed when ouabain binding to a Na^+,K^+-ATPase preparation is examined over a prolonged period of time in the presence of a combination of ATP, magnesium, sodium, and potassium. The amount of ouabain eventually bound equals the amount bound in the presence of ATP plus magnesium plus sodium in the absence of potassium (27). A similar result was obtained when binding was carried out in the presence of magnesium plus inorganic phosphate (395). The level of binding ultimately achieved with potassium was the same as that without potassium. These results suggested that the

effect of potassium on glycoside interaction was to decrease the rate of complex formation between the drug and receptor. Later studies suggested that potassium does not induce dissociation of ouabain from its receptor (10, 24). A similar type of result has been reached by employing much lower concentrations of glycoside and determining the amount of drug bound when the association reaction reached equilibrium (249). In this case, the data were analyzed by Scatchard plots so that dissociation constants could be derived. Potassium was found to increase the dissociation constant for the drug-receptor interaction.

The chemical reaction between a drug and its receptor, in general, is described as follows:

$$D + R \underset{k_b}{\overset{k_f}{\rightleftharpoons}} DR \cdot [k_b \text{ is sometimes written: } k_{-1}]$$

(f = forward; b = backward)

As stated above, k_b for the dissociation of the cardiac glycoside-receptor complex is quite slow at 37°C when dissociation is measured in the presence of a condition favorable for binding. When free ouabain remains in excess such that its concentration is essentially unaltered by the amount of drug bound to its receptor, the rate of binding follows a kinetic pattern for a pseudo first-order reaction (fig. 7; refs. 61, 396). The reaction is a true second-order type, however, since a plot of pseudo first-order rate constants *versus* ouabain is rectilinear. Also, such a plot does not plateau at higher concentrations of the drug, which suggests that a rapidly formed and easily dissociable complex does not precede a pseudo irreversible complex (396).

Rate constants for chemical reactions reflect the product of two probability factors: a) that the molecules have sufficient energy for reaction; and b) that the molecules have the appropriate geometry for reaction. As discussed above, the energy of activation for the association of ouabain with its receptor is identical for three different ligand conditions. This suggests

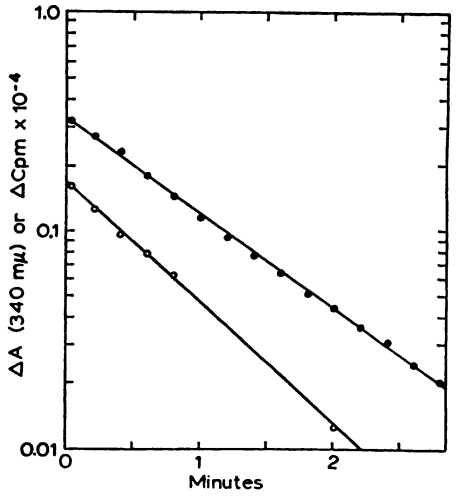

FIG. 7. Kinetics of ouabain binding and inhibition at 37°C, pH 7.0, and ionic strength 0.3 M. Kinetics of binding for 0.5 μM [^{3}H]ouabain-3 mM Tris-ATP-3 mM $MgCl_2$-0.1 M NaCl (0); kinetics of inhibition, conditions are 0.1 M NaCl-0.01 M KCl-3 mM Tris-ATP-3 mM $MgCl_2$-20 units/ml of pyruvate kinase-2 units/ml of lactic dehydrogenase-0.15 mM NADH-1.5 mM phosphoenolpyruvate, pH 7.0, ionic strength 0.3 M, at 37°C and 3.33 μM ouabain (●). [From Barnett (61).]

that ligands which modulate the rate do not do so by altering the number of molecules that are energetically in a state required for the reaction. Thus, if there is only one species of receptor present, the ligands must affect the rate by modulating the geometry of the reactants. As far as is known, the modifying ligands do not alter the geometry of ouabain. Their influence on the rate, therefore, is probably due to an alteration in the geometry or conformation of the receptor which can react with the drug. It seems likely, in fact, that modulation of glycoside binding does predominantly reflect different steady-state concentrations of the receptor conformation required for binding. The possibility that slightly different ligand-induced conformations react with the drug at substantially different rates seems remote.

Potassium and cardiac glycosides are antagonistic to each other with respect to their actions on the sodium pump of intact transporting systems and to the Na^+,K^+-

ATPase activity of broken membrane preparations (151, 219, 275, 488). A similar potassium-glycoside antagonism is well recognized clinically. This antagonism, at least with respect to the pump, was originally thought to reflect a competition between the glycoside and potassium for the potassium activation sites (2, 3, 36, 151, 494). It should be recalled that the potassium activation sites are located on the external surface of the membrane system and that cardiac glycosides appear to interact with the pump only when the drug is in the external medium (see above). Also, the responses shown in figure 8 appear to reflect competition between ouabain and potassium for the potassium activation sites. This interpretation, however, did not completely explain other observations. In 1965, Schatzmann (559) showed that a third factor, sodium, was also involved. In resealed erythrocyte ghost preparations, he observed that potassium could not completely overcome the effect of external ouabain and that ouabain appeared to enhance the inhibitory effect of external sodium with respect to potassium interaction with the potassium activation sites. A year later, Matsui and Schwartz (433) reported a kinetic analysis of the effects of ouabain, sodium, and potassium on a beef heart Na^+,K^+-ATPase preparation and concluded that the sensitivity of catalytic activity to ouabain was dependent upon the ratio of sodium to potassium. A similar conclusion was reached for a beef brain preparation (396). Also, Hoffman (276) observed that cesium, which can activate the pump through a potassium activation site, could not alter the inhibition of the pump by digitoxin. This led him to conclude that the glycoside interacts with a site that is different from the potassium-activation site on the external surface of the membrane.

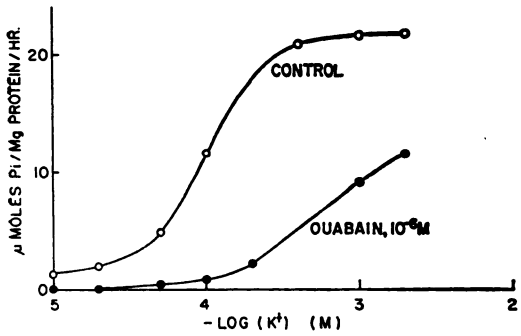

FIG. 8. Heart Na^+,K^+-ATPase: Effect of K^+ on ouabain activity. Concentrations of $MgCl_2$ and NaCl were 5 mM and 100 mM, respectively. The basic Mg^{2+}-dependent activity was subtracted from the activity in the presence of Na^+ and K^+. [From Schwartz and Matsui (579).]

In more recent years, the application of ^3H-glycoside binding techniques has allowed a detailed probe of the antagonism between potassium and cardiac glycosides. In the presence of magnesium plus ATP, sodium stimulates and potassium retards the rate of ouabain binding, but potassium does not favor dissociation of drug from the receptor. It is also recognized that the ouabain-receptor complex is quite stable (18, 61, 377, 435, 565, 580), at least for those enzyme preparations derived from species sensitive to cardiac glycosides. These results encouraged a reinterpretation of the data presented in figure 8. In the presence of low concentrations of ouabain, potassium appears to have two effects. One is to activate catalysis, and the second is to retard the rate of ouabain interaction. Thus, activities observed at higher concentrations of potassium approach those observed in the absence of ouabain, because the drug cannot interact to a significant extent over the period of the assay for ATP hydrolysis. At high concentrations of ouabain, however, some drug will bind, so that the V_{max} at saturating concentrations of potassium decreases, and the apparent dissociation constant for potassium (or the concentration of potassium required for a velocity equal to one-half of V_{max}) appears to increase, which is consistent with a classification of the antagonism as of the "mixed" type. The classifications of competitive antagonism

for a low concentration of ouabain and mixed antagonism for a high concentration of ouabain are both incorrect since ouabain binds in a pseudo-irreversible manner. As discussed below, it does seem apparent that potassium retards glycoside binding by interacting with a potassium activation site. It is not possible at this time to draw a conclusion about either the location of the glycoside receptor site, *i.e.*, whether the receptor encompasses in part a potassium activation site or is completely separated from it in a physical sense or whether bound glycoside makes a potassium site inaccessible to monovalent cations.

In 1970, Barnett (61) presented a mathematical relationship to describe the effects of sodium and potassium on the rate of ouabain interaction.

I

$$\frac{k_i}{k_p} = 6.6 + 1.5\frac{[Na^+]}{[K^+]} + 4.58 \times 10^{-3}\frac{[Na^+]}{[K^+]^2}$$

k_i is the second-order rate constant for enzyme inhibition (or ouabain binding), and k_p is the turnover number of the enzyme. Barnett explained this relationship in terms of the following sequence for the catalytic turnover cycle of the enzyme.

II

$$E_1 \cdot MgATP \cdot Na_m \underset{k_{-c}}{\overset{k_c}{\rightleftharpoons}} E_2\text{-}P \cdot Na_r \longrightarrow \cdots \longrightarrow$$

$$E_2\text{-}P \cdot Na_xK_y \cdot \longrightarrow \cdots \longrightarrow E_2\text{-}P \cdot K_m$$

$$\overset{k_c'}{\longrightarrow} E_1 \cdot MgATP \cdot Na_m$$

$E_2\text{-}P \cdot Na_xK_y$ designates states of (E_2-P) for which sodium and potassium compete for binding. k_{-c} was assumed to be negligible, and Barnett further assumed that ouabain binds only to or predominantly with (E_2-P). Further analysis suggested that the principle states of $E_2\text{-}P \cdot Na_xK_y$ are those in which $x = 1, y = 0$ and $x = 1, y = 1$. He then assumed that the second-order rate constant for ouabain binding was independent of the form $E_2\text{-}P \cdot Na_xK_y$. Sodium, in this case, would favor binding by promoting the synthesis of E_2-P, and potassium would retard binding by promoting hydrolysis of E_2-P, presumably through the form $E_2\text{-}P \cdot Na_xK_y$, where $x = 0$ and $y = 2$. For arguments against some of these conclusions and assumptions, see reference 396.

In 1971, Siegel and Josephson (601) suggested that 17 to 22 mM sodium yielded half-maximum rates of glycoside binding in the presence of ATP and magnesium. Low amounts of potassium increased the amount of sodium required. These authors pointed out that this sodium effect must be mediated at a site with lower affinity than that site which must bind sodium for phosphorylation of the enzyme with ATP. Their study did not employ an exact quantitative index of binding rates, but the results from this study roughly agree with those from a study independently carried out in our laboratory (396). [Siegel and Josephson (601) allowed their reactions to proceed to something less than 20% saturation of the sites by ouabain. This is beyond the amount of reaction which could be taken as an initial rate of the reaction, since the free receptors disappear exponentially with time in the presence of excess free ouabain.] In the presence of excess free ouabain (relative to total receptor sites), the interaction of ouabain with its receptor follows pseudo first-order kinetics:

$$R = R_o e^{-k_1 t} \qquad \text{III}$$

where R is the free receptor concentration, R_o is the total receptor concentration, t is the time of exposure to the drug, and k_1 is the apparent pseudo first-order rate constant for the reaction. We found that sodium and potassium modulated the rate of ouabain binding (*i.e.*, in the presence of magnesium and ATP) by competing for a common site or conformer of the system (396).

$$k_1' = \frac{k_f[\text{ouabain (M)}]}{1 + \frac{13.7}{[Na^+]}\left(1 + \frac{[K^+]}{0.213}\right)} \qquad \text{IV}$$

where k_f is the second-order rate constant

at infinite sodium (*i.e.*, 4–5 × 10^4 M^{-1} sec^{-1} at 37°C), and 13.7 and 0.213 are the dissociation constants (mM) of the modulating site for sodium and potassium, respectively. Since the number of glycoside binding sites correlates with the specific catalytic activity of Na^+,K^+-ATPase (18, 580), the amount of ATP hydrolyzed over a given assay period is proportional to the product of the total number of free receptors present (*i.e.*, macromolecules available for catalytic activity) and the assay time (18, 574). Thus, in the absence of ouabain, the amount of ATP hydrolyzed is proportional to the area defined by the product of R_o and the assay time, whereas in the presence of ouabain, the amount is proportional to the area under the curve describing the disappearance of free receptor, R. The latter is given by the following expression:

$$A_1 = \frac{R_o}{k_1'}\left(1 - e^{-k_1 t}\right) \qquad V$$

Note that in the absence of ouabain, $k_1' = 0$, and the integration yields $R_o t$, which is set to A_2 for control. Thus $(A_1/A_2) \times 100$ gives the predicted percentage of control catalytic activity at various sodium, potassium, and ouabain concentrations, where k_1' is defined by equation IV. When compared to observed percentages of control catalytic activity, agreement with the predicted values was observed for the following ranges: 10 to 200 mM sodium; 0.5 to 10 mM potassium; a ratio of 4 to 400 sodium to potassium; and 0.01 to 10 μM ouabain for a 10-minute assay at 37°C. Longer assays with the lower concentration of ouabain would be expected to deviate from the predicted values, since a significant portion of the receptors would not be bound to drug at equilibrium. The reason this discrepancy is not seen in 10-minute assays is that the fraction of sites bound to ouabain over this time is far removed from that which would be reached at equilibrium. Also the discrepancy that may exist is small and lies within the experimental error of the techniques used to measure Na^+,K^+-ATPase activity. The agreement between observed and predicted values reinforces the conclusion that the relationship between ouabain bound to its receptor and the inhibition of Na^+,K^+-ATPase activity by the drug is absolute.

The relative dissociation constants of the modulation sites (equation IV) for sodium and potassium suggested that this site may be a potassium activation site. Further analysis of potassium activation of Na^+,K^+-ATPase isolated from beef brain showed that the modulation site could function as a potassium activation site if there existed a second potassium activation site with different affinities for the cations (398).

$$v = \frac{V_m}{\underbrace{\left[1 + \frac{0.213}{[K^+]}\left(1 + \frac{[Na^+]}{13.7}\right)\right]}_{\text{Site I}} \underbrace{\left[1 + \frac{0.091}{[K^+]}\left(1 + \frac{[Na^+]}{74.1}\right)\right]}_{\text{Site II}}} \qquad VI$$

The fact that two sites appear to be required agrees with the probable stoichiometry of the pump, which is three sodium ions pumped out per two potassium ions pumped in per molecule of ATP hydrolyzed (see above).

As discussed in a previous section, there is considerable evidence that the turnover cycle of the pump consists of at least two intermediate states, one with high affinity for sodium and the second with high affinity for potassium. Also, we have reviewed evidence that the receptor conformation required for glycoside interaction exists transiently within the transport cycle of the pump. We emphasize that a series of partial reactions may lead to the existence of a state which "reveals" the potassium activation sites. Potassium interaction with these sites induces a new enzyme state that

does not have the site (*i.e.*, or one in which the affinities of the sites for potassium have drastically decreased). Thus, the immediate interpretation of sodium and potassium modulation of ouabain binding is that the state or conformation possessing the potassium activation sites coincides with the receptor conformation required for glycoside interaction. Potassium at the potassium site causes the enzyme state and receptor conformation to disappear (*i.e.*, in steady-state terms, potassium reduces the percent of the receptor in the appropriate structure for drug interaction). Sodium at the site prevents further reaction, increasing the steady-state concentration of the appropriate receptor conformation.

If there are two potassium activation sites, however, there must be some explanation for the apparent lack of modulation at the second site. An examination of equation VI suggests that the ratio of the affinities for potassium *versus* sodium is about 814 for site II compared to a ratio of 64 for site I (*i.e.*, the "modulation site"). Sodium at either site I or II should stabilize the binding conformation, but potassium must be present at both sites to induce a nonbinding conformer of the receptor. Thus, the binding conformer could be stabilized by sodium at site I and potassium at site II, which is consistent with one component of the model proposed by Barnett (61), as outlined above (*i.e.*, $E_2\text{-}P \cdot Na_xK_y$, where $x = 1$ and $y = 1$).

The rate of ouabain binding in the presence of ATP plus magnesium plus low or zero sodium is much slower than in the presence of high sodium. Also, the enzyme catalyzes a sodium-dependent ATPase activity in the absence of potassium and presence of low sodium which is inhibited by high sodium (but see above) or cardiac glycosides. This activity is much lower than the Na^+,K^+-ATPase activity but suggests that in the presence of low sodium and zero potassium, the pump does turn over at a slow rate. Again, high sodium would further stimulate the rate of ouabain binding by stabilizing the binding conformation of the receptor.

There is a question as to whether the drug can bind to the form of the enzyme, $(E_2\text{-}P) \cdot$potassium. There may be a very low but finite steady-state level of the potassium-phosphoenzyme complex which corresponds to a low but significant existence of the binding conformation. A study in 1974 by Wallick *et al.* (685) from this laboratory suggested that glycosides may act with a potassium form of the system. They examined the antagonism between ouabagenin and potassium with respect to inhibition of Na^+,K^+-ATPase activity. Ouabagenin, as opposed to ouabain, reacts with the system in a freely reversible manner (685, 721, 722, 724, 725). At low concentrations of potassium (up to 10 mM) and in the presence of a constant concentration of sodium, there appeared to be a competitive type of antagonism between ouabagenin and potassium, with a dissociation constant, K_i, for the drug of less than 0.1 μM. At higher concentrations of potassium, however, the antagonism required a K_i of about 0.9 μm for ouabagenin, and inhibition became relatively independent of potassium. This is consistent with a competition between ouabagenin and small amounts of potassium for a common state or conformation of the system, *i.e.*, potassium reduces the steady-state concentration of the form reactive to ouabagenin. At higher concentrations (saturating) of potassium, the steady-state level of this conformer would be reduced to a minimal value (*i.e.*, a level which would not change with more potassium) which would be associated with a decrease in the sensitivity of the system to the glycoside. [The potassium-induced reduction in the steady-state level, in effect, reduces the probability for ouabagenin's interaction such that the dissociation constant is correspondingly increased ($K_d = k_b/k_f$).] Hansen and Skou (249) also suggested that ouabain may bind to a

potassium form of the enzyme when binding is supported by magnesium plus inorganic phosphate. They observed that increasing potassium could not reduce ouabain binding to zero but rather that the binding reached a constant (reduced, but significant level for constant assay times. This is also consistent with data from our laboratory (679).

One of the limitations of the modulation model (equation IV) is encountered when a low concentration of sodium is present, suggesting that the model is valid only when sodium saturates the sodium activation sites to maintain the steady-state level of the binding conformer at its highest possible level (396). Inagaki *et al.* (294) found evidence that a site different from the modulation site described by equation IV might be involved in regulating the rate of ouabain interaction. This proposed second site had about a 10-fold greater affinity for sodium than the other site, which was consistent with the conclusion that the former is a sodium activation site. Other experiments, however, made this conclusion questionable. The binding rates supported by low concentrations of sodium were quite sensitive to potassium. Since the sodium activation sites were thought to have a low affinity for potassium, the relationship of the second site to sodium activation was perplexing. A second complication was an antagonism between Mg·ATP and potassium with respect to the rate of ouabain binding. As described above, Mg·ATP promotes a slow rate of ouabain binding in the absence of added sodium and the rate is retarded by potassium. In the presence of 2.5 mM magnesium plus ATP, the concentration of potassium required to cause a 50% reduction in the rate of binding (I50 for potassium) was about 2 mM. In the presence of 0.25 mM magnesium plus ATP, however, the I50 was reduced to about 0.5 mM. It was of interest to find that potassium inhibited a ouabain-sensitive, magnesium-dependent ATPase catalyzed by the highly purified enzyme preparation when the assays were carried out in the absence of added sodium. The sensitivity of this reaction to potassium was the same as the sensitivity of ouabain binding to potassium under the same conditions, *i.e.*, in the presence of magnesium plus ATP. One explanation for these results is that Mg·ATP and sodium bind to one conformer of the enzyme and potassium stabilizes another conformer, where the two conformers exist in equilibrium with each other. The conformer, reacted with sodium plus ATP (E_1-P?), would convert to the state that can react with the glycoside (E_2-P?). Conversely, the potassium form of the enzyme (E_1 or E_2) would be unreactive. Examination of ouabain binding rates at various concentrations of Mg·ATP (393) revealed that the rates respond in a saturable manner to increasing Mg·ATP. This response was characterized by a rectangular hyperbola with a dissociation constant for Mg·ATP of 10 to 20 μM (fig. 9). Potassium did not appear to alter the binding rate at saturating concentrations of Mg·ATP but did increase the apparent dissociation constant for Mg·ATP to about 40 μM in 0.4 mM KCl, 400 to 500 μM in 1.0 mM KCl, and greater than 1000 μM in 2 to 5 mM KCl. These responses are consistent with the conclusion

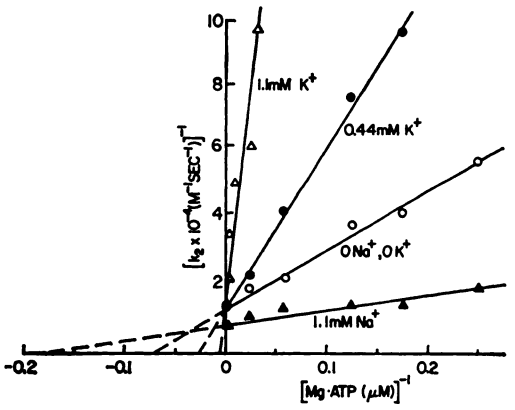

FIG. 9. Double-reciprocal plot of second-order rate constant for ouabain binding *versus* the concentration of Mg·ATP. [From Lindenmayer *et al.* (393).]

that potassium and Mg·ATP compete with each other and that potassium binds to more than one site, since the dissociation constant for Mg·ATP increases through some type of power function with respect to increasing potassium. Sodium, as opposed to potassium, increased the rate constant for ouabain binding at saturating Mg·ATP (compared to that observed in the absence of sodium). A second effect of sodium was to decrease the apparent dissociation constant for Mg·ATP (in the absence of added sodium) from 10 to 20 μM to about 6 μM at 1 mM NaCl, 0.7 μM at 2 mM NaCl, and between 0.1 to 0.1 μM at 10 and 100 mM NaCl. Thus, the decrease in the apparent dissociation constant for Mg·ATP appeared to be mediated through a power function of increasing sodium and also appeared to be a saturable function of sodium. In terms of modulation, neither sodium nor potassium seemed to alter the rectangular hyperbolic nature of the response of the association rate constants for ouabain interaction to increasing Mg·ATP. These responses are consistent with the hypothesis that Mg·ATP and sodium react with a conformer on the pathway which leads toward the macromolecular state that binds ouabain, whereas potassium in this case reacts with a conformer which is unreactive toward ouabain. These two conformers should be in equilibrium with each other, since Mg·ATP and potassium appear to be antagonistic in a competitive sense. It is also apparent that sodium is not an absolute requirement for binding supported by Mg·ATP. We feel that sodium is a modulator of ouabain-enzyme interaction.

As discussed in the previous section, there is evidence that the phosphoenzyme derived from ATP exists in at least two forms, designated as (E_1-P) and (E_2-P). It is suggested that the former is sensitive to ADP and the latter to potassium. There is some reason to believe that (E_2-P) more closely corresponds to the state of the receptor required for glycoside interaction. Post *et al.* (499) showed that the enzyme in the form (E_2-P) could react with ouabain, whereas the enzyme in the form (E_1-P) was relatively insensitive to the drug. In 1973, Tobin *et al.* (664) reported that under certain conditions, calcium favors the existence of (E_1-P) over (E_2-P), in that the phosphoenzyme formed in the presence of calcium is sensitive to ADP and relatively resistant to potassium and that ouabain reacted poorly when the system was in E_1-P configuration. These concepts are summarized in figure 10. Cardiac glycosides in the presence of Mg·ATP would bind to the macromolecular conformation represented by (E_2-P). The steady-state level of (E_2-P)·K^+ would always be low because of the instability of this state. The rate of glycoside binding would depend on the level of (E_2-P). Thus, sodium would stimulate binding through two pathways: a) driving the enzyme from E_2 toward (E_2-P) through interaction with a site of high sodium affinity; and b) stabilization of (E_2-P) through interaction with a site with low affinity for sodium and high affinity for potassium. Potassium would reduce the rate of binding through two pathways: a) reduction of (E_2-P) through interaction with a site with high affinity for potassium; and b) induction of an increase in the steady-state level of a

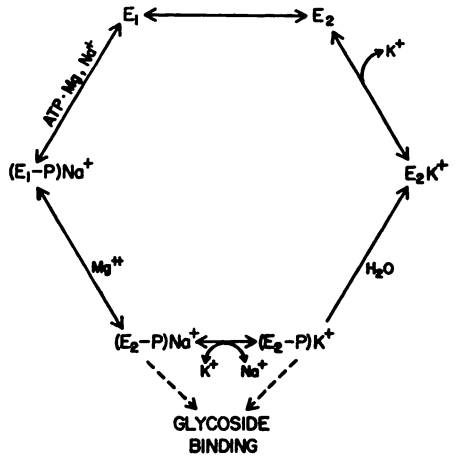

FIG. 10. Scheme for Na^+ and K^+ modulation of ouabain interaction.

nonbinding conformer (*e.g.*, E_2K^+ and/or E_1K^+). The level of (E_2-P) in the presence of Mg·ATP but absence of sodium would be low, consistent with the observed slow rate of glycoside binding.

The conclusion that all glycoside binding in the presence of nucleotide must occur through interaction with (E_2-P) is challenged by the apparent lack of nucleotide specificity for binding as compared to a much greater nucleotide specificity for nucleotide hydrolysis. Matsui and Schwartz (435) found that ATP, inosine 5′-triphosphate (ITP), GTP, cytidine triphosphate (CTP), UTP, and ADP supported significant ouabain interaction to a beef heart Na^+,K^+-ATPase preparation in the presence of magnesium and sodium. Hoffman (277) in 1969 confirmed these results; ATP, ITP, CTP, and ADP stimulated binding to erythrocyte membrane preparations. (Also, see the section V D and VII K on NEM.)

Tobin *et al.* (666) in 1972 re-examined the nucleotide specificity of a rat brain preparation with respect to hydrolysis, phosphorylation of the enzyme, and support of glycoside binding. They reported that the initial rates of hydrolysis of ATP, ITP, UTP, and ADP in the presence of sodium and magnesium (*i.e.*, potassium was absent; thus the activity measured was a sodium-stimulated nucleotide triphosphatase) correlated with the initial rates of ouabain binding when the latter were examined under the same conditions. Furthermore, ATP, CTP, ITP, and UTP were found to be indirectly or directly capable of phosphorylating the Na^+,K^+-ATPase. Higher concentrations of ADP inhibited both phosphorylation of the enzyme by ATP and ATP-dependent glycoside binding. These results strongly suggest, at least with respect to the Na^+,K^+-ATPase in fragmented membrane preparations, that the receptor conformation for glycoside binding either coincides with the phosphorylated enzyme or with a state subsequent to it, *when binding is carried out in a medium containing nucleotide, sodium, and magnesium.* Second, cardiac glycosides inhibit nucleotide binding to Na^+,K^+-ATPase preparations (259, 469). Hansen *et al.* (248) showed that ADP displaced ouabain bound to an ox brain preparation. This result appears inconsistent with the interaction of both nucleotide and glycoside with the same conformation of the system. In 1973, Tobin *et al.* (665) and in 1974, Tobin (662) reported that an analog of ATP, adenylylmethylenediphosphonate, inhibited the binding of ouabain and the interaction of ATP with a rat brain Na^+,K^+-ATPase preparation. This analog did not appear to be hydrolyzed by the enzyme. Since the analog did occupy the nucleotide site, these data may support the concept that phosphorylation, as opposed to nucleotide binding, is required for ouabain binding. Alternatively, the analog may simply distort the macromolecular conformation so that glycoside interaction cannot occur.

Hoffman (278) in 1973 interpreted the effects of nucleotides on glycoside interaction in terms of a two-state model for the pump. The two states would be in equilibrium, $A \rightleftharpoons B$, and glycosides would bind only to the state B. Hoffman suggested that the nucleotide, by binding to an internal site, results in a shift to the right in the equilibrium, $A \rightleftharpoons B$. This shift favors glycoside interaction. The effect of internal sodium would be to phosphorylate the pump which shifts the equilibrium away from B, and, thereby, inhibits binding. In fact, it was suggested that any increase in the activity of the pump would shift the equilibrium away from B, reducing glycoside interaction. The predominant argument in favor of such a model was that the pyrimidine nucleotides could not be broken down by the system (278). This model appears to be in disagreement with the studies reviewed in the preceding paragraphs.

The interaction of cardiac glycosides with the sodium pump in intact transport-

ing systems also appears to be modulated by the cationic composition of the bathing medium. Baker and Willis (53) found that in mammalian cell preparations (HeLa and Girardi), potassium reduced the rate of glycoside interaction but did not alter the rate of dissociation. The potassium effect seemed to be mediated through a potassium activation site, since a reciprocal relationship appeared to exist between the reduction in rate of binding and activation of the pump. Furthermore, the sensitivity of the glycoside binding reaction to potassium was increased by replacing external sodium with choline. Baker and Manil (51) reported that in squid axon, ouabain binding required external sodium but was relatively insensitive to potassium. This suggested that the glycoside binding may be facilitated by the interaction of sodium with an external site on the pump. Wu and Sjodin (715) found that the affinity of the pump for strophanthidin in frog sartorius preparations was about 20 times greater in a sodium medium (potassium-free) than in a potassium medium (sodium-free). Consistent with the above observations, the rate of inhibition was dependent upon the external sodium concentration and was an inverse function of external potassium.

We have referred above to Schatzmann's work (559) in 1965 on erythrocyte preparations, in which he reported that cardiac glycosides and sodium act in some synergistic manner with respect to potassium activation of the pump. Beaugé and his colleagues (66, 67) found that a sodium-rubidium antagonism modulated the rate of inhibition produced by ouabain in human erythrocyte preparations. Rubidium, an effective substitute for potassium with respect to activation of the pump, inhibited the rate of inhibition while sodium increased the rate. On the other hand, these authors found that removal of sodium did not prevent inhibition, although a requirement for sodium as a cofactor for binding was not completely eliminated as a possibility (*e.g.*, contaminating sodium?). Gardner and Conlon (197) found that neither external sodium nor external potassium altered the maximum amount of ouabain which could bind to human erythrocyte preparations. Sodium and potassium, however, modulated the affinity of the receptor for ouabain. They interpreted their results in terms of two sites, one being the glycoside receptor and a second being a cation binding site. Occupation by sodium of the latter increased the affinity for ouabain, whereas occupation by potassium decreased the affinity. These studies are consistent with the interpretation put forward above, derived from studies on the isolated fragmented Na^+,K^+-ATPase. In other words, there is a state of the pump that coincides with a reactive conformer of the glycoside receptor. Reaction of external sodium stabilizes the state or at least prevents potassium from converting it to a different pump state with which the receptor cannot react. Furthermore, these studies on intact transporting systems are consistent with the existence of these states within a sequence requiring internal sodium, prior to external potassium, to react with the pump (fig. 10).

Hoffman (278) has summarized some unpublished experiments which challenge this concept. In human erythrocyte ghost preparations containing internal magnesium plus ATP plus constant sodium plus potassium, increasing the concentration of external sodium increased the rate of glycoside interaction, whereas an increase in the concentration of external potassium depressed the rate; but at constant external sodium and potassium, raising the internal sodium or potassium also decreased the rate of glycoside binding. This internal effect of sodium is inconsistent with internal sodium interacting with the pump to produce a conformer reactive with the glycoside. Furthermore, Hoffman states that inorganic phosphate promoted binding when present on the *inside* and not on the outside. The rate of binding supported by internal magnesium and

inorganic phosphate was increased by external sodium by competition with external potassium, but the rate was decreased by raising internal sodium. These data are seemingly at variance with data obtained from fragmented membrane preparations. In the latter case, low, intermediate or high concentrations of sodium stimulated the rate of glycoside binding in the presence of ATP and magnesium, but sodium inhibited binding supported by magnesium plus inorganic phosphate. This discrepancy may be, as Hoffman suggests, due to the "sidedness" of the erythrocyte ghost preparation *versus* the existence of equivalent environments found at both surfaces of the pump in fragmented membrane preparations. If it is "sidedness," it seems likely that the crucial factor is the exposure *versus* nonexposure of something besides sodium and potassium at both surfaces, perhaps magnesium (?) or calcium (?).

There is a final complexity regarding the binding conformer of digitalis: if (E_1-P) does not react with glycoside, occluding the enzyme with N-ethylmaleimide should yield a preparation that does not bind glycoside. Studies reported by Siegel *et al.* (602) and by Banerjee *et al.* (58) and preliminary experiments by us and by Hegyvary (personal communication) are inconsistent with that concept, *i.e.*, ^{3}H-ouabain binds to an N-ethylmaleimide-treated enzyme (see section II). Banerjee *et al.* (58) did state that they observed ^{3}H-ouabain binding to a NEM-treated enzyme which was supported by ATP, magnesium plus sodium, magnesium plus phosphate, or magnesium alone, although the amounts bound were less than for the control preparation. Also, Siegel *et al.* (602) reported ^{3}H-ouabain binding to a NEM-treated preparation that was supported by magnesium.

F. Simultaneous versus Sequential Interaction of Sodium and Potassium

A number of models for the sodium pump, in addition to those found in the papers reviewed above, have been published (61, 102, 176, 227, 265–269, 410, 443, 476, 481, 498, 515, 530, 538, 617, 640, 710). Many of these models have similarities, but the large number points out the fact that no one model explains all aspects of the system. In the last five years, however, several studies have appeared that limit the number of mechanisms which could apply to the sodium pump.

Three laboratories have presented evidence that the sodium and potassium activation sites are independent. In one experiment with squid axons, Baker *et al.* (49) reported in 1969 that the potassium activation curve at low internal sodium was within the range of a curve observed at higher internal sodium. This is consistent with the suggestion that the affinity of the potassium activation sites is independent of the internal sodium concentration and, as such, the sodium activation sites. In 1971, P. G. Hoffman and Tosteson (281) observed that alteration in the internal medium in sheep erythrocyte preparations changed the rate of pumping when external potassium was saturating but did not alter the affinity of the potassium activation sites. Although these authors examined models of sequential interaction that could account for their data, they concluded as follows (281):

> "Thus, our investigation of the kinetic characteristics of sequential models has found that mathematical solutions do exist which provide for independence of internal and external activation curves. These solutions require that physically unlikely fortuitous relationships must obtain among the rate coefficients of the various reactions in the sequence, and the concentrations of external Na and internal K. Moreover, independence requires the presence of external Na. These theoretical requirements are not compatible with the experimental data on sheep red cells. Evidence provided above . . . is consistent with the conclusion that the pump fluxes of Na and K were independent both in the presence

and absence of external Na. The absence of significant K-K exchange was indicated by Tosteson and Hoffman (1960), and more recently by Ellory and Lew (1970). In the light of these considerations, we believe that it is unlikely that the K + Na pump in sheep red cell operates by a sequential mechanism."

The essential difference between the consecutive or sequential and simultaneous models is diagrammed in figure 11. In the former, sodium reacts first, initiating a chemical reaction followed by a potassium interaction presumably with an altered conformation of the same "carrier molecule." In the latter, all ligands interact in a more or less simultaneous manner with some type of pump "unit."

Garay and Garrahan in 1973 showed in human erythrocyte preparations that the affinity of the sodium activation sites appears to be independent of the cation composition of the external medium, and the affinity of the potassium activation sites appears to be independent of the internal cation composition. These authors concluded the following (196):

"This strongly suggests that, as already proposed by Hoffman and Tosteson (1971) for sheep red cells, active ion translocation in human red cells is the consequence of the *simultaneous* reaction of inner and outer cations with a pump unit [see also Baker & Stone (1966) and Skou (1971)]. It would seem, therefore, that sequential models for active transport (see for instance, Shaw 1954, Garrahan & Glynn, 1967a, Stone 1968, and Caldwell, 1970) do not provide an adequate explanation for the molecular mechanism of the Na pump in red cells, since in these models inner and outer sites do not exist at the same time on the same pump unit."

As stated above, sodium and potassium activation of a beef brain Na^+,K^+-ATPase preparation is consistent with the simultaneous existence of three-equivalent sodium activation sites and two-nonequivalent potassium activation sites, all of which must be occupied by their preferred cations for the hydrolysis of one molecule of ATP (398).

These results and conclusions form this paradox: a) the phosphoenzyme (discussed above) appears to have all of the characteristics required of an intermediate in a sequential mechanism for sodium-potassium exchange; and b) the kinetics of sodium and potassium activation of the sodium pump are consistent with a mechanism for simultaneous interaction of the cations. If the sodium pump does not operate through a sequential mechanism, it is

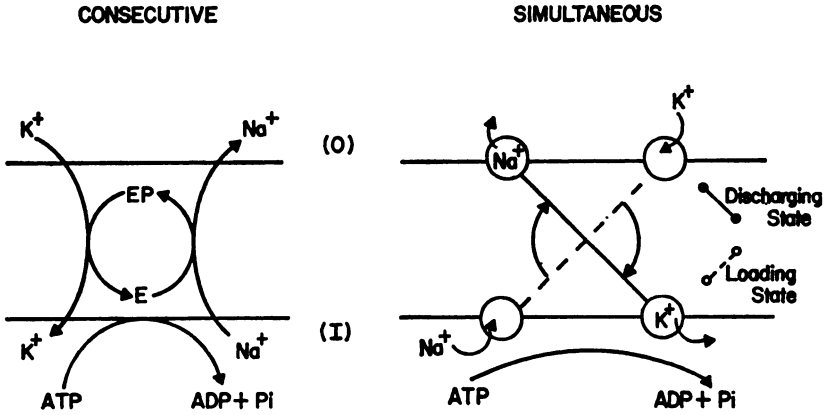

FIG. 11. Two different mechanisms proposed for the sodium pump.

possible to conclude that the phosphoenzyme is not an intermediate in the reaction sequence for sodium-potassium exchange. Rather, it may a) serve as an intermediate in an *alternative pathway* for ATP hydrolysis, b) exist as a product of a *side reaction* (613, 620), or c) it may be an *artifact* of the procedures used to detect its existence (577). The current evidence, however, does not eliminate the phosphoenzyme as an intermediate conformation in the function of the pump (see below).

It now appears that sodium and potassium activation of the sodium pump and the Na^+,K^+-ATPase is consistent with the rapid equilibrium and random interaction of sodium and potassium with two species of independent binding sites. A critical question is whether there are specific mechanisms of sequential interaction which can mimic the responses predicted for a simultaneous interaction. A second and equally important question is whether such mechanisms are plausible in a macromolecular sense. Baker and Stone (52) examined this problem and found one type of reaction sequence which mimicked the responses predicted by a simultaneous model. This was of the form, $Na^+ \underset{\rightarrow}{\rightleftarrows} K^+$. In reviewing the latter paper, Hoffman and Tosteson (281) pointed out that, as written, the reaction sequence requires potassium to react in a nonsaturable manner, a response in obvious disagreement with the observations. Hoffman and Tosteson (281) considered a second mechanism but concluded that it was unlikely since it required improbable relationships among various rate constants and also required that external sodium be greater than zero.

In 1973, Stein *et al.* 638a) proposed a different type of model which was based on the assumption that the pump is a tetramer. [See section IV of this review; a functional molecule of the system, composed of two "catalytic" and two glycoproteins, does account for the maximum levels of ATP or ouabain bound, if there is only one effective binding site per functional enzyme molecule. Also, the molecular weight of such a tetramer is in the range of that estimated for Na^+,K^+-ATPase, with *in vacuo* irradiation studies (342).] Stein *et al.* (638a), employing analogies of "half-of-the-sites-reactive" enzymes, suggested that there may be two nucleotide binding sites per functional Na^+,K^+-ATPase molecule but that only one of the sites (at a time) can be phosphorylated by the nucleotide. A second consideration in the development of their model was that the internal sites for sodium activation and the external sites for potassium activation exist simultaneously. Their model for active sodium and potassium transport is presented in figure 12, where H and L represent cation binding sites with high sodium affinity-low potassium affinity and low sodium affinity-high potassium affinity, respectively. The *alpha* subunit presumably would represent the higher molecular weight polypeptide, while the *beta* subunit would represent the glycoprotein. The authors suggest that the upper and lower subunits in figure 12 are phosphorylated by ATP in an alternate fashion and that the upper and lower sets are 180° out of phase. Since cardiac glycosides interact from the outside, their receptor would presumably reside on the glycoprotein.

The model is quite attractive in a general sense (see below). In more specific terms, however, evidence renders the model presented in figure 12 questionable. First, there is disagreement about the molar ratio of the "catalytic" protein to the glycoprotein. Kyte (371) found a ratio of 0.5; Lane *et al.* (377) observed a ratio of one; and Hokin *et al.* (285) and Jørgensen (317) reported a ratio of two. [Hokin (283, 285) suggested that the discrepancy in these ratios may reflect the use of different techniques for polyacrylamide gel electrophoretic separation of

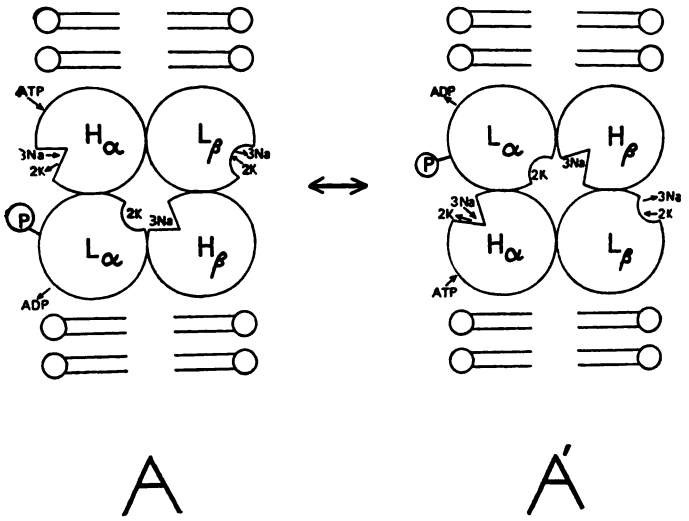

FIG. 12. Model for active transport of Na^+ and K^+ ions as mediated by an $\alpha_2\beta_2$ tetrameric enzyme. H, subunit having a high affinity for Na^+ and low affinity for K^+; L, the converse situation. Each binding site can, intrinsically, be either H or L. (For simplicity, release of inorganic phosphate from H is not shown.) Active transport results from the sequential flipping between the isoenergetic conformations A and A'. [From Stein *et al.* (638a).]

the polypeptides. Alternatively, he suggested that there may be multiple species of polypeptides in one of the bands. While the former of these two explanations appears to be the more reasonable, the discrepancy does raise the question of whether the glycoprotein is part of the enzyme or is a contaminant in the preparation.] Furthermore, Nakao and his coworkers (458–461) have reported the absence of a glycoprotein in their "purified" preparation. Finally Hart and Titus (254, 255) could find no evidence that the exposure of sulfhydryl residues on proteins below 90,000 daltons was affected by the various reactive states of the Na^+,K^+-ATPase. Repke (523) and Repke and Schon (525) presented a modified version of the "half-of-the-sites-reactive" mechanism for the sodium pump. They pointed out that it is not necessary to have a tetramer since a dimer would suffice for this type of mechanism.

Second, Jørgensen (317) reported in 1974 that the maximum level of phosphorylation from ATP is twice the maximum level of ATP or ouabain binding to a highly purified Na^+,K^+-ATPase. This is not consistent with the specific model presented by Stein *et al.* (638a).

Third, one feature of the model in figure 12 is that there is a state in which the cations are occluded within the pump (*i.e.*, the occluded cations are not able to exchange with cations in the media bathing the membrane). Garrahan and Garay (201) stated in 1974 that on the basis of studies employing erythrocyte ghosts, the number of occluded conformations must be negligible. Also, they suggested that the affinity of the sodium activation sites must be constant during the transport cycle. These findings further restrict the type of mechanisms that may apply to the sodium pump. If we adhere to the concept that a phosphoenzyme complex must be an intermediate in a sequence, it would appear that only two states in the sequence should have significant steady-state levels—one with high affinity for sodium and the other with high affinity for potassium. This concept could

meet this and the other requirements by employing the "half-of-the-sites-reactive" mechanism, as suggested by Stein *et al.* (638, 638a) and later by Repke (523) and Repke and Schon (525). In this case, one of the subunits of a dimer would exist in a state with high affinity for sodium, while the second would exist in the state with high affinity for potassium. The transport cycle would consist of a cooperative oscillation of the two subunits between the two states; the existence of the sodium activation sites and the potassium activation sites would appear to be invariant during the transitions of the cycle, since they would always exist on one or the other of the two subunits. Furthermore, variations in the external and internal concentrations of sodium and potassium would not alter the ratio of the high-sodium affinity state to the high-potassium affinity state, since they would remain at equivalent steady-state levels. Thus, the two species of sites would exist simultaneously and in a functionally independent manner.

VI. Potassium-dependent Phosphatase (K^+-Pase)

As discussed in a previous section, the overall hydrolysis of ATP consists of:

$$1)\ ATP + E \overset{Mg^{++},\ Na^+}{\rightleftharpoons} E\text{-}P + ADP$$

$$2)\ E\text{-}P \overset{K^+}{\rightarrow} E + Pi$$

In 1962, Judah *et al.* (323) showed that red cell membrane preparations which contained a Na^+,K^+-ATPase also exhibited a potassium-activated *p*-nitrophenyl-phosphatase, which, like the ATPase, was inhibited by ouabain. Since that time, by using a number of artificial substrates, the presence of this K^+-Pase has been demonstrated in a variety of tissues (530, 537, 554).

Yoshida and co-workers (304, 452, 728) showed that K^+-Pase catalyzed the hydrolysis of a variety of organic phosphates, including carbamylphosphate and acetylphosphate but not *o*-phosphorylserine or α- or β-glycerol phosphate. A close similarity was found in a time course of heat inactivation of K^+-Pase, with carbamylphosphate and *p*-nitrophenylphosphate (PNPP) as protective substrates. The potassium-dependent activity of carbamylphosphate hydrolysis was far greater than PNPP hydrolysis, and the K_m value for carbamylphosphate suggested that it was a better substrate for the K^+-Pase than PNPP. Yoshida and co-workers also showed that the subcellular distribution of the K^+-Pase and the Na^+,K^+-ATPase were similar. Furthermore, the specific activities of both preparations were increased by exposure to sodium iodide and were decreased by pretreatment with protamine or direct treatment with *p*-chloromercuribenzoate, protamine, or igrosin. Potassium, which stimulated the phosphatase, could be replaced by rubidium, ammonium, or cessium. A similar lack of specificity has been recognized for the potassium activation sites of the Na^+,K^+-ATPase (see above). Bader and Sen (43) observed that the following characteristics of K^+-Pase and Na^+,K^+-ATPase were identical: a) K_m values for magnesium, rubidium, ammonium, and lithium; b) K_i (inhibitory constants) values for ouabain and for calcium, mercury, and fluoride ions; c) pH optimum; and d) the energy of activation.

Thus, while early studies of K^+-Pase were consistent with the hypothesis that K^+-Pase activity reflects the second half of the Na^+,K^+-ATPase catalytic cycle (537), subsequent studies have raised a number of questions. Israel and Titus (302) found that K^+-Pase was less sensitive to N-ethylmaleimide than was Na^+,K^+-ATPase, and Albers and Koval (19) reported that the two systems had different sensitivities to magnesium. Both groups concluded that K^+-Pase and Na^+,K^+-ATPase were different entities.

Israel and Titus (302) also noted that the experiments of Bader and Sen (43) employed different ratios of sodium and potassium in experiments designed to determine the sensitivity of K^+-Pase and

Na^+,K^+-ATPase to cardiac glycosides. Israel and Titus (302) found that the sensitivity of the two systems was considerably different (*i.e.*, the apparent sensitivity of K^+-Pase was about 20 times greater than that of Na^+,K^+-ATPase) when identical ionic conditions were employed for the comparison.

Yoshida *et al.* (728) demonstrated that ouabain sensitivity of the phosphatase was significantly increased if the assay was carried out in the presence of ATP and sodium. Ouabain (10 μM) inhibited the K^+-Pase in the absence of sodium or ATP by about 50%. However, the addition of 5 mM potassium, 20 mM sodium, and 0.1 mM ATP increased inhibition to 65%. Raising the sodium concentration to 100 mM increased the level of inhibition induced by ouabain to 90%. It is interesting that the effect of the increasing sensitivity to ouabain in the presence of sodium was specific for ATP. These studies suggested that sodium and ATP modulate the macromolecular determinants for K^+-Pase activity.

ATP or ADP in the absence of sodium inhibit K^+-Pase activity, perhaps through competition with organic phosphates for an active site (452). In the presence of sodium plus low potassium, however, ATP *stimulates* K^+-Pase (304, 452, 542, 728). Analysis of the response of K^+-Pase activity to increasing potassium showed that ATP plus sodium reduced the amount of potassium required for half-maximum activation of K^+-Pase (304, 352). The K^+-Pase activity in the presence of saturating potassium (*i.e.*, V_{max}), however, is somewhat decreased when sodium or ATP are present (531). CTP, ITP, or ADP could substitute for ATP (728) with respect to nucleotide stimulation of K^+-Pase activity, but no other monovalent cation could substitute for sodium.

In a 1973 report, Robinson (542) proposed a model to account for the sodium stimulation of the potassium phosphatase. An alternative enzymatic form is induced by Na^+ and ATP, permitting catalysis over the entire Na^+,K^+-ATPase pathway, instead of just the K^+-Pase pathway. This hydrolytic route would occur in addition to the more conventional phosphatase route. Robinson further suggested that the increase in the apparent affinity for potassium that occurs with sodium plus a phosphorylating substrate was due to this alternate pathway. He also showed that a phosphorylating substrate was necessary, since nitrobenzyl phosphate, a nonphosphorylating substrate which bound to the active site, was ineffective in increasing potassium affinity. This second pathway would reflect steps of the entire ATP hydrolysis scheme, *i.e.*, sodium-dependent hydrolysis. As a result, in the presence of both sodium and potassium, both reactions would be available, and the direction would be regulated by the relative concentrations of sodium and potassium.

ATP alone (*i.e.*, without sodium) has been reported to stimulate K^+-Pase activity, but this effect appears to require a cofactor, presumably calcium (507). The results suggested that K^+-Pase activation in the presence of ATP and potassium required the presence of a substrate which can be chelated by ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetate (EGTA). The reappearance of activation by addition of calcium suggests that this cation is the activating substance. There are parallels between this effect, the proposed mechanism of hydroxylamine release of a heavy metal bound to an inhibitory site on the Na^+,K^+-ATPase (40, 45) and stimulation of the enzyme activity by amino acids and EGTA (631, 659, 684).

Data published in 1972 (519) suggest that the calcium plus ATP stimulation of the K^+-Pase is asymmetrical, since calcium plus ATP stimulated potassium-dependent hydrolysis of PNPP only when placed on the inside of red cell ghosts.

This type of asymmetrical activation of the K^+-Pase suggests that the entire system has specific membrane orientation. It is

possible that common sites for ATP plus calcium may be shared by the K^+-Pase and the calcium-stimulated membrane-bound magnesium-ATPase (calcium plus magnesium ATPase) earlier demonstrated by Schatzmann and Vincenzi (561). It is an attractive concept that these three entities, K^+-Pase, calcium plus magnesium-ATPase, and Na^+,K^+-ATPase, may be part of the same structural system and that the particular activity expressed depends upon the ligands present.

In 1970, Inturrisi and Titus (299) showed that K^+-Pase isolated from beef brain could be phosphorylated by *p*-nitrophenylphosphate when ouabain was present in the reaction medium. This ouabain-dependent reaction required magnesium but not sodium or potassium. The authors suggested that ouabain places the K^+-Pase in a conformation that allows *p*-nitrophenylphosphate or inorganic phosphate to label the same site on the enzyme. Robinson (540) subsequently showed that phosphorylation by *p*-nitrophenylphosphate could be stimulated by potassium or sodium in a manner independent of ouabain.

By using a system of continuous monitoring with umbelliferone as substrate, Pitts and Askari (485) showed a time-dependent activation by ouabain of a phosphatase activity in the presence of zero or low potassium. The ouabain-dependent activation was at a maximum under conditions also maximal for ouabain binding to the Na^+,K^+-ATPase, (*i.e.*, ATP, magnesium, sodium, and low potassium). Because of these similarities, they suggested that ouabain exerted its activating effect by binding at the same site as it does when it exerts its inhibitory effect on the Na^+,K^+-ATPase. This apparent paradox, *viz.*, activation of phosphatase and inhibition of Na^+,K^+-ATPase, will be discussed in further detail below, but ouabain, in the presence of ATP, magnesium, sodium, and potassium could promote the hydrolysis of phosphatase substrates in preference to ATP, perhaps by changing the accessibility of catalytic centers for nucleotide and organic phosphate esters. More recently, Pitts (484) showed that the sodium plus ATP stimulation of phosphatase activity may be associated with effects on ouabain binding. Both reactions were stimulated maximally by 10 mM sodium, had K_m values below 1 μM for ATP, and lacked nucleotide specificity. He suggested that the sodium-dependent phosphorylation of these preparations by ATP may occur through sodium interaction with the same site as that involved in the stimulation of both K^+-Pase and ouabain binding.

An important question with respect to K^+-Pase is its role, if any, in the transmembrane movements of sodium and potassium. In 1969, Askari and Rao (32) reported that substrates for the K^+-Pase could support active cation movements in resealed erythrocyte ghost preparations. They concluded that the K^+-Pase resided on the external surface of the membrane and was the primary ion translocator; a second enzyme on the internal side of the membrane supplied a physiological substrate (*e.g.*, the phosphorylated intermediate?) for use by the translocator phosphatase. In 1971, Askari and Rao (33) reported that sodium efflux was not stimulated if *p*-nitrophenylphosphate was inside the erythrocyte ghosts.

The study by Askari and Rao (33) showed a good correlation between K^+-Pase activity and sodium efflux and gave further support to one of the major conclusions of the initial efflux study (32). In the previous paper, the authors had suggested that the potassium-dependent breakdown of *p*-nitrophenylphosphate was sufficient for the stimulation of sodium efflux. That conclusion was justified for the relatively simple case of efflux into a sodium-free medium. In the sodium-containing medium, however, the need for a nucleoside phosphate to be present in the medium caused complications. Although it had been shown that ATP could be used

for ion movements only when it was on the inside of the membrane, it was possible that in the presence of *p*-nitrophenylphosphate, ATP was in some manner being used to stimulate sodium efflux. The studies with oligomycin (31) appear to eliminate this possibility, since the inhibitory effect of external sodium on the K^+-Pase and sodium efflux was overcome either by ATP or by oligomycin. Thus, it is presumably the substrate for K^+-Pase and not ATP in the external medium which is hydrolyzed in association with sodium efflux. The results also indicated that either the sodium efflux associated with K^+-Pase activity is independent of the internal sodium concentration or that the efflux is more sensitive to increasing sodium concentrations than is the metabolically stimulated sodium efflux. The question as to whether the response of the K^+-Pase-mediated sodium efflux to internal sodium is drastically different from that of physiological sodium efflux, however, is unanswered. The above studies suggested to Askari and Rao that the sodium efflux, which is activated by *p*-nitrophenylphosphate, is not an artifact and represents a function of the sodium pump. They (33) also concluded that any proposed mechanism for the physiological operation of the sodium pump must explain the K^+-Pase-sodium efflux phenomenon. At the present time, their findings may be incorporated into several equally plausible models; their working hypothesis is outlined as follows: a) most sodium and potassium membrane translocations are associated with the K^+-Pase segment of the Na^+,K^+-ATPase complex; b) the primary role of the transphosphorylation segment (from internal ATP to the enzyme) is the production of a substrate for the translocator phosphate; and c) it is step b) which is controlled by the internal sodium concentrations.

Garrahan and Rega (209) claimed that they could not repeat the studies of Askari and Rao and reported that PNPP could not support any ouabain-sensitive sodium efflux into either a sodium-containing or a sodium-free medium. In fact, PNPP inhibited ATP-supported, ouabain-sensitive sodium efflux, presumably by combining with an ATP site located at the inner surface of the membrane. However, it should be emphasized that Garrahan and Rega used intact red cells whereas Askari and Rao employed a specific type of resealed ghost preparation. Therefore, it is inappropriate to conclude that a definite controversy exists until identical preparations are used by the different laboratories.

On the other hand, Garrahan *et al.* (207, 208) did state that the K^+-Pase and Na^+,K^+-ATPase are part of the same system and that the phosphatase is part of the translocator system. These results, together with the fact that sodium competitively inhibited the effect of potassium, but only when present externally, suggested that the potassium site of the membrane phosphatase was located at the outer surface of the cell membrane.

An additional aspect of the K^+-Pase should be mentioned regarding HK (high potassium- and LK (low potassium)-containing sheep erythrocytes, which have been shown to possess different numbers of ouabain-sensitive pump sites and Na^+,K^+-ATPase activities, according to their potassium transport requirements. Whittington and Blostein (79, 700) carried out an analysis of the HK/LK ratios for partial reactions catalyzed by the Na^+,K^+-ATPase. They found ratios of 10 for Na^+-ATPase (*i.e.*, no potassium), 13 for Na^+,K^+-ATPase, 2.7 for the ADP-ATP exchange rate, and 7 for the sodium-dependent phosphorylation of the system by ATP. They were unable, however, to show any significant K^+-Pase activity. It is interesting that hyman erythrocytes do exhibit a high K^+-Pase activity; the magnesium-dependent phosphatase was 40% higher in HK red cells than LK, but neither was stimulated by potassium. If the K^+-Pase is a part of the overall Na^+,K^+-ATPase reaction, it

would seem crucial to demonstrate species differences in this activity that are consistent with differences in other partial reactions of the catalytic sequence.

However, in 1974 Ellory and Lew (160) demonstrated a small K^+-dependent *p*-nitrophenylphosphatase activity in LK-type red cell ghosts. This potassium-dependent portion showed a K_m for PNPP of 3.3 mM and showed substrate inhibition as well. The ratio of potassium stimulation compared to the usual magnesium phosphatase activity was much lower in the LK cells (1.1) as compared to HK cells (2.8). Furthermore, the ratio of potassium phosphatase to Na^+,K^+-ATPase was also much lower for LK cells (0.1) when compared to HK cells (0.4).

These authors also showed that a specific sheep blood group antibody from LK red cells (anti-L) which stimulates potassium transport and the Na^+,K^+-ATPase of LK cells also stimulates the potassium phosphatase activity of the LK cells, without changing the affinity for potassium. They were unable to determine whether the antibody acts by unmasking new LK- or HK-type pumps or by changing the reactivity of LK-type pumps towards sodium and potassium, bringing them nearer to HK-type pump behavior. The finding of such potassium phosphatase activity in LK cells is very important in assessing the role of potassium phosphatase in overall pump activity.

Tanaka (644) compared the K^+-Pase activity with the Na^+,K^+-ATPase activity of a membrane fraction derived from cerebral cortex. He observed that K^+-Pase activity after phospholipid extraction had altered the Na^+,K^+-ATPase so that the latter now required addition of lipid for activity. A greater variety of phospholipids were effective in activating the K^+-Pase than was the case with the Na^+,K^+-ATPase, and with each phospholipid, the extent and pattern of the former was different from that of the latter. During treatment with deoxycholate, the pH optimum of the K^+-Pase was shifted independently from the shift in the pH optimum for the Na^+,K^+-ATPase, and the ratio of K^+-Pase activity to Na^+,K^+-ATPase activity was not constant during purification. However, the two enzymes could not be separated by ammonium sulfate fractionation. Tanaka (644) suggested that the K^+-Pase may be a different entity from the Na^+,K^+-ATPase or, alternatively, K^+-Pase activity may result from a portion of the Na^+,K^+-ATPase molecule(s), and he suggested that the lipid requirement for this activity is less specific than the requirements for the overall catalytic sequence of Na^+,K^+ATPase.

Since Jørgensen and Skou (320) and Lane *et al.* (377) have demonstrated a K^+-Pase in highly purified Na^+,K^+-ATPase which contain only a protein of ~90,000 daltons and a glycoprotein of ~55,000 daltons (see section IV) and both components are required for enzymatic activity, the K^+-Pase most probably is a part of the overall hydrolytic reaction.

Oligomycin, which inhibits Na^+,K^+-ATPase activity, does not affect the potassium-dependent component *per se* of K^+-Pase (298, 359). Askari and Koyal (29), however, found that oligomycin blocked the stimulation of the activity induced by sodium and ATP. In the presence of sodium and low concentrations of potassium, oligomycin stimulated the phosphatase. This stimulation, similar to the activating effect of ATP, was accompanied by an increase in the apparent affinity of the system for potassium. Although the response of enzyme activation to increasing concentrations of oligomycin had a sharp peak, the maximum activation obtained with oligomycin never approached that obtained with ATP. It was concluded that sodium-dependent binding of either ATP or oligomycin to the modifying site affects the interaction of potassium with the system. Higher concentrations of oligomycin block the stimulation of K^+-Pase induced by ATP or lower oligomycin concentrations. Askari and Koyal (29) concluded

that the demonstration of similar modifying effects of oligomycin and ATP on the K^+-Pase supports the proposal of Skou and Hilberg (620), *viz.*, sodium-dependent ATP binding to the enzyme complex (as opposed to phosphorylation of the enzyme by ATP) may be the primary event in producing certain conformational changes in the membrane and perhaps in the process of sodium translocation through the membrane.

VII. Modifying Agents on Na^+,K^+-ATPase

Compounds which affect Na^+,K^+-ATPase activity are varied in their mechanisms because of the complex nature of the enzyme system. These compounds may inhibit or stimulate by actions at a number of specific sites in the reaction sequence or perhaps by a generalized type of membrane action. The same end result may occur, but the mechanism may be totally different. This section will cover effects of many compounds on the enzyme system but will exclude cardiac glycosides and related compounds, since this aspect will be discussed in a separate section (see sections V and VIII).

Compounds of three general types will be discussed: first, those compounds with a therapeutic or toxic mechanism of action that might involve an interaction with the Na^+,K^+-ATPase; second, a group of drugs which act as "tools" in that they may reveal aspects of the mechanism of the overall hydrolysis of ATP by the enzyme system, even though they are of no therapeutic significance and; third, those drugs which simply have an effect on the enzyme system but as yet have not produced data useful in determining specific mechanism or therapeutic effect.

A. Mercurial Diuretics

Infusion of cardiac glycosides into a renal artery causes natriuresis, and it has been shown that this effect is closely correlated with Na^+,K^+-ATPase inhibition (291, 427, 464). In 1950, Goth *et al.* (237) showed that both mersalyl and mercuric chloride in nephrotoxic doses *in vitro* inhibited an ATPase. Although they did not at the time recognize that the enzyme activity was Na^+,K^+-ATPase, in retrospect the data are consistent with this possibility (A. Goth, personal communication). Jones *et al.* (315) demonstrated that a number of diuretic and nondiuretic organic mercurials inhibited the enzyme system *in vitro*. Although the mercurial diuretics inhibited the enzyme system and also caused a diuresis, it was essential to explain why the enzyme system could be inhibited by nondiuretic organic mercurials or, in turn, why inhibition of the enzyme system by these compounds did not cause a diuresis. Compounds of both classes inhibited ATPase *in vitro*, but only the diuretic compounds were effective when administered *in vivo*. These workers postulated that mercurials prevented hydrolysis of ATP by reacting with the ionic sites on the enzyme. Although this work suggests an involvement of the enzyme in the mechanism of action of mercurials, it raises too many issues to be conclusive.

Cafruny (101) suggested that there was a lack of correlation between *in vitro* and *in vivo* data. He felt that the data warranted a rejection of the ATPase as the active receptor for mercurial diuretics. Because of conflicting data, Nechay *et al.* (463) examined some of the problems and suggested that mercurial diuretics may act by inhibiting the Na^+,K^+-ATPase. As in the rat, both diuretic and nondiuretic mercurials inhibited ATPase activity in dogs *in vitro*, but there were very important differences between the species. Most importantly, none of the mercurials affected the activity of ATPase *in vivo* in the dog. Still, these authors refrained from excluding an ATPase mechanism by suggesting a redistribution of the mercury compound during microsomal isolation. However, since large amounts of the mer-

cury compound were still found in the microsomal fraction, it is unlikely that redistribution would account for the negative results obtained.

At present, the role of the ATPase system in the kidney with respect to drug action is unresolved. Furthermore, the only strong evidence at this time which could implicate Na^+,K^+-ATPase as the possible receptor site for the action of mercurial diuretics is the consistent finding that the mercurial diuretics do inhibit the enzyme system *in vitro* (656). But it must be pointed out that many mercurial compounds that inhibit the enzyme system *in vitro* have no diuretic effect. While the mercuric chloride had a preferential effect on the Na^+,K^+-ATPase-stimulated activity, it also inhibited the basic magnesium-ATPase activity. Sulfhydryl-reacting compounds, iodoacetate and iodoacetamide, did not inhibit the enzyme nor did the diuretic compounds, caffeine, theobromine, theophylline, and chlorothiazide (656).

B. Ethacrynic Acid

Since the work of Duggan and Noll in 1965 (149), tests have shown that ethacrynic acid could inhibit Na^+,K^+-ATPase, and it has been suggested by many authors that this inhibition might possibly be the mode of action of this class of diuretics. However, while the evidence is equivocal concerning such a mechanism of action, interesting work has come out of the interaction between ethacrynic acid and Na^+,K^+-ATPase. It has been suggested by Duggan and Noll (149) and others that the mechanism of inhibition *in vitro* involves the sulfhydryl groups, which are known to be required for enzyme activity. In 1970, Davis (139) showed that sulfhydryl reagents such as *p*-chloromercuribenzoate (*p*CMB) and 2,4-dinitrofluorobenzene inhibit the Na^+,K^+-ATPase, as do organic mercurials. These effects can be prevented by prior addition of cysteine. The data on cysteine blockade of ethacrynic acid inhibition of the enzyme supported the earlier suggestion by Duggan and Noll that ethacrynic acid may act through enzyme sulfhydryl groups. However, a weakness in this argument is that cysteine could simply be decreasing the amount of the free ethacrynic acid available for interaction with the enzyme, which may or may not involve a sulfhydryl inhibition.

There are a number of limitations to the concept that renal Na^+,K^+-ATPase is the site of diuretic action of ethacrynic acid. The magnitude of renal Na^+,K^+-ATPase inhibition by ethacrynic acid is quite similar *in vitro* in the dog, guinea pig, and rat (291). However, the drug produces marked diuresis in the dog and little in the guinea pig and rat. Furthermore, the *in vitro* dose-response relationship of ethacrynic acid on the enzyme system is quite narrow, and the enzyme system is never totally inhibited by concentrations of the drug as high as 10^{-3} M. In 1965, Hook and Williamson (292) showed additional inconsistencies between ethacrynic acid and another diuretic, furosemide. Furosemide is a potent diuretic in man, dog, and rat, whereas ethacrynic acid is a potent diuretic in man and dog only. Both agents were found to inhibit the Na^+,K^+-ATPase activity after *in vivo* infusion into the rat kidney. Furosemide produced a significant diuretic response in the rat, whereas ethacrynic acid did not unless perhaps if administered in very high doses. Therefore, there appears to be no correlation between diuretic activity and enzyme inhibition with these agents.

Recent experiments regarding the possible involvement of Na^+,K^+-ATPase on the mechanism of action of ethacrynic acid action have been both positive (150, 462) and negative (295). Duggan and Noll (150) have studied the problem arising from the fact that the concentrations of ethacrynic acid bound to isolated kidney membranes during development of diuresis are far below I50 values for the *in vitro* inhibition of Na^+,K^+-ATPase by

the drug. They infused ^{14}C-ethacrynic acid both intravenously and directly into the renal artery, subsequently isolated cortical microsomes and determined Na^+,K^+-ATPase activity and the quantity of ^{14}C-ethacrynic acid which remained bound to the enzyme system. The concentration of ethacrynic acid found in the cortical microsomes was 500 pmol/mg. The isolated microsomes showed a significant decrease (48%) in Na^+,K^+-ATPase activity. They then compared the effectiveness of ethacrynic acid as an inhibitor under different experimental procedures and measured the drug concentration present at the time of final assay during each procedure. When added directly to the assay medium, the concentration of drug required for inhibition (3.4×10^{-4} M) was considerably higher (about 10^4 times) than the inhibition measured from drug bound *in vivo* to the microsomes (7.6×10^{-8} M). The latter concentration caused no measurable inhibition of control enzyme *in vitro*. Their conclusion was that the intact organ can irreversibly concentrate the drug at appropriate receptors but that addition to an *in vitro* system would not necessarily induce an appropriate response. Duggan and Noll (150) further concluded, ". . . the disparity between respective concentrations of ethacrynic acid required for inhibition *in vitro* and prevailing *in vivo* does not, of itself, preclude involvement of membrane ATPase in the mechanism of action of ethacrynic acid."

Nechay and Contreras (462) also studied the effect of an infusion of ethacrynic acid on urine output and Na^+,K^+-ATPase activity both *in vitro* and *in vivo* and concluded that the drug may act *via* interaction with the enzyme *in vivo*. Furthermore, the rat kidney Na^+,K^+-ATPase was as sensitive *in vitro* to ethacrynic acid as was dog enzyme, but *in vivo*, very little diuresis or enzyme inhibition was observed. They concluded from their data that the natriuretic receptor for ethacrynic acid may well be the Na^+,K^+-ATPase.

However, Inagaki *et al.* (295) were unable to show any relationships between infused ethacrynic acid and either cortical or medullary Na^+,K^+-ATPase activity. It was demonstrated that ethacrynic acid binding to Na^+,K^+-ATPase *in vitro* was neither specific nor saturable. The drug bound quite readily to either denatured microsomes or to a partially purified Na^+,K^+-ATPase preparation. Furthermore, nonspecific binding to other isolated subcellular particles occurred after ^{14}C-ethacrynic acid incubation with kidney slices.

Landon and Fitzpatrick (373), Klahr *et al.* (349), and Daniel *et al.* (136) have reported effects of ethacrynic acid on energy metabolism in various tissues. Daniel *et al.*, by using isolated rat uteri, concluded that ethacrynic acid affected ion movements mainly by inhibiting the liberation of energy and that it acted on both oxidative as well as glycolytic sources, even though it inhibited Na^+,K^+-ATPase of uterine microsomes. Martinez-Maldonado and Schwartz (unpublished observations) found that ethacrynic acid depressed respiratory control of isolated kidney mitochondria, and Eknoyan *et al.* in this laboratory (159a) reported similar inhibitory effects on oxygen consumption.

Ethacrynic acid has an interesting effect on the portion of the sodium pump of erthrocytes which is cardiac glycoside-insensitive [Hoffman and Kregenow (279 and Welt (692)]. Proverbio *et al.* (512) related the residual active cation transport in guinea pig cortex slices to the magnesium ATPase activity remaining after inhibition of Na^+,K^+-ATPase by ouabain. This residual ATPase activity, in the absence of sodium and potassium but in the presence of magnesium, was completely insensitive to ouabain, as has been shown by many other workers, but can be inhibited by high doses of ethacrynic acid.

These experiments suggest that two pumps are involved in sodium extrusion from the kidney cortex cell. One involves exchange for external potassium and

derives its energy from the ATPase. The other, which should be more effective in cell volume regulation, expels sodium accompanied by chloride without the involvement of Na^+,K^+-ATPase. It is obvious that the exact mechanism of action of the diuretics discussed above is unclear. Before an explanation of the involvement of these diuretics with Na^+,K^+-ATPase can become meaningful, the mechanism of cation transport in the kidney tubules requires elucidation. There is some indication that Na^+,K^+-ATPase is involved. However, no qualitative or quantitative data are available.

Attempts have been made to elucidate the mechanism of ethacrynic acid interaction with Na^+,K^+-ATPase at a molecular level. Charnock *et al.* (118) found that ethacrynic acid inhibited microsomal preparations of the enzyme obtained from guinea pig cortex and that the degree of inhibition was influenced by the concentration of potassium ion present and was greatest when potassium ion was low. Furthermore, ethacrynic acid did not inhibit sodium-dependent phosphorylation. However, there was considerable inhibition of the release of inorganic phosphate from this complex upon the addition of potassium. They demonstrated a significant antagonism between potassium and ethacrynic acid in both the incorporation of ^{32}P into the phosphorylated intermediate and the amount of ^{32}P liberated. These experiments indicate that ethacrynic acid inhibits the potassium-dependent dephosphorylation step in the overall hydrolysis of ATP.

In a series of papers, Bannerjee *et al.* (55, 56) found two modes of inhibition of Na^+,K^+-ATPase by ethacrynic acid. First, ethacrynic acid blocked the phosphorylation of the enzyme but had an insignificant effect upon the dephosphorylation step when the sodium-potassium ratio was 10:1. The drug also prevented the ADP-ATP exchange reaction. The degree of enzyme inhibition was closely correlated with the degree of inhibition of phosphorylation and ADP-ATP exchange. Second, ethacrynic acid seemed to stabilize the spontaneous disappearance of the phosphorylated intermediate and slightly decreased the apparent affinity of the enzyme for potassium with respect to hydrolysis of the intermediate.

Stabilization of the phosphorylated intermediate seems to be important. Banerjee *et al.* (55, 56) ascribed pharmacological significance to this reaction. If this type of inhibition occurred in the whole animal, no decrease of Na^+,K^+-ATPase activity would necessarily be observed *in vitro* at a sodium-potassium ratio of 4:1, which has been used by most workers. However, the investigators suggested that the enzyme may turn over much more slowly *in vivo* since the sodium-potassium ratio in the extracellular milieu is 36; under those conditions, inhibition by ethacrynic acid may be much more readily observed.

In a series of experiments in which various ligands were preincubated with the enzyme in the presence of ethacrynic acid, Banerjee *et al.* (56) showed that the inhibition of enzyme activity by the drug was antagonized by preincubation with ATP, ADP, and potassium. ATP with or without ouabain did not reverse the acceleration or retardation of the rate of inhibition of enzyme activity by ethacrynic acid in the presence of sodium or potassium. This suggests that stabilization of a phosphorylated intermediate by ethacrynic acid could be an important factor in the *in vivo* effect of this drug as a diuretic. However, in view of the fact that currently the role and possible *in vivo* importance of a phosphorylated intermediate is not clear, it is impossible to evaluate the validity of these speculations. A major suggestion in their paper is that ethacrynic acid seems to exert an effect in the presence or absence of ouabain, and in the presence of magnesium and phosphate. Lindenmayer *et al.* (394), as well as many

other workers, have shown that a phosphorylated intermediate is formed in the presence of magnesium, $^{32}P_i$, and ouabain. Thus, the results of Banerjee *et al.* seem to be in dispute, since they find no difference in effect of ethacrynic acid in the presence or absence of ouabain, magnesium, and phosphate.

It is apparent that the specific mechanism of action of ethacrynic acid as a diuretic is unresolved at present.

C. Diphenylhydantoin (DPH)

In 1955, Woodbury (714) suggested that diphenylhydantoin exerted its anticonvulsant action by facilitating the active transport of sodium out of the cell. Since the movement of ions in nervous tissue is closely associated with Na^+,K^+-ATPase, several workers suggested that this drug could act by stimulating the transport enzyme system. In 1968, Rawson and Pincus (518) suggested that this could not be the mode of action of the drug since there appeared to be a consistently significant inhibition of Na^+,K^+-ATPase isolated from brain homogenates of both guinea pig and man.

Recent data supports the notion that the Na^+,K^+-ATPase may be involved in the mechanism of action of DPH. Under very specific ionic conditions, Festoff and Appell (175) reported that at a concentration of 50 mM sodium and 0.2 mM potassium, DPH stimulated phosphate release by an average of 92% above the control. A rat brain synaptosomal preparation was used. The *ratio* of sodium-potassium rather than the absolute level of these ions was, according to these investigators, critical in determining the effect of DPH. DPH produced significant stimulation of enzyme activity at a high sodium-potassium ratio of 25 to 50:1. However, at ratios of 5 to 10:1, DPH produced little or no effect, and at low sodium-potasium ratios, *i.e.*, less than 5:1, DPH inhibited the Na^+,K^+-ATPase. Under all ionic conditions examined, DPH produced no apparent change in the affinity of the enzyme for ATP.

The effect of a number of anticonvulsant agents such as DPH and phenobarbital on cardiac arrhythmia has been known for quite some time although only recently was this thought to be of clinical significance. However Conn (128) showed in 1965 that DPH could specifically affect the positive inotropic effect and toxicity induced by digitalis.

In 1969, Boyd and Williams (83) published an extensive study on the effect of DPH on the positive inotropic action of ouabain. Given intravenously over 5 minutes during the infusion of ouabain, the drug significantly reduced the augmentation in left ventricular contractile force which had been produced by the glycoside. The magnitude of the reduction in the duration of the effect was similar to that which occurred in 10 animals given DPH alone. The same interaction, however, was found when DPH was infused during the administration of isoproterenol. These workers concluded that DPH transiently reduced the positive inotropic action of ouabain but that the effect was not directed specifically at the inotropic effect of the glycoside and apparently occurred as a result of direct myocardial depressant properties of the drug. However, because of the presumed stimulatory effect of DPH on Na^+,K^+-ATPase (see above) and the suggestion that ouabain inhibition of the enzyme system is related to the positive inotropic effect, some investigators now feel that DPH acts specifically on digitalis-induced arrhythmias by overcoming the digitalis effect on Na^+,K^+-ATPase. It is thought that DPH, in stimulating the Na^+,K^+-ATPase, overcomes the inhibitory effect of ouabain.

However, more recent work of Godfraind *et al.* (230), Spain and Chidsey (630), and Goldstein *et al.* (233) suggests that the specific action of DPH in reversing digitalis-induced toxicity is unrelated to the Na^+,K^+-ATPase. The first group

showed with guinea pig atria that ouabain toxicity could be prevented by simultaneous administration of DPH. Although the drug did slightly reverse the effects of toxic doses of ouabain, it did not alter the tissue binding characteristics of radiolabeled glycoside. These workers concluded that any ionic alteration caused by DPH would be due to effects other than those directly on membrane ATPase [see for example, Basset and Hoffman (64a)] and a stimulation of sodium–potassium exchange pumping.

Spain and Chidsey (630) studied the interaction of DPH and ouabain on cardiac Na^+,K^+-ATPase both *in vitro* and *in vivo*. DPH stimulated enzyme activity *in vitro* at a sodium-potassium ratio of 50:0.2. However, the drug did not stimulate enzyme activity when the enzyme was isolated from a dog into which ouabain had been infused to the point of toxicity. Furthermore, the drug, while effective in reversing ouabain-induced arrthymias *in vivo*, did not alter the enzyme activity which had been inhibited by ouabain and was ineffective in preventing partial or complete enzyme inhibition produced by ouabain *in vitro*. They concluded that DPH must have reversed the toxic effects of ouabain by a mechanism other than that involving the Na^+,K^+-ATPase.

An extension of this type of approach was reported by Goldstein *et al.* (233) who studied the effect of DPH and digoxin, alone and in combination, on canine myocardial contractility, potassium efflux, and Na^+,K^+-ATPase. DPH alone did not alter Na^+,K^+-ATPase activity but did depress contractility. Digoxin produced the expected effects, *i.e.*, increased contractility, depressed Na^+,K^+-ATPase activity, and a net potassium loss. When DPH was infused in combination with digoxin, onset of the toxic effect was delayed by about 13 minutes but the contractile force was increased and Na^+,K^+-ATPase was inhibited to a greater extent than with digoxin alone. When, however, digoxin and DPH were infused in combination up to the point at which digoxin if given alone would be expected to produce toxicity, the Na^+,K^+-ATPase activity was higher than with digoxin infused alone, and the increase in contractile force was not as great. So DPH did appear to retard concomitantly the digoxin-induced increase in contractility and Na^+,K^+-ATPase depression. Nevertheless, DPH plus digoxin ultimately produced a *greater* increase in contractile force, and greater Na^+,K^+-ATPase inhibition and potassium efflux *prior* to toxic manifestations than digoxin alone. These results suggest that the antiarrhythmic effects of DPH cannot be entirely due to a decrease in the digoxin-induced inhibition of Na^+,K^+-ATPase.

Finally, we have been unable to observe any significant effect of DPH on enzyme activity or on any phase of ^3H-ouabain association-dissociation with partially purified Na^+,K^+-ATPases from a variety of sources—beef brain, dog heart, and kidney —and in the presence of a wide range of sodium–potassium ratios. Therefore, at the present time, there seems to be little evidence of a direct involvement of the Na^+,K^+-ATPase (64, 169, 687).

D. Oligomycin

Oligomycin is a specific inhibitor of oxidative phosphorylation in mitochondria, acting at a phosphorylated site distal to the electron transport system. In 1963, however, Jöbsis and Vreman (313) showed almost total inhibition of rabbit brain Na^+,K^+-ATPase by a concentration of oligomycin of 10 μg/ml. Since oligomycin inhibited the hydrolysis of ATP in the presence of sodium plus potassium plus magnesium, it is possible that the reaction sequence may involve a "high-energy intermediate" similar to one postulated earlier for oxidative phosphorylation. Oligomycin inhibits the Na^+,K^+-ATPase isolated from many different tissues: brain [Jöbsis and Vreman (313), Järnefelt

(310)]; red cell membranes [Van Groningen and Slater (678); Whittam *et al.* (699)]; electric organ of the electric eel [Glynn (221)]; and calf heart muscle [Matsui and Schwartz (432)].

Fahn and co-workers (171) showed that although oligomycin inhibited the overall Na^+,K^+-ATPase of the electric eel preparation, it had no effect on the exchange reaction (ADP-ATP) indicating that the effect of oligomycin may be on a partial sequence of the ATPase which occurs subsequent to the locus of transphosphorylation. Furthermore, it did not inhibit the phosphorylation of the microsomes by $AT^{32}P$. In 1971, Robinson (539), in a kinetic analysis of the effect of oligomycin on the Na^+,K^+-ATPase from rat brain microsomes, reported that the inhibition was incomplete even at high oligomycin concentrations; Dixon plots were concave in a downward direction. As the temperature was decreased, inhibition increased and elevated magnesium chloride concentrations antagonized the inhibition. Relative inhibition was also less at lower ATP concentrations than at higher ones. There appeared to be a relationship between the effects of oligomycin and those of ouabain. In the presence of ouabain, the relative efficacy of the inhibition produced by oligomycin decreased. Oligomycin also diminished the interaction between sodium plus potassium and the enzyme, and reduced the half-maximum activation concentration for sodium chloride. The potassium-dependent phosphatase was inhibited in a similar manner. Robinson suggested that oligomycin inhibits ATPase activity by affecting an equilibrium between two different allosteric forms of the enzyme. He suggested the existence of an alternative reaction scheme with co-existing interconvertible forms. The interesting work of Askari and Koyal (29, 30) on oligomycin sensitivities of the Na^+,K^+-ATPase and its related potassium phosphatase is covered under the section concerning the partial reactions.

E. Hydroxylamine

In 1965, Hokin *et al.* (290) and Nakao *et al.* (455), using hydroxylamine, suggested that an acyl phosphate was an intermediate involved in the overall hydrolysis of ATP (see section V, B, D) Both of these groups showed that hydroxylamine after denaturation of the protein could induce the hydrolysis of the phosphorylated intermediate which had been formed in the presence of $AT^{32}P$, magnesium and sodium, thus acting in a manner similar to potassium. It was these experiments that first suggested that the phosphorylated intermediate found was an acyl phosphate. The work of Hokin *et al.* (290) showing that incubation of the "intermediate" with hydroxylamine or with acyl phosphatase could liberate most of the label as inorganic phosphate was supported by Schöner *et al.* (568), and by Chignell and Titus (124). However, the latter reasoned that the effect of hydroxylamine on the overall hydrolysis of ATP should be due to the blocking of the acceptor group and, if this were so, hydroxylamine should irreversibly inhibit the overall activity of the ATPase. None of these postulates agreed with the experimental data. Na^+,K^+-ATPase was not inhibited, even at concentrations of 0.8 M hydroxylamine. Furthermore, even in the presence of hydroxylamine, optimal rates of ATP hydrolysis depended on the presence of potassium ion. Chignell and Titus excluded, therefore, the role of the enzyme-bound acyl phosphate in the overall hydrolysis of ATP.

Bader and Broom, in 1967 (40), pointed out further complications of the effect of hydroxylamine on the mechanism of Na^+,K^+-ATPase, and Chignell and Titus also failed to obtain an inhibition of the enzyme system by hydroxylamine. Bader and Broom (40) suggested that calcium was required for hydroxylamine-induced inhibition.

Since the enzyme system could be inhib-

ited by incubation in the presence of calcium, ATP, and hydroxylamine, Bader suggested that hydroxylamine interfered with the active site of the enzyme by forming a stable hydroxamate of the intermediate. Bader also concluded that calcium in low concentrations may play a role in the activity of the Na^+,K^+-ATPase, possibly by regulating access of substrate or inhibitor to the enzyme.

Charnock *et al.* (117) also found a potassium-like effect of hydroxylamine on the hydrolysis of the phosphorylated intermediate. Because of the very complicated involvement of hydroxylamine, and the interaction with calcium and Na^+,K^+-ATPase, Bader *et al.* (45) carried out studies on the effect of hydroxylamine, divalent metals, chelators, and mercaptans on a possible control mechanism of Na^+,K^+-ATPase. According to Bader, if the divalent cation can be chelated, *i.e., by* 8-hydroxyquinoline chelating copper and EGTA-chelating calcium, hydroxylamine will have an effect. Furthermore, hydroxylamine in the presence of calcium did not split the phosphorylated intermediate of the ATPase system but seemed to prevent the sodium-dependent phosphorylation of the enzyme from $AT^{32}P$. In addition, the mercaptans, 2,3-dimercaptopropanol and 2-mercaptoethanol, were involved in the presence and absence of calcium. These drugs inhibited at low concentrations (10 to 100 μM) and activated at high concentrations, and the inhibition was increased in the presence of 30 μM calcium. Both mercaptans reversed calcium-induced inhibition of hydroxylamine at concentrations above 1 mM. N-ethylmaleimide (NEM) also reversed the calcium-induced inhibition of hydroxylamine at calcium concentrations between 10 and 100 μM, if calcium was incubated with the enzyme before hydroxylamine. The reactivation is apparently due to alkylation of a sulfhydryl group through which NEM inhibits the ATPase.

Bader *et al.* (45) also presented a possible control mechanism for the Na^+,K^+-ATPase. An intrinsic heavy metal is bound to a high affinity storage site on the enzyme. Hydroxylamine is known to increase or decrease the affinity of Na^+,K^+-ATPase for different essential cations. Hydroxylamine may attack on or near this proposed storage site, decreasing its affinity for the intrinsic metal. If a chelating agent is present, it would bind the intrinsic metal, and the metal would not be available for enzyme inhibition. However, if the chelating agent is bound by excess calcium, the endogenous metal will attach to its inhibitory site. This inhibitory site is without significance for the activity of Na^+,K^+-ATPase until it forms a complex with the intrinsic metal and thereby prevents sodium-stimulated phosphorylation. The intrinsic metal ion must, in some way, be released by hydroxylamine; the inhibitory site could be a sulfhydryl group. If this sulfhydryl group is not available, for instance, due to protection or due to alkylation by NEM, no inhibition results.

Thus, hydroxylamine has provided some insight into a possible control mechanism of Na^+,K^+-ATPase. Bader *et al.* (45) further suggest that the above model could be utilized *in vivo* to control the transport of sodium and potassium through the cell membrane by turning on or off different numbers of sites, with the intrinsic heavy metal as the "switch."

Sachs *et al.* (553) found additional conflicting results with the effect of hydroxylamine. They treated pig brain microsomes with *gamma*-labeled $AT^{32}P$ in the presence of magnesium plus sodium and found that the phosphorylation was inhibited by 80% or more when microsomes were preincubated with ATP plus hydroxylamine, whereas hydrolysis was only slightly reduced. They argued, therefore, that hydroxylamine was capable of reacting with the native enzyme and concluded that the breakdown of ATP must be limiting, allowing 80% inhibition of formation of "E-P" without affecting the overall hydrolysis of ATP.

Work by Formby (181) suggests that the hydroxylamine effect may be due to a physical alteration of microsomal membranes. Turbidity studies showed that the compound induced significant particle aggregation. Such an effect could explain enzyme inhibition, which may be due to limited activation and substrate access to specific sites.

F. Chlorpromazine

Chlorpromazine inhibits transport of a variety of substances across cell membranes (243). It has been suggested that a possible mechanism of action of chlorpromazine as a tranquilizer might involve a general "stabilizing" effect upon the cell membrane (243). Since that time, many workers have attempted to involve Na^+,K^+-ATPase in the mechanism of action of chlorpromazine as an antidepressant agent. Most studies, however, have indicated that Na^+,K^+-ATPase is relatively insensitive to chlorpromazine. Many investigators have reported that in the range of 0.1 to 0.5 mM (concentrations of drug presumed to exist in the brain after therapeutic doses), chlorpromazine inhibited less than 50% of the enzyme activity, although Squires (632) observed almost total inhibition when the enzyme and the inhibitor were first incubated together for 30 minutes at 37°C in the absence of sodium and potassium. Robinson *et al.* (533) showed an almost 50% inhibition of the enzyme by 0.1 mM chlorpromazine, and Davis and Brody (140) attempted to correlate the therapeutic efficiency of a series of phenothiazines with the degree of Na^+,K^+-ATPase inhibition. A thioxanthine derivative of chlorpromazine, chlorprothixene, a potent antipsychotic agent, also inhibits Na^+,K^+-ATPase (156).

In a series of interesting papers, Akera and Brody (7–9, 11) studied the *in vitro* mechanism of action of chlorpromazine-free radical on Na^+,K^+-ATPase inhibition and showed that inhibition of enzyme activity was minimal when the experiments were carried out in the dark. However, significant inhibition of Na^+K^+-ATPase activity was observed when the chlorpromazine-free radical was added to the enzyme mixture after it had been generated by one of three methods: ultraviolet (UV) radiation, chemical oxidation of chlorpromazine with sulfuric acid, or when the free radical was generated in the ATPase system assay in the simultaneous presence of peroxidase and hydrogen peroxide. The UV exposure I50 was 40 μM when carried out in the absence of enzyme. Even greater inhibition was observed when the chlorpromazine-free radical was generated by UV radiation in a mixture containing enzyme where the I50 was 3.5 μM. At that time, the authors concluded that a semiquinone radical of chlorpromazine rather than chlorpromazine itself could be responsible for the inhibition of the enzyme system *in vitro*.

Further experiments by this group showed that washing of the inhibited enzyme preparation with buffer did not restore the activity of the preparation, but treatment with various sulfhydryl-containing compounds could. It appeared kinetically that the inhibition by chlorpromazine-free radical was of either the pseudo-irreversible or the irreversible type. Inhibition of the enzyme activity by chlorpromazine-free radical and PCMB was reduced, while inhibition by ouabain for comparison was enhanced at low KCl concentrations. It is clear that the mechanism by which chlorpromazine-free radical and PCMB inhibits the Na^+,K^+-ATPase is different from that of ouabain. Enzyme treatment with drug inhibited the potassium phosphatase reaction in the same manner as the Na^+,K^+-ATPase, while the magnesium ATPase activity was unaffected.

The effects of the free radical were evaluated on the free sulfhydryl concentration and on the ability of the enzyme to bind tritiated ouabain. Enzyme treatment with chlorpromazine-free radical resulted in

inhibition of the activity, while proportional decreases in free sulfhydryl concentrations by chlorpromazine did not affect the sulfhydryl concentration or the enzyme activity. When the enzyme was incubated in the presence of ouabain with sodium plus magnesium plus ATP at 37°C for 20 minutes, irreversible inhibition of enzyme activity resulted, while free sulfhydryl concentration was not affected. Furthermore, prior treatment with ouabain did not prevent chlorpromazine-free radical from reacting with sulfhydryl groups of the enzyme preparation. Prior treatment with chlorpromazine-free radical also did not prevent ouabain, but did prevent PCMB, from reacting with the inhibited enzyme, thereby suggesting that chlorpromazine-free radical and PCMB may inhibit the enzyme by interacting with free sulfhydryl groups. It is clear that ouabain acts at a different site. The ability of chlorpromazine-free radical to inhibit ^{3}H-ouabain binding to the enzyme was less in magnitude than its ability to reduce ATPase activity. The chlorpromazine-free radical inhibits enzyme activity by interacting with sulfhydryl groups on the ATPase at a different site from ouabain. The chlorpromazine-free radical had less inhibitory effect when incubated with cholinesterase than with Na^+,K^+-ATPase and had little effect on either reduced nicotinamide dinucleotide (NADH)-cytochrome c reductase or the magnesium ATPase mentioned above. However, it must be noted that NADH-cytochrome c reductase contains reactive sulfhydryl groups, and that at the point Na^+,K^+-ATPase was totally inhibited by chlorpromazine, at least 25% of its sulfhydryl groups were unaffected. These experiments suggest that the semiquinone form of chlorpromazine is the active form of the tranquilizing agent. Akera and Brody (8, 9) calculated that 50 mg of chlorpromazine in a 70-kg man can elicit a pharmacological response. The estimated concentration of the drug in the brain would be around 10^{-6} to 10^{-5} M, assuming equal distribution of the drug in the brain. The inhibition of key metabolic pathways by these concentrations of chlorpromazine has not been demonstrated. However, it is also possible that the free radical could be generated at a specific site within the brain, and it has been shown that the free radical can be formed enzymatically by oxidative metabolism *in vivo* or nonenzymatically by photo-oxidation. A 1972 report (245) has suggested that the monodesmethyl metabolite of both chlorpromazine and promazine are more effective inhibitors of Na^+,K^+-ATPase than their parent compounds.

A 1970 report indicates that an examination of the electron-donating properties of phenothiazines and related compounds has been determined by measuring the maximum of the charge transfer band on complexation with tetracyanobenzene (74). It was concluded that phenothiazine did not differ significantly in this capacity from any of the other compounds tested, which had no tranquilizing effect, and that the N and not S is largely responsible for the observed moderate electron-donating properties. The results of these studies suggest that the unique role of S in tranquilizers may be associated with a molecular flexibility inferred on the molecule, thus allowing a better "fit" to the receptor. Akera and Brody (7) have shown that UV treatment of the drug in the presence of the enzyme augments inhibition. It also seems apparent that sulfhydryl groups may be involved in the action of the drug. The S could be essential for proper conformation involved in drug binding to its receptor and may be responsible for its affinity. However, the efficacy of the drug may, in turn, be due to the electron-donating capacity of the N group. Thus, after binding occurs, electron donating begins as the free radical is formed.

G. Fusidic Acid

Fusidic acid is a GTPase inhibitor which blocks the translocation of pepti-

dyl-tRNA to ribosomes. Matsui *et al.* (431) have shown that fusidic acid inhibits both Na^+,K^+-ATPase and the phosphatase with a K_i in each case of about 5×10^{-4} M (431). However, unlike ouabain (and other cardiac glycosides), the K_i of fusidic acid was not influenced by potassium. Furthermore, fusidic acid did not interfere with the binding of a cardiac glycoside to Na^+,K^+-ATPase when supported by ATP plus magnesium plus sodium [(complex I), see section V E]. However, glycoside binding supported by magnesium plus inorganic phosphate (complex II) was slightly but significantly depressed by high concentrations of fusidic acid. Furthermore, sodium-dependent phosphorylation of the protein by $ATP^{32}P$ was unaffected by the compound, while ouabain-stimulated phosphorylation by ^{32}P was significantly inhibited by the drug. The suggestion has been made by a number of workers that the ouabain binding conformations are significantly different, depending on ligand conditions, *i.e.*, complex I and complex II (see section V E). It is possible that this compound may be able to *distinguish* between complexes I and II.

H. Dimethylsulfoxide (DMSO)

In 1969, Burgess *et al.* (96) studied the effects of DMSO, DMF, and DMA (all dipolar aprotic solvents) on a number of membrane systems, including canine myocardial Na^+,K^+-ATPase. They found a 57% inhibition of the enzyme at 20% (v/v) of DMSO but also a 41% inhibition of the accompanying magnesium ATPase, with no alteration of the effect of ouabain on the Na^+,K^+-ATPase-stimulated portion. They did not detail a specific mechanism, but the DMSO effect was completely reversible while the DMF and DMA effects were not.

In 1970, Mayer and Avi-Dor (439) found that both glycerol and DMSO reversibly (by dilution) inhibited Na^+,K^+-ATPase but stimulated the potassium phosphatase. They suggested that the activation was more complex than the ATP plus sodium-induced activation of potassium phosphatase, which appears only to increase the enzyme affinity for potassium, while the solvents appear to increase the V_{max} of the reaction as well as the affinity for potassium. However, their suggestion was that the increase in binding affinity of the phosphatase towards potassium was probably connected with the solvation of potassium rather than an effect on the active site. It also was concluded that DMSO had a specific effect on the active site of the enzyme, since it enhanced the inhibitory effect of ouabain on the potassium phosphatase. If the compound were simply enhancing potassium binding, it would probably decrease the affinity for ouabain due to the well known potassium-ouabain antagonism. These investigators did not study the interaction of DMSO on ouabain-Na^+,K^+-ATPase interaction.

Robinson (541) reiterated that the disparate effect on the ATPase (inhibition) and phosphatase (stimulation), respectively, seemed to be specific since two other lipolytic agents, Lubrol-W and propanol, did not show the effect. Along with ATPase inhibition, he showed decreases in V_{max} and K_m for both ATP and sodium, a decrease in V_{max} for potassium but an increase in the K_m for potassium, suggesting that DMSO affected reaction stages that exist after interaction of the enzyme with sodium plus ATP, *i.e.*, that the reaction sequence is altered subsequent to phosphorylation. The stimulating effect of DMSO on the potassium phosphatase supports this since it reflects an increase in affinity for the substrate, *i.e.*, V_{max} is unchanged but K_m is decreased. The conclusion reached is that DMSO facilitates the availability of the potassium phosphatase substrate to the active site, while hindering the hydrolysis of the normal substrate, ATP. Thus, DMSO must hinder the conversion of E_1-P to E_2-P and subsequent potassium hydrolysis, if E_1-P is stimulated by ATP plus sodium.

The drug probably enhances E_2-P formation only if E_2-P is formed *via* a phosphatase substrate.

Albers and Koval (19) studied further the effect of DMSO and glycerol on the partial reactions of the ATPase. As found earlier, Na^+,K^+-ATPase was reversibly inhibited, potassium-PNPPase was reversibly stimulated, but potassium-acetylphosphatase was inhibited by concentrations of DMSO which stimulated the potassium PNPPase. Furthermore, DMSO had no effect on E-P formation from magnesium plus ATP plus sodium, nor could its inhibitory effect on Na^+,K^+-ATPase be explained by inhibition of potassium-induced hydrolysis, since DMSO did not inhibit the potassium-induced decrease in phosphoryl enzyme to a large enough extent. The compound, however, significantly inhibited the sodium dependent (low magnesium) ATP-ADP phosphotransferase (90% inhibition by 25% DMSO). The sodium dependent ATP-ADP phosphotransferase reaction is presumably a measure of the steady-state rate of the protein phosphorylation reaction, and the effect of DMSO on it could mirror an effect on the phosphokinase reaction. These workers suggested that the effects of both DMSO and glycerol could result from an interference with the interaction of the phosphoryl acceptor with proximal kinase or distal phosphatase sites. In view of previous work, substrate (ATP) inaccessibility to the phosphatase site would increase PNPP substrate availability to its hydrolytic site. The data also suggest that acetyl phosphate is hydrolyzed *via* a site intermediate between the phosphatase and the ATPase sites, since it is stimulated by potassium and inhibited by DMSO.

I. Phlorizin

Phlorizin, a well known inhibitor of sodium-dependent glucose transport (577) also has complex actions on the Na^+,K^+-ATPase. It stimulates potassium phosphatase but inhibits Na^+,K^+-ATPase (194). Robinson (536) found that the concentration of phlorizin required for half-maximum stimulation of the phosphatase was 0.05 mM, and the concentration required for half-maximum inhibition of the Na^+,K^+-ATPase was 0.06 mM. Phloretin, a hydroxylated aromatic without a β-D-glucose on the benzene ring, also stimulated the phosphatase. Phlorizin (0.05 mM) decreased the concentration of potassium required for half-maximum stimulation of the phosphatase from 1.92 to 1.17 mM but had no effect on V_{max}. Sodium inhibited the phosphatase, and phlorizin increased the K_i for sodium from 6 to 12 mM. The compound decreased the K_m for potassium for Na^+,K^+-ATPase from 0.74 to 0.48 mM and increased the K_m for sodium from 5.0 to 10.5 mM. The positive co-operativity of sodium was converted to a negative allosteric effect, and the compound inhibited the sodium-dependent phosphorylation of the enzyme. Robinson suggested that phlorizin acts as an allosteric modifier in a similar way in both systems, increasing and decreasing the affinity for potassium and for sodium, respectively.

When the overall effects of phlorizin are compared to those of DMSO, there is a similarity, *i.e.*, inhibition of Na^+,K^+-ATPase and stimulation of potassium phosphatase. However, closer examination of various kinetic parameters reveals significant differences between the two compounds. Most important is the fact that DMSO alters the V_{max} of the ATPase (decrease) and phosphatase (increase) with a concomitant decrease in substrate K_m. Phlorizin has no effect on V_{max} of either reaction but affects the affinity of the cationic modifiers. Thus, as Robinson points out (see above), DMSO affects the "rate of substrate entry" on both reactions, while phlorizin does not affect the active site, only its cationic activation.

J. Ethanol

In 1971, Järnefelt (309) showed that ethanol inhibits brain Na^+,K^+-ATPase *in vitro*. A number of workers have suggested that interaction of the drug with

the enzymes *in vivo* may be partially responsible for the effects of chronic ethanol administration. Ethanol can inhibit active transport of sodium and potassium across cell membranes. Since red blood cells of chronic alcoholics may show an increased capacity for cation transport, Israel *et al.* (301) proposed that adaptive changes in the transport system may be responsible for the effects of chronic ingestion. They reported an increase in brain Na^+,K^+-ATPase activity of rats chronically treated with ethanol (303).

Akera *et al.* (16) found that despite the development of tolerance to the depressant effects of ethanol on behavior, neither the Na^+,K^+-ATPase activity nor the ^3H-ouabain binding in brain homogenates or microsomal fractions were altered during either treatment or withdrawal periods. With cat brain, Knox *et al.* (353) showed that chronic treatment with ethanol induced small but significant increases in Na^+,K^+-ATPase activity in the frontal cortex, association cortex, and hippocampus but not in the caudate nucleus, reticular formation, or amygdala.

Thus, at present, no definitive conclusion can be reached regarding the *in vivo* consequences of chronic administration of ethanol in relation to Na^+,K^+-ATPase, despite the well known *in vitro* inhibition of the enzyme. It appears unlikely that if alteration of the pump does occur as a result of chronic administration of ethanol that it is due directly to a specific effect on Na^+,K^+-ATPase.

K. Sulfhydryl Reagents

The inhibiting effect of sulfhydryl (SH) reagents on Na^+,K^+-ATPase has been well known since Skou and Hilberg (620) demonstrated inhibition of the overall enzymatic activity by both NEM and PCMB and protection by ATP. Shortly after, Fahn *et al.* (170) examined the effect of NEM more closely and showed that the drug specifically inhibited the transphosphorylation of the terminal phosphate from ATP and ADP [exchange reaction, Skou (610)]. However, the NEM inhibitory effect which required magnesium could be restored by the addition of sodium, while the untreated particles were either unaffected or only slightly inhibited by sodium.

Fahn *et al.* (173) expanded these experiments with an additional study of the effect of NEM and the other SH reagents on the sodium-stimulated ATP-ADP exchange reaction. They previously showed that lowering the magnesium concentration from 3 mM to 0.3 mM induced a sodium-stimulated ATP-ADP exchange, whereas at higher magnesium levels, sodium had little or no effect (171). Treatment with NEM or N-butylmaleimide (but not PCMB or PCMBS) produced the same overall effect on the exchange as lowering the magnesium level, *i.e.*, sodium stimulation. Furthermore, they showed additional similarities between the NEM-treated microsomal sodium-dependent ADP-ATP exchange and the "native" microsomal exchange (low magnesium) reaction. Both reactions required adenine nucleotides, sodium ions, and a divalent cation, magnesium, and both were inhibited by calcium, ouabain, and potassium but were unaffected by oligomycin.

Post *et al.* (499) determined that NEM treatment "prevented" conversion of E_1-P to E_2-P. Their data were similar to that of Fahn *et al.* (173), namely that NEM-treated microsomes or low magnesium could reveal a sodium-stimulated ATP-ADP exchange, presumably occurring through E_1-P, and the native Na^+,K^+-ATPase demonstrated a potassium-dependent dephosphorylation, presumably occurring through E_2-P. Thus, NEM may "freeze" or occlude the enzyme in an "E_1-P" conformation.

$$Mg\cdot ATP + E \overset{Na}{\rightleftharpoons} E_1\text{-}P + ADP$$

$$E_1\text{-}P \overset{Mg}{\rightarrow} E_2\text{-}P \quad (\text{NEM blocks})$$

$$E_2\text{-}P \overset{K^+}{\rightarrow} E_2 + P_i$$

Banerjee *et al.* (58,59) showed that the effects of NEM depended upon both ligand conditions and inhibitor concentration and suggested both that NEM reacted at two different sites on the enzyme and that the reactivity depended on the particular conformational state induced by ligand conditions (*i.e.*, E_1 or E_2). NEM treatment of the kidney microsomes in the presence of ATP with either sodium or potassium stimulated exchange, thereby suggesting that ATP binds to the E_1 form. The E_2 form induced by magnesium alone could be converted back to E_1 by ATP. This suggested that phosphorylation was necessary to induce the E_2 conformation and not just ATP occupation of a modifier site (see section V).

Hart and Titus (254, 255) studied specific ligand influences on different NEM binding conformations, by the use of double-labeled NEM-^{14}C and NEM-^{3}H. They treated identical aliquots of a partially purified preparation from the outer medulla of rabbit kidney with NEM-^{14}C and NEM-^{3}H in the absence and presence of various combinations of ligands (sodium, magnesium, ATP, potassium) and inhibitor drugs (ouabain and oligomycin). The pairs were then combined and submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis. When no physiological ligands were present (E_1), a 98,000 dalton peptide containing sulfhydryl groups was protected by sodium plus ATP. In the presence of magnesium (E_2), sulfhydryl groups on the same 98,000 dalton peptide were protected by potassium or ouabain. However, when a phosphorylated state was induced by sodium plus magnesium plus ATP, additional sulfhydryl groups were exposed to the alkylation by NEM, and both ouabain and oligomycin further increased the exposure of the sulfhydryl groups during this phosphorylation. Ouabain, however, protected sulfhydryl group reactivity when phosphorylation occurred in the presence of magnesium plus inorganic phosphate which is reminiscent of an earlier suggestion of Akera and Brody (10) and Allen *et al.* (24) that ouabain binding conformations of the enzyme may be different depending on the ligand conditions of phosphorylation (*i.e.*, ATP plus magnesium plus sodium or magnesium plus inorganic phosphate). These data directly involve different sulfhydryl groups reactivity in designation of particular conformations of the enzyme, *i.e.*, E_1 or E_2, depending on ligand conditions.

An interesting physiological effect of sulfhydryl reagents has been documented by Fromm *et al.* (190) who showed that NEM induced positive inotropism in cardiac tissue. This work was extended to PCMBS, which, by having one sulfhydryl combining site, is similar to NEM (although as noted above, it has different effects on partial ATPase reactions than does NEM). Fromm and Probstfield (191) showed that at lower concentrations, PCMBS significantly increased contractility of guinea pig left atria, which was not blocked by propranolol. The inotropic effect was biphasic, since at higher drug levels, a decrease in contractility always occurred. A similar type of effect was noted by these workers with ethacrynic acid (191). It is possible, therefore, that these agents may induce positive inotropism through a Na^+,K^+-ATPase inhibition, a suggested mechanism of action of the cardiac glycosides (see section VIII). However, these preliminary experiments are in no way conclusive.

If NEM "occludes" the Na^+,K^+-ATPase in the E_1-P conformation, and ouabain affects the enzyme by combining either with E_2-P or KE_2, then ouabain should not bind to an NEM-treated enzyme. We treated a heart and kidney Na^+,K^+-ATPase with NEM and with oligomycin under conditions in which enzyme activity was significantly inhibited. No effect on the binding of total ^{3}H-ouabain was observed. This surprising finding has been independently observed by Hegyvary (personal communication) whose

data and statement are reproduced with his permission below:

Pseudo first-order rate constants for ^{3}H-ouabain binding under different ligand effectors

Enzyme	$k \times 10^2$ (sec^{-1})
Native	6.4
NEM-treated	4.1
Oligomycin-treated	7.1

"Thus, E_1-P does bind ^{3}H-ouabain . . . ratio of moles of ouabain binding sites to moles of E_1-P was 1:1 in (either) native or NEM or oligomycin-treated membranes. NEM or oligomycin-treated membranes can be phosphorylated from $^{32}P_i$. . . . These findings clearly contradict the dogma that E_1-P is a necessary precursor of E_2-P . . . and that ouabain binds to E_2-P preferentially. . . ."

We agree with Hegyvary's new findings and concept with regard to ouabain binding to intermediary conformations associated with the Na^+,K^+-ATPase.

L. Suramin

Suramin, a trypanocidal drug, has been shown to inhibit Na^+,K^+-ATPase (575). However, it may have significant utility in studies of the actual pump since it is relatively impermeable to the red cell membrane, and its structure (*i.e.*, naphthalene trisulphonate groups) resembles certain fluorescent probes (186, 187).

In 1973, Fortes *et al.* (186) studied the effect in more detail on various aspects of the sodium pump and associated enzymatic reactions of red blood cells. They were able to show a significant inhibition of both human red cell ghost Na^+,K^+-ATPase, with a K_i of about 50 μM, and the enzyme from electric eel microsomes (80 μM). Furthermore, the effect of up to 1 mM suramin could easily be reversed by a simple wash, and it was concluded that since the drug did not cause inhibition of another membrane enzyme, acetylcholinesterase, its effect may be site-specific, even though ouabain-insensitive red blood cell ATPase was also inhibited ($K_i \sim 15$ μM). The presence of up to 1 mM of suramin in the medium had no effect on pump Na^+,K^+ fluxes in either red cells or hypotonically resealed ghosts.

Despite technical difficulties involving binding of suramin to hemoglobin, the drug appeared to inhibit ouabain sensitive sodium efflux into sodium media containing either 0 or 10 mM potassium in ghosts hemolysed and resealed in the presence of 50 μM suramin.

In addition, the drug inhibited potassium phosphatase, and ATP plus magnesium plus sodium and magnesium plus inorganic phosphate supported ^{3}H-ouabain binding to red blood cell ghosts. While still unclear, it is suggested that these reactions occur on the outside of the cell membrane. Fortes *et al.* (186) concluded that suramin may be useful in studies involving the sodium pump since it affects ATPase, potassium-phosphatase and ^{3}H-ouabain, yet seems to inhibit pump activity from the inside only.

M. Adrenal Steroids

Extensive work has been carried out to clarify the role of Na^+,K^+-ATPase in urine concentrating and diluting mechanism. Many workers have shown a significantly higher enzyme content in renal medullary areas than in cortical areas, although there appears to be no significant kinetic differences between the regional enzyme preparations. Indeed, many workers have begun using medullary tissue as a prime source of enzyme for extensive purification studies (319, 377) (see section IV).

It is clear that inhibition of Na^+,K^+-ATPase by cardiac glycoside perfusion can cause a significant diuresis (427). However, the role of the enzyme in basal urine concentrating and diluting mechanisms is still unclear. Some workers have suggested that certain diuretics may exert their therapeutic effect through inhibition of this

enzyme system (see section VII). Since the enzyme may be involved in kidney sodium (and potassium) transport, it is reasonable to assume an adaptive role as well.

In 1966, Landon *et al.* (374) showed that renal Na^+,K^+-ATPase activity was markedly decreased when isolated from adrenalectomized rats and that administration of aldosterone subsequent to surgery could prevent the decrease. Since the level of steroid required was far in excess of that necessary to produce an electrolyte response in adrenalectomized animals, these workers suggested that no correlation existed between the salt-retaining activity of aldosterone and the maintenance of Na^+,K^+-ATPase activity. At the same time, Chignell *et al.* (121) and Chignell and Titus (122) showed that while there was a decrease in Na^+,K^+-ATPase activity after adrenalectomy, it was not due to the alteration of K_m values for ATP, sodium or potassium. Furthermore, they could *not* show recovery of enzyme activity with aldosterone but could with corticosterone, the main steroid secreted by the rat adrenal.

Jørgensen (316), however, reproduced the findings of Landon *et al.* (374) in that aldosterone could counteract the decrease in enzyme activity resulting from adrenalectomy. He also found that the decrease was more marked in the outer medulla than in the outer cortex. He concluded that although aldosterone could partially reverse the drop in Na^+,K^+-ATPase activity, the change occurred *subsequent* to the reversal of the ionic effects caused by the adrenalectomy. He suggested that aldosterone may regulate Na^+,K^+-ATPase activity by first increasing internal sodium concentration which results in increased sodium binding to internal enzyme sites. This may be feasible, since postadrenalectomy $[sodium]_i$ is 14 mM, and $[potassium]_i$ is 136 mM; the saturation of the sodium site by sodium is low (10–20%) at those concentrations and increases sharply with increases in sodium. This is called the acute phase of adaptation. The chronic adaptation phase occurs some 24 hours after repeated injections of aldosterone and results from the sustained increase in the functional demands on the enzyme. Thus, it is clear that while a specific adaptive role of Na^+,K^+-ATPase to aldosterone administration is possible, its functional aspect, *i.e.*, role in Na^+,K^+ transport, is still unclear.

Edelman (157) has suggested that the induction of protein synthesis at the transcriptional level may mediate the regulation of sodium transport by aldosterone. He has been studying an aldosterone binding protein (ABP) of rat kidney. However, Hendler *et al.* (261) have shown that effects of adrenalectomy and subsequent administration of methylprednisolone are specific for Na^+,K^+-ATPase, while no changes were observed in either 5′-nucleotidase or adenyl cyclase, two additional membrane-bound enzyme systems. They found a significant decrease only in the renal medullary Na^+,K^+-ATPase and not in the cortical enzyme. Furthermore, as other workers have also shown, sodium deprivation did not affect the Na^+,K^+-ATPase, even though circulating aldosterone levels were probably high.

An intriguing result was reported by Silva *et al.* (603) in response to potassium adaptation in the *absence* of adrenal glands. Large increases in dietary potassium intake increased both medullary and cortical Na^+,K^+-ATPase levels, while enzyme levels in brain, liver, and muscle were unaffected. In this study, it is clear that adrenal hormones may not play a direct role in Na^+,K^+-ATPase synthesis, but the potassium increase in some way may alter renal Na^+,K^+-ATPase levels. In general, the interactions of Na^+,K^+-ATPase with sodium, potassium, and aldosterone levels are unexplained.

In 1975, however, Schmidt *et al.* (563) re-examined the possibility that aldoster-

one does induce synthesis of Na^+,K^+-ATPase. They used a very sensitive microassay for renal tubular ATPase and showed that administration of aldosterone did result in an increased enzyme activity suggestive of synthesis. Pretreatment with inhibitors of protein synthesis prevented the aldosterone-induced changes in the Na^+,K^+-ATPase. It appears that the possibility of a direct involvement of aldosterone in control of Na^+,K^+-ATPase requires reconsideration.

N. Stimulation of Na^+,K^+-ATPase

For a number of years, it had been suggested that ouabain could stimulate Na^+,K^+-ATPase under certain conditions (93, 135, 390, 477, 521). The majority of laboratories working in the area have indicated that these results were inconclusive, yet the effect may be real under very specific circumstances with specific preparations. For example, Palmer *et al.* (477) showed a biphasic effect (inhibition at high concentrations, 10^{-4} to 10^{-7}, and stimulation at low, 10^{-7} to 10^{-15} M) on a chicken kidney and rabbit brain Na^+,K^+-ATPase preparation. However, potassium did not affect the stimulatory effect of ouabain but did, in the usual way, partially prevent the inhibition. Dal Pra and his co-workers (135) have shown a stimulatory effect of relatively high (10^{-5} M) concentrations of ouabain on frog heart microsomal Na^+,K^+-ATPase and tried to correlate this to the uptake of ^{3}H-ouabain into isolated frog hearts.

Because of the lack of reproducibility of the stimulatory effect of cardiac glycosides on Na^+,K^+-ATPase by other laboratories, further discussion of the effect will be limited here. However, since other compounds have been shown to stimulate the enzyme, the ouabain stimulatory effect, although unresolved, is still of some interest even though the significance of a stimulatory effect is obscure. Schwartz, in ref. 702a, postulated that an enhancement of Na^+,K^+-ATPase by digitalis could be related to a mechanism of digitalis action. Upon reflection, however, he is unable to relate this response to a positive inotropic action. We have not observed stimulation of Na^+,K^+-ATPase from cardiac muscle by any concentration of cardiac glycoside. Furthermore, Repke (522) does not feel that his initial observation has relevance to a mechanism of digitalis action.

In 1975, Bihler (74a) and Bihler and Sawh (74b) reported that very low concentrations of ouabain (10^{-9} M) stimulate the sodium "pump" associated with rat atria and with rat hemidiaphragm. It is noteworthy that this concentration of ouabain did not affect cardiac contractility.

There is recent interest in other agents that may stimulate Na^+,K^+-ATPase activity in the presence of EGTA or absence of added calcium. Bader *et al.* (45) demonstrated a slight stimulation of the enzyme by hydroxylamine. The most significant effect noted was a decrease in the K_m for sodium for the enzyme. These results were explained by the possible presence of an intrinsic heavy metal ion which could bind to a storage site. When the enzyme was treated with hydroxylamine, the heavy metal was then released from the storage site and was bound to an inhibitory site. If a chelator was present, the metal would not bind to the inhibitory site, but added calcium would allow inhibition to occur, since it would tie up the chelator.

The idea of an endogenous heavy metal in controlling enzyme activity conformation is now being explored in detail. Ting-Beall and Wells (660) and Ting-Beall *et al.* (659) suggested that copper and calcium were involved in the activity of the enzyme (482, 483). However, these workers were more concerned with *added* copper than with the endogenous ion. Their concept that both EDTA and EGTA were stimulatory because of calcium chelation has been disputed by Wallick *et al.* (684) in this laboratory who showed that

at physiological pH, EDTA binds only magnesium which induces an inhibition subsequently overcome by addition of more magnesium. EGTA, on the other hand, did stimulate the enzyme but only when added before the reaction was begun (*i.e.*, prior to enzyme addition). When added during enzymatic turnover (the reaction was continually monitored by a spectrophotometric measurement), stimulation did not occur, *i.e.*, after enzyme turnover had begun, EGTA did not stimulate.

A number of laboratories have reported that certain amino acids stimulate Na^+,K^+-ATPase from Ehrlich ascities cells [Forte *et al.* (183)], rabbit kidney medulla [Specht and Robinson (631)], and calf brain [Wallick *et al.* (684)]. Forte *et al.* concluded that a tightly bound metal exerts a controlling effect on the enzyme, while Specht and Robinson favored metal contamination of the reagents as being the cause of the "endogenous" inhibition of the enzyme. It is apparent from these studies that trace metals endogenous to the enzyme itself have not been eliminated.

A 1974 paper (391a) reports the stimulation of vascular smooth muscle Na^+,K^+-ATPase by a large number of "vasodilating" agents: Minoxidil, diazoxide, hydralazine, isoproterenol, norepinephrine epinephrine, prostaglandin E_1, prostaglandin E_2, and prostaglandin A_2. The first three compounds are indeed vasodilators, but in each case the indicated concentration (1 μM) which stimulated activity is different from the reputed dose *in vivo*. The catecholamine agents probably are not vasodilators and the prostaglandins have varying effects on vascular contractility. Furthermore, there are discrepancies regarding minoxidil which in one case stimulates the enzyme by 250% at a 1 μM concentration and has no effect in another case at 10 μM (2 μg/ml). In addition, there are difficulties in interpretation of the data, since these authors state in one place that basal MgATPase (in the absence of $Na^+ + K^+$) is 2.7 μmol P_i/mg/hour and in another place 6.5 μmol P_i/mg/hour. Therefore, although the authors give their total control ATPase activity as 10.5, we calculate from their own data that the control Na^+,K^+-ATPase varies between 7.8 and 4.0. As a result, it is difficult to conclude that any of these compounds do stimulate the enzyme. Assessment of these data are important, since enzyme stimulation occurs only in limited cases. Perhaps there is a significant difference between arterial Na^+,K^+-ATPase and all others, but this paper is not critical or detailed enough for such conclusions.

This section on modifying agents was not meant to be inclusive. We have omitted many compounds which have been shown to have no effect on Na^+,K^+-ATPase as well as some compounds that may inhibit, but are either of no current clinical interest or are not useful chemical tools. The compounds included either may be of some therapeutic value and may exert their effect *via* Na^+,K^+-ATPase interaction, or may possibly function as "tools" in the dissection of mechanism. Some additional compounds are briefly discussed in table 3.

VIII. Na^+,K^+-ATPase: Pharmacological Receptor for Digitalis?

A. Inhibition in Situ of Na^+,K^+-ATPase

The question we are concerned with in this section is whether the binding site(s) for cardiac glycosides associated with the Na^+,K^+-ATPase is the receptor responsible for the therapeutic and/or toxicological effects of digitalis at the tissue level or whether the binding is unrelated to any action *in situ* of digitalis.

Variations in the sensitivity of various species of animals to cardiac glycosides has been known for a number of years (144). Human, beef, and dog hearts are sensitive to the drugs, whereas the rat heart is insensitive, and the guinea pig and rabbit

TABLE 3

Additional modifiers of Na^+,K^+-ATPase

Modifier	Definition and/or Discussion[a]
1. Amiodarone	An antiarrhythmic agent with no inotropic action. It inhibits guinea pig cardiac Na^+, K^+-ATPase [a] with a K_I of 0.065 mM, but is without effect on magnesium ATPase. The authors conclude that it is a competitive inhibitor with ATP (91).
2. Ascorbic acid	A general inhibitor of both Na^+,K^+-ATPase and magnesium ATPase [Glynn (222)]. Inagaki (293) showed that 10^{-4} M inhibited rat brain magnesium ATPase about 35% but inhibited Na^+,K^+-ATPase about 80%. Frey *et al.* (188) found significant inhibition of rat, guinea pig, and beef brain, but no effect on rat heart, beef heart, and human red cells. K_I for rat brain Na^+,K^+-ATPase and potassium phosphatase was 5×10^{-5} M, with maximum inhibition of 0.5 mM. The inhibition was essentially irreversible.
3. Azasteroids	(Quindonium bromide and analogs). Brown (94) showed that the compounds that were active as inotropic agents inhibited rabbit cardiac Na^+,K^+-ATPase, whereas the inactive compounds had no effect. He suggested a similarity of mechanism of action between cardiac glycosides and azasteroids. For example, the *cis*-isomer of quindonium bromide had both an inhibitory effect on the enzyme and inotropic effect on intact heart, while the *trans*-isomer had neither.
4. Basic proteins	Schwartz and Lasetar (576) have suggested that a crude factor isolated from most tissues and which has a preferential effect on certain aspects of Na^+, K^+-ATPase may act similarly to partially purified *histone, viz.*, to inhibit the magnesium ATPase component (ouabain-insensitive). Yoshida *et al.* (726, 727) have shown a specific inhibitory effect of protamine during preincubation with the enzyme, which could be overcome by the presence of either sodium or potassium. Because of this effect and protamine inhibition of L-dopa transport in brain slices, the authors suggested involvement of Na^+,K^+-ATPase in L-dopa uptake.
5. Potassium canrenoate	The compound is a major metabolite of spironolactone and has been reported to reverse cardiac glycoside-induced arrhythmias (719, 720). Baskin *et al.* (63), however, were unable to show any effect on ouabain-induced toxicity in the guinea pig heart Langendorff preparation, although alone the drug did depress myocardial contractility. The drug had no effect on any parameter of rat brain Na^+,K^+-ATPase.
6. Carbodiimides	(R − N = C = N − R). These compounds are carboxyl group activating agents, but the effect they have on Na^+,K^+-ATPase may be different depending upon the relative lipid or water solubility of the substituent groups. Godin and Schrier (230a), with a water soluble derivative, EDAC, showed that both magnesium ATPase and Na^+,K^+-ATPase activities of red cell ghosts were inhibited by EDAC. Furthermore, ATP as well as nonsubstrate nucleotides protected against the inhibition. Alterations in the molecule also allowed for potassium phosphatase inhibition; these workers concluded that the carbodiimide attack on the erythrocyte membrane may well involve a selective structural alteration. Schöner and Schmidt (569) and Schöner *et al.* (570), however, used DCCD, a lipid soluble derivative and found different effects on ox brain cortical Na^+,K^+-ATPase. They found temperature-dependent inhibitory effects

TABLE 3—*Continued*

Modifier	Definition and/or Discussion
	which could be blocked by sodium. Furthermore, DCCD inhibited sodium-dependent phosphorylation. The sodium-protected sites could be labeled and partially purified. These authors concluded that DCCD acts at very specific membrane areas near or at the active site. Contessa and Bruni (129) found very little effect of DCCD on partially purified Na^+,K^+-ATPase from rabbit kidney.
7. Insecticides	At approximately the same time, two laboratories, Koch *et al.* (354, 355) and Matsumura and Narahashi (436, 437), showed rabbit and rat brain Na^+,K^+-ATPase could be inhibited by DDT and chlorinated hydrocarbons. This is consistent with a suggested mechanism of action of the drugs as well as a mechanism of toxicity. Both Davis and Wedemeyer (141) and Janicki and Kinter (306) working with various tissues of teleosts suggested that Na^+,K^+-ATPase may be involved in chronic exposure; they quoted a concentration of DDT found in fish of 2.75 ppm, a drug level which gave 25% inhibition of the intestinal mucosa of the white flounder. A more detailed study of insecticides and some analogs on a partially purified rat brain preparation carried out by Akera *et al.* (12) revealed that Na^+,K^+-ATPase inhibition was not specific to the active form P,P′-DDT. Less active analogs such as o,P′-DDT, and P,P′-DDE were equally potent as enzyme inhibitors. Furthermore, these workers also showed no temperature effect with chlordane and reduced effects with the DDT group at lower temperatures. They did indicate, however, that a lack of significant relationship between enzyme inhibition and insecticide activity did not rule out an *in vivo* relation, since the latter depends on pharmacological disposition and perhaps species selectivity. Sachs *et al.* (554a) showed that DFP (as well as methane sulfonyl chloride and diethyl *p*-nitrophenyl phosphate) inhibit Na^+,K^+-ATPase, and specifically proposed a mechanism involving sulfonation or phosphorylation of a serine residue present in the enzyme. This was disputed for DFP by Lahiri and Wilson (372) who noticed a lag period before development of inhibition. From this observation, they suggested that the inhibition may be due to F^- contamination and continual hydrolysis of DFP. They simultaneously measured F^- concentration occurring from DFP hydrolysis with enzyme inhibition and found that the two parameters were directly related. Furthermore, various characteristics of DFP inhibition were similar to those observed for F^- inhibition alone and both required magnesium. ^{14}C-DFP labeling of the enzymes occurred to nonspecific proteins in the absence of magnesium as well as in its presence. Even though insecticides may inhibit Na^+,K^+-ATPase *in vitro*, no definite conclusions can be reached regarding their *in vivo* mechanism of action.
8. Erythrophleum alkaloids (erythrophleine and cassaine)	Bonting *et al.* (81) compared the effects of these compounds to ouabain on Na^+,K^+-ATPase activity of a number of different tissue sources because of certain similarities found in inhibition of active potassium transport in red blood cells (329). These workers found that the inhibition curves and I50 values for these compounds were very similar to those for ouabain. They further showed a similarity between the ability of potassium to overcome or prevent the enzyme inhibition caused by erythrophleum and ouabain. In view of the earlier demonstrated positive inotropic action of the erythrophleum alkaloids (418) it would be of considerable interest to study further these compounds on more highly purified preparations.

TABLE 3—*Continued*

Modifier	Definition and/or Discussion
9. Diketocoriolin B	This is a sesquiterpene antitumor antibiotic which was shown to inhibit Na^+,K^+-ATPase activity of Yoshida sarcoma cells, rat brain, rat liver, and rat red cells (363, 364). The compound did not, however, inhibit potassium phosphatase activities from rat brain or erythrocyte ghost, nor did it inhibit residual magnesium ATPase or 5′-nucleotidase activity from these tissues. It was also of interest that these workers demonstrated inhibition of DNP-stimulated mitochondrial ATPase as well. In later work, Kunimoto and co-workers (363, 364) examined the mechanism of diketocoriolin B inhibition. They showed that it appeared to compete with ATP for a single binding site, while inhibition with respect to sodium and potassium was uncompetitive. These results could be obtained with particulate Na^+,K^+-ATPase of Yoshida sarcoma cells, rat brain, and a DOC-solubilized enzyme from the latter tissue. With the particulate enzyme, inhibition was reduced by phosphatidyl serine and with the solubilized enzyme, the inhibition was actually reversed. However, the use of particulate and soluble Na^+,K^+-ATPase revealed differences in the mode of drug inhibition. Two moles of diketocoriolin reacted with the soluble enzyme and only one with the particulate preparation and the potassium phosphatase of the soluble preparation required phospholipid for activity and was sensitive to the drug, while the potassium phosphatase of the particulate preparation did not require phospholipid and was insensitive to the drug. These workers concluded that diketocoriolin B is a unique Na^+,K^+-ATPase inhibitor in that it competitively interferes with the ATP binding site, presumably by competing with phospholipid. Furthermore, they felt that their data suggested a scheme for hydrolysis of ATP which required a specific phospholipid to be bound in order to achieve initial ATP binding to the enzyme.
10. AY 22,241 (Actodigin)	In a search for shorter acting and more readily reversible cardiac glycosides, Pastelin and Mendez (479) described the effects of a semisynthetic glycoside, AY 22,241. This compound possesses standard glycoside inotropic effects, but differs in its effects on Purkinje fiber significantly in that toxicity develops very quickly, but is readily reversible. Gliklich *et al.* in 1973 (217) confirmed these data with microelectrode techniques. In 1975, studies from this laboratory (Thomas *et al.*) show that this compound may indeed be a more readily reversible inhibitor of Na^+,K^+-ATPase. Wallick *et al.* (685) and Yoda (721) have shown that genins (aglycones) inhibit Na^+,K^+-ATPase in a reversible manner. For example, the inhibition is *not* time dependent as with the complete glycoside. The inhibition by AY 22,241 is also more readily reversible, suggesting that there is reason to involve directly the cardiac effect and Na^+,K^+-ATPase.
11. Harmaline	Canessa *et al.* (105a) reported that this hallucinogen is an inhibitor of Na^+,K^+-ATPase, specifically the Na^+-dependent phosphorylation step. The concentrations were high, 0.3 to 3 mM and these same amounts blocked sodium efflux from squid giant axons. It is thought that this drug is a competitive inhibitor at sodium sites and it can be used as a "tool" to show that entrance of sodium into the enzyme system may occur prior to formation of E-P.

[a] The abbreviations used are: ATP, adenosine triphosphate; dopa, 3,4-dihydroxyphenylalanine; EDAC, 1-ethyl-e-(3 dimethyl-aminopropyl) carbodiimide hydrochloride; DCCD, N,N′-dicyclohexyl carbodiimide; DDT, dichlorodiphenyltrichloroethane; DDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene; DFP, diisopropyl fluorophosphate; DNP, 2,4-dinitrophenol; DOC, deoxycholate.

hearts exhibit intermediate sensitivity. In 1963, Repke (521) observed a significant relationship between the I50 for digitoxin and K-strophanthoside on inhibition of red cell ATPase and lethal doses in man and rat. He stated that ". . . differences in glycoside sensitivity of man and rat well reflect the corresponding differences in glycoside susceptibility of the respective ATPase preparations." Thus began attempts to correlate digitalis receptor interaction and the Na^+,K^+-ATPase. Repke (521) was the first to postulate that the glycoside receptor associated with the Na^+,K^+-ATPase was responsible for the cardiotonic effects of the digitalis-like glycosides; he later suggested that a moderate inhibition of the enzyme was responsible for the cardiotonic effect of the glycosides. At first, however, Repke thought that a stimulation of Na^+,K^+-ATPase by very low concentrations of digitalis might be related to inotropism but later retracted this concept when he and others showed that this stimulatory effect was nonspecific.

The relationship between glycoside-induced inhibition of Na^+,K^+-ATPase and species sensitivity was further studied in this laboratory and others (14, 26, 166, 574). We found that the effect of ouabain on Na^+,K^+-ATPase from either rat heart or rat kidney was independent of time. This differed significantly from ouabain-induced inhibition of beef, dog, or human cardiac or renal Na^+,K^+-ATPase which show a time-dependent inhibition (18, 521, 574) and significantly lower I50 values for ouabain than is the case with the rat. Furthermore, it was also found that ^3H-ouabain after binding could be readily dissociated from the rat enzyme preparation at 0°C, with a concomitant restoration of Na^+,K^+-ATPase activity; on the other hand, the drug remained tightly bound to beef or dog heart Na^+,K^+-ATPase. This suggested that the affinity of cardiac glycosides for a binding site on Na^+,K^+-ATPase preparations could be responsible for the different sensitivities of the various enzyme preparations to the drugs as well as the different sensitivities to the drugs among various animal species *in vivo*. Further studies showed that the time-dependency of the ouabain-induced inhibition and the apparent affinity of ouabain for various Na^+,K^+-ATPase preparations (*i.e.*, human, beef, dog, rabbit, guinea pig, and rat) varied directly with sensitivity *in situ* (*i.e.*, high sensitivity was coincident with high affinity of the enzyme preparation for the drug and significant time-dependency). The relationship between species sensitivity and cardiac Na^+,K^+-ATPase-ouabain interaction was extended further by Akera *et al.* (13), who showed a direct relationship between dissociation *in vitro* of ouabain from Na^+,K^+-ATPase and the washout of the pharmacological effect of the drug on isolated hearts from the dog, cat, rabbit, and guinea pig. The data concerning the similarity of rates of onset of inotropism and the *in vitro* ouabain binding to Na^+,K^+-ATPase are consistent with the concept of Allen and Schwartz (26) and Tobin and Brody (667) that dissociation rates (*i.e.*, as opposed to *association* rates) are related to species sensitivity to the glycosides. Our recent studies, however, indicate that dissociation alone may not be the sole factor involved (685).

Akera *et al.* (14) have shown a specific *in vivo* relationship between heart rate, blood pressure, and ECG changes (*i.e.*, P-Q interval) and the *in vitro* I50 for ouabain on cardiac Na^+,K^+-ATPase from dog, sheep, and guinea pig. Addition of potassium during infusion of ouabain prevented arrhythmias but failed to influence the positive inotropic effects and the inhibition of cardiac Na^+,K^+-ATPase activity. They concluded that the cardiac Na^+,K^+-ATPase activity is specifically inhibited during the positive inotropic response to ouabain which is in agreement with the original concept of Repke (see 1963 reference quoted above). It is interesting that potassium appeared to prevent the *toxic* but not the inotropic effect or inhibition of Na^+,K^+-ATPase. This suggests that the

toxic effects of the drug may be due to loss of potassium from the intracellular space but that the inotropic response is mediated by some other change (see below) secondary to inhibition of the Na^+,K^+-ATPase. [It is possible that the potassium loss involves mitochondria; (388)]

The correlation of sensitivity *in situ* to cardiac glycosides with the sensitivity *in vitro* of Na^+,K^+-ATPase constitutes an argument that the receptor associated with the enzyme is responsible for the inotropic effect. A second argument for this conclusion is the relationship between the development of the inotropic response and the development of an inhibition of the Na^+,K^+-ATPase, a correlation that was originally found in this laboratory (72). We infused 1 μM ouabain through a modified dog heart Langendorff preparation and measured left and right ventricular contractility with a strain gauge arch and intraventricular balloon. At the peak of the inotropic effect, the heart was removed and three major cell fractions, Na^+,K^+-ATPase, sarcoplasmic reticulum, and mitochondria were isolated and assayed for the functional biochemical parameters of each. The only system consistently affected was the Na^+,K^+-ATPase. The ATPase inhibition and its relationship to positive inotropism was confirmed by Akera et *al.* (15).

During the development of the inotropic response, ouabain was bound to the receptor associated with Na^+,K^+-ATPase and remained bound during the isolation of the enzyme (22). We showed a direct correlation between inhibition of the Na^+,K^+-ATPase, binding of ^{3}H-ouabain to the Na^+,K^+-ATPase preparation and the development of the inotropic response over a range of ouabain concentrations from 0.05 to 1.0 μM (25a).

Additional interesting experiments with a frog heart were reported by Brooker and Thomas (92). By use of a Straub cannula, they were able to measure continuously reaction products in the perfusate and correlate these with contractility. They found that 3 μM ouabain significantly inhibited Na^+,K^+-ATPase during development of the positive inotropic response, and the inhibition increased as the inotropic response proceeded to the toxic level.

More recently, we have been able to localize specifically ^{3}H-ouabain to the enzyme, Na^+,K^+-ATPase, after development of the positive inotropic response. In these experiments, 0.5 μM ^{3}H-ouabain was infused until a peak inotropic effect was obtained, and the subcellular systems listed above were then isolated and assayed. Mitochondria contained no radioactivity. The sarcoplasmic reticulum preparation did have some radioactivity (*i.e.*, about 10 pmol/mg of protein), but all measurable biochemical parameters were at control levels and the drug was easily washed off at low temperature. On the other hand, the Na^+,K^+-ATPase preparation contained over 30 pmol of drug per mg of protein which was tightly bound and the enzyme activity was reduced to about 70% of control (25a).

It is important to discern the nature of this isolated enzyme-drug complex. If the bound drug was actually responsible for enzyme inhibition (as well as the inotropic response), the characteristics of the *in vivo*-formed complex should be identical with those of a Na^+,K^+-ATPase-glycoside complex formed *in vitro*. A number of laboratories have shown that the *in vitro* Na^+,K^+-ATPase-ouabain complex formed in the presence of ATP plus magnesium plus sodium is readily dissociable at 37°C and can be stabilized by potassium or by other monovalent cations in the dissociation medium (10, 23, 24). We found that the dissociation characteristics of the complex formed *in vivo* were the same as the complex formed *in vitro*.

These studies have now been extended by infusing radioactive ouabain into cat and rabbit heart Langendorff preparations. At the peak of the inotropic response, the three subcellular components referred to earlier were isolated and assayed. In the cat heart (as earlier shown

with dog heart), a significant amount of latter two organelle systems exhibited ATPase with concomitant enzyme inhibition, significantly less was bound to the sarcoplasmic reticulum and none was found in the mitochondrial fraction. The latter two organelle systems exhibited normal biochemical parameters (sarcoplasmic reticulum: calcium binding, uptake; mitochondria: rates of oxygen consumption, ADP:O ratio, and respiratory control). As in the canine experiments, the ^{3}H-ouabain dissociated readily at 37°C from cat heart Na^+,K^+-ATPase in buffer only, and the rate of dissociation was hindered by addition of potassium to the dissociation medium. When the inotropism was "washed out" and contractility of the isolated cat heart returned to normal, the Na^+,K^+-ATPase activity was restored to the control level. Then upon reinfusion of ouabain, a second inotropic effect developed and again the isolated Na^+,K^+-ATPase was inhibited (573a).

The experiments with rabbits yielded different results. The development of the inotropic response was easily established (and was reversible) but a higher dose of ouabain was required than for the cat (0.25 *vs.* 0.04 μM). After isolation of the Na^+,K^+-ATPase at the peak of the inotropic effect, however, no inhibition of enzyme activity or bound, labeled drug was observed. This was not surprising, since the rabbit may be less sensitive to ouabain than the cat (144). For example, the *in vitro* I50 is higher than for the cat, and the *in vitro* rate of dissociation of drug from the Na^+,K^+-ATPase preparation is much greater for the rabbit than for the cat. The lack of inhibition suggested that the drug readily dissociated from the rabbit heart Na^+,K^+-ATPase during the isolation procedure. These data are in contrast to the work of Okita (473) who showed an inhibition of rabbit heart Na^+,K^+-ATPase after isolation from a ouabain-perfused rabbit Langendorff at the time of inotropism. He reported, however, that the inotropic response was dissociated from inhibition of the Na^+,K^+-ATPase. When the inotropic effect was washed out, the activity of the isolated enzyme remained inhibited compared to control levels. It should be emphasized that in their report, the inotropic response they observed was always accompanied by enzyme inhibition during initial drug administration. These studies were extensions of earlier work by Okita's group (551), in which strophanthidin-3-bromoacetate, a derivative of the parent glycoside, was perfused into rabbit hearts. There are at least three aspects of these experiments which we emphasize: a) Perfused rabbit hearts were used; this species possesses intermediate sensitivity to cardiac glycosides (see above); thus, the native cardiac glycoside would probably dissociate, at least to some extent, from the enzyme during isolation. b) The use of strophanthidin-3-bromoacetate is inadequate because of its relatively nonspecific alkylating effect, alluded to by Hokin and Dahl (284) and Frick and Klaus (189). Tobin *et al.* (668) re-examined the effect of the bromoacetate derivative on Na^+,K^+-ATPase and concluded (opposite to that of Okita) that ". . . .rapid dissociation of strophanthidin-3-bromoacetate from Na^+,K^+-ATPase" does occur, and thus it is not an "effective site-directed inhibitor (affinity label) for Na^+,K^+-ATPase." c) The control Na^+,K^+-ATPase activity reported by Okita *et al.* was significantly lower than what we routinely measure. It is difficult to be secure in a complex comparative enzymatic study when the control activity is low. Small but significant changes would not be picked up unless a large number of experiments were to be carried out. We conclude that Okita and his colleagues challenge the Na^+,K^+-ATPase concept with questionable data. They have proposed the hypothesis that the Na^+,K^+-ATPase is an "arrhythmogenic receptor" only, something that appears to us to be vague and undocumented, though certainly possible (473, 474).

Frick and Klaus (189) studied potassium interaction and the glycoside bromo-

acetate derivative in papillary muscle, isolated, perfused guinea pig heart and in cardiac Na^+,K^+-ATPase, and compared the effects to k-strophanthidin and digitoxin interaction with potassium. In the experiments with the isolated papillary muscles, the most potent effects were produced by digitoxin at all potassium concentrations. Increasing the potassium concentration in the bathing medium caused a shift in the dose-response curve toward higher digitoxin concentrations and a similar effect was observed with strophanthidin. However, this type of potassium dependence was *not* found with strophanthidin-3-bromoacetate. Similar results were obtained with isolated Na^+,K^+-ATPase, and the most potent effects at all potassium concentrations were produced by digitoxin. Increasing the potassium concentration *reduced* significantly the inhibitory effect along with the depression of contractility. Contrary to this phenomenon, the action of strophanthidin-3-bromoacetate on Na^+,K^+-ATPase was not influenced by potassium, except at very high concentrations. Frick and Klaus concluded that there was a direct correlation between the inhibition of the cardiac Na^+,K^+-ATPase and the inotropic action of digitalis and indicated that the effects of strophanthidin-3-bromoacetate are not consistent with the suggestion. It appears that this drug produces an irreversible inhibition of the Na^+,K^+-ATPase by about 30% under certain conditions, and yet complete reversibility of the positive inotropic effect in washout experiments on isolated papillary muscles was obtained.

It is very difficult to assess the relationships between the kinetic characteristics of drug-receptor interaction and the resulting pharmacological effect (39). If we assume that the following scheme represents the sequence

$$D + R \rightarrow (DR) \longrightarrow \text{effect}$$

with an indeterminant number of steps between combination of the drug with its receptor and the final measured effect, the association-dissociation kinetics for the drug-receptor interaction may easily differ from the *apparent* kinetics of association-dissociation as determined for the *end result* of the drug-receptor interaction. Specifically, in the case of Na^+,K^+-ATPase, we may assume that the enzyme activity and various membrane phenomena reflect the formation of the drug-receptor complex and the immediate direct effect. However, the end result of this interaction presumably occurs after many intermediate steps at the contractile protein level (as calcium availability to troponin increases). It would not be surprising, therefore, to find that the drug-receptor complex may remain intact, with a change in membrane properties and a decrease in Na^+,K^+-ATPase activity, while the specific final event (*i.e.*, increasing calcium availability) may be more readily reversible. It is recognized that this description is oversimplified, but if at least one step is interposed between the formation of the drug-receptor complex and the final drug effect, a difference in the kinetic characteristics for the initial response and for the final effect could be observed. With these reservations, however, our recent experiments employing an isolated cat preparation (see p. 91) in no way eliminate a direct relationship and in fact support a correlation between inhibition of the Na^+,K^+-ATPase and positive inotropism. The recent papers by Dutta *et al.* (154, 155) and by Daniel and his coworkers (451) are, however, inconsistent with the concept that the Na^+,K^+-ATPase is the "pharmacological receptor" for digitalis. The studies by Daniel and his co-workers (451) require comment. These workers showed that while ouabain reversibly potentiated acetylcholine-induced contractions in rabbit myometrium, ^3H-glycoside binding to a plasma membrane fraction was not readily reversed in 10 minutes. Further, they were unable to show any inhibitory effect

of glycosides on sodium "pumping" from sodium-loaded myometrium during a 10-minute exposure, the length of time during which the glycosides potentiated the acetylcholine-induced contracture. There are a number of problems with this study. Firstly, the workers never directly measured Na^+,K^+-ATPase activity. This difficulty is magnified by the fact that the specific glycoside-induced "inotropic" effect measured consisted of a potentiation of acetylcholine-induced contractions; the Na^+K^+-ATPase related parameters, *i.e.*, ^{3}H-glycoside binding to a "plasma membrane" fraction, and lack of inhibition of a sodium pumping activity from sodium-loaded myometrium were *not* measured in the presence of acetylcholine. Furthermore, the conditions of the three related parameters are not coincident and all three parameters were never measured together in the same tissue. Indeed the parameters presumed to reflect Na^+,K^+-ATPase activity were never assessed during the potentiation process, and it appears that these workers equated the *potentiation* of acetylcholine contractures by cardiac glycosides with glycoside-induced positive inotropism, an extrapolation that appears to be overextended. These workers suggest by indirect means only that the glycosides are binding at all to Na^+,K^+-ATPase: 1) highest binding occurs to a plasma membrane fraction (from which Na^+,K^+-ATPase has only been poorly demonstrated in this tissue); and 2) total tissue binding and binding to isolated fractions both seem to be inhibited by potassium.

B. Uptake and Localization of Glycosides in Myocardial Tissue

Almost all subcellular organelles have been implicated in the mechanism of action of ouabain (389). In particular, the sarcoplasmic reticulum has been emphasized from which ouabain could perhaps either cause a release of calcium or an increased exchange of calcium. Fujino *et al.* (192) found most of the ^{3}H-ouabain in their experiments bound to isolated sarcoplasmic reticulum and some to the cell membrane. The experiments, however, of Kuschinsky *et al.* (365–367) indicate that the amount of cardiac glycoside bound to a *superficial* site is directly associated with positive inotropism, while that amount of drug bound inside the cell is not associated with therapeutic action. In an interesting study, Kim *et al.* (347) found that there was a significant correlation between the positive inotropic effect and the content of ^{3}H-digoxin in a crude microsomal fraction but not in mitochondrial or nuclear fractions. A significant fraction of the bound drug was released into the supernatant when the microsomal pellet was shaken in solution and recentrifuged. There was a greater correlation of the inotropic effect with ". . . the loosely bound drug than with the total microsomal binding" (90a, 347). There was no correlation between contractility and the drug remaining in the microsomal pellet. It is possible that the loosely bound material was associated with the cell membrane containing Na^+,K^+-ATPase, although there are no published data on this point (Bailey and Dresel, personal communication).

There have been a considerable number of studies investigating the influence of extracellular potassium, hyperkalemia, and hypokalemia on the myocardial uptake as well as inotropic effect of ^{3}H-digoxin and other cardiac glycosides. While the results certainly are not conclusive, there is some indication that in the presence of a potassium deficiency, there is an inhibition of the early myocardial binding of ^{3}H-digoxin (253). Once digitalis is bound to the cell, hyperkalemia does not appear to alter its retention (425). Deutscher *et al.* (145) found that the time of onset and the peak of hemodynamic effects of ^{3}H-digoxin occurred prior to initial *uptake* and maximal *uptake* of the drug, respectively, suggesting that the *total* myocardial uptake of drug may not be the sole determinant of

the rate of onset and development of the inotropic effect of digoxin. These authors suggest that this lag can be explained by the drug occupying its specific receptor on the membrane and/or entering a specific intracellular compartment such as the microsomal region (154). An active transport process for the various digitalis compounds has been suggested by Dutta *et al.* (154) and Dutta and Marks (155) who indicate that nonpolar glycosides such as digitoxin demonstrate the same ion-dependent accumulation mechanism as do the more polar glycosides such as digoxin and ouabain. The low accumulation of all cardiac glycosides by rat heart in comparison with the guinea pig heart may be due to the formation of unstable complexes between the cardiac membranes in the rat heart and the various cardiac glycosides used in this study. While the evidence supporting an active transport process for the cardiac glycosides is not totally convincing, the concept is certainly of interest. In a study attempting to relate extracellular potassium concentrations with the myocardial uptake as well as the inotropic effect of ^{3}H-digoxin, Prindle *et al.* (511), with an isolated papillary muscle preparation, compared the effects of varying concentrations of potassium on the above parameters on isolated papillary muscle. The authors concluded that the rate of development of the inotropic response to digoxin was significantly altered by potassium in an inverse manner. Although there were no differences in peak inotropic responses achieved at different concentrations of potassium, a significant positive correlation between digoxin content and the increment in active tension was observed. They explained their results on the basis of the Na^+,K^+-ATPase hypothesis outlined in this review. Very recently, Goldman *et al.* (231) reproduced these findings but included a relationship with the Na^+,K^+-ATPase and binding of digitalis.

A major difficulty in the past has been that during glycoside-induced inotropism, the suggested changes in ion movements resulting from pump inhibition have not been measurable (389). Clearly, however, at toxic levels of drug administration, ion movements were detectable (389). A possible explanation of these results could be that the inotropic effect is unrelated to pump inhibition, *i.e.*, only when the drug concentration reaches a toxic level does pump inhibition occur and this results in increases of tissue sodium and decreases of tissue potassium. An alternative explanation is that the degree of pump inhibition occurring during inotropism is small, and detection of small but significant ion movements and changes are technically difficult.

In 1970, Langer and Serena (381) reported the first positive evidence that the sodium pump may be inhibited to a small degree during development of a positive inotropic response. They measured the mechanical responses and the ionic exchange characteristics in a rabbit interventricular septum perfused arterially with digitalis. The positive inotropic response occurred concomitantly with a net uptake of cellular calcium, a loss of potassium and a gain of sodium, which was also accompanied by alterations in pump activity. A very important aspect of this work is that during these ion changes, there was no reported evidence of toxicity. However, if perfusion was continued, further altering ion content in the same direction, toxicity ensued. These data add important conceptual dimensions to the glycoside-Na^+,K^+-ATPase interaction. If the drug acts via pump inhibition with a significant inotropic but nontoxic effect, enough of the pump must remain intact to insure proper cell function. It is possible that mechanisms other than inhibition of the sodium-potassium pump may be responsible for toxicity. Specific antibody components to "catalytic" and to "digitalis binding conformations" may resolve these important questions (see section V).

A clinical demonstration of ionic movements associated with positive inotropism *in the absence of toxicity* was reported in 1972 by Brennen et al. (85) who studied the loss of myocardial potassium after administration of ouabain in 14 patients. They showed a significant coronary sinus-arterial potassium difference *prior* to a maximum increase in left ventricular contractility, thus suggesting that pump inhibition occurs prior to evidence of a positive inotropic effect. This indicates, according to Brennen *et al.* (85), that cellular potassium loss occurs in the absence of any toxic manifestation of digitalis administration in man.

A general comment should be made regarding the toxicity of drugs especially in laboratory procedures. It is probably misleading to assign a particular concentration of a drug as being a "toxic dose." Such a designation can only be determined *after* the production of a toxic effect. Since pharmacological effects adhere to dose-response and sensitivity (i.e., for species) curves, a glycoside level of 0.5 μM, for example, may be toxic in one preparation but not in another. Certainly, there are doses of the drug which would almost always produce toxic manifestations, but, again, these are statistical chances. On the other hand, the experiments designed to test the relationship between Na^+,K^+-ATPase activity and inotropic action almost always employ a preparation not perfused with blood. Therefore, it is impossible to extrapolate the dosage used in such a condition to the clinical situation and indeed even concentrations of cardiac glycoside as low as 10^{-8} M may produce toxic effects.

The antagonism between potassium and cardiac glycosides is manifested at the tissue level and at the enzyme level. As discussed in a previous section, we developed a mathematical expression which defines the relationship between sodium and potassium concentrations, and the rate of ouabain interaction with a Na^+,K^+-ATPase preparation (396). A comparison of some predictions of this model with the data of Prindle *et al.* (511; see above) for digoxin-induced inotropism in a cat papillary muscle preparation is of interest. Prindle *et al.* (511) examined the response to digoxin in three media containing: a) 142 mM sodium and 7.5 mM potassium; b) 145 mM sodium and 4.4 mM potassium; and c) 140 mM sodium and 1.5 mM potassium and found ratios for the initial rates of inotropic development of 1.00:1.72:3.43 for a): b): c), respectively. The model for the Na^+,K^+-ATPase predicted a ratio of 1.00:1.48:2.51 (396). Also, our model predicts rate constants for ouabain interaction with a Na^+,K^+-ATPase preparation in the range of the rate constants obtained for ouabain uptake by guinea pig atrial preparations as reported by Kuschinsky *et al.* (366). The ouabain uptake appears to reflect a ouabain interaction with the cell surface, as opposed to intracellular accumulation of the drug.

The equilibrium or affinity constant of the receptor associated with the Na^+,K^+-ATPase is about 2.9×10^8 M^{-1} in the presence of 150 mM sodium and 2 mM potassium. If the potassium concentration is raised to 6 mM, the equilibrium constant decreases to about 1.6×10^8 M^{-1}. This suggests, therefore, that an increase in the extracellular potassium concentration from 2 to 6 mM would reduce the affinity of the Na^+,K^+-ATPase-associated receptor by one-half for ouabain. Thus, changes in extracellular sodium and potassium modulate the degree of cardiac glycoside interaction in two ways. First, in the presence of high concentrations of the glycoside, sodium and potassium would modulate the *rate* of glycoside interaction with the system and conceivably the *rate* of developed inotropism [e.g., as in the study by Prindle *et al.* (511)] and possibly the time of exposure to the drug required to achieve toxicity. However, sodium and potassium would not alter the magnitude of glycoside interaction, since the rate of binding would remain much greater than

the rate of dissociation of the glycoside receptor complex (*i.e.*, the receptor would approach saturation with the drug regardless of the sodium and potassium concentrations). In the clinical setting, however, the blood levels of the digitalis glycosides remain low and the time of exposure to the drug (to the sodium pump sites) is long, so that the pump does not become completely inhibited. Rather, the glycoside receptor complex reaches equilibrium with free drug and free receptor. Therefore, a second way that extracellular sodium and potassium would modulate glycoside action is through the equilibrium *constant*, which equals the ratio of the association rate constant/dissociation rate constant. Sodium increases and potassium decreases the association rate constant, while neither cation alters the dissociation rate constant at high ionic strength (*i.e.*, presumably the physiological case).

The very tight pseudo-irreversible binding of cardiac glycosides with the receptor associated with Na^+,K^+-ATPase suggested that if the enzyme has a significant physiological role, prolonged *in situ* exposure to the drug might lead to a synthesis of more Na^+,K^+-ATPase molecules (392). Such an effect was reported in 1972 by Vaughn and Cook (680). They found that the half-time for dissociation of ouabain from sites associated with the sodium pump in HeLa cells was 16 hours. In other experiments, after partial blockade of the pump in these cells, they observed the appearance of new ouabain binding sites concomitant with total restoration of sodium pump function within 3 hours. Cycloheximide prevented the recovery, which suggests that restoration of the cation transport was due to protein synthesis. Interestingly, total inhibition of the pump by ouabain prevented recovery. These authors suggested that the stimulus to synthesis may be an alteration in intracellular electrolyte content since cells grown in a low potassium medium exhibited higher titers of Na^+,K^+-ATPase. [Other studies have shown that the rate of Na^+,K^+-ATPase synthesis is responsive to changes in environment. The rate is increased in kidney by aldosterone or potassium loading (for example, see ref. 603 and below).] Clinically, therefore, the rate of Na^+,K^+-ATPase synthesis may be enhanced by glycoside administration. In a more general sense, alteration in the state of the myocardium (*e.g.*, ischemia, overload, failure, thyroid state, *etc.*) may modulate the equilibrium of [free glycoside] [free pump units]/[glycoside-pump units] by changing the rates of synthesis and/or degradation of the Na^+,K^+-ATPase (turnover).

C. Possible Mechanisms of the Inotropic Response to Digitalis

If the Na^+,K^+-ATPase is involved in the mechanism of digitalis action, then calcium must also be in some way controlled by Na^+,K^+-ATPase. Skou (609, 610) was the first to show that calcium inhibited a Na^+,K^+-ATPase preparation from crab nerve. Järnefelt (310) confirmed these studies and found that calcium inhibition was "hardly observable in the absence of sodium and potassium, whereas in their presence already small concentrations of calcium (μM) are inhibitory with a half-maximal inhibition of about 0.5 mM calcium." Therefore, the part of the ATPase activity that is specifically inhibited by low concentrations of calcium is a sodium plus potassium-induced component. A number of laboratories have now documented calcium inhibition of the Na^+,K^+-ATPase (579, 664).

In a series of preliminary experiments, we observed an interesting "substitution" effect of low concentrations of calcium (less than 25 μM) for sodium in the ATP plus magnesium-dependent ouabain binding reaction. However, these results were quite variable due to, we feel, the variation in endogenous calcium in each preparation, considering that the preparations

used were only about 5 to 10% pure. The "substitution" effect was published recently by Schön *et al.* (564).

In an interesting study in 1971, Schatzmann and Rossi (560), by studying a calcium plus magnesium-activated membrane ATPase that is ouabain insensitive and probably related to cation transport, concluded that the sodium plus calcium plus magnesium-activated ATPase and the potassium plus calcium plus magnesium-activated ATPase are the results of an *uncoupling* of calcium on the Na^+,K^+-ATPase which is responsive to sodium and potassium transport. The investigators speculated that calcium or calcium-ATP on the internal surface of the membrane disrupts the combined action of sodium and potassium.

In this context, some data from this laboratory are worth mentioning (165). A sarcoplasmic reticulum preparation isolated in a bicarbonate-azide medium readily binds calcium, is unaffected by ouabain, and contains little or no Na^+,K^+-ATPase activity. Conversely, a sarcoplasmic reticulum preparation isolated in a sucrose medium binds calcium and 30% of the ATPase activity is ouabain-inhibitable. However, ouabain increases calcium exchange in this preparation. Furthermore, if a Na^+,K^+-ATPase preparation (no calcium binding, 95% ouabain inhibitable) is added to the azide-bicarbonate sarcoplasmic reticulum preparation, ouabain enhances ^{45}Ca exchange. While these experiments are crude and of a preliminary nature, nevertheless, they do suggest that a proper juxtaposition of the receptor, Na^+,K^+-ATPase, with the "effector," sarcoplasmic reticulum, may be analogous to a cellular event involving digitalis and calcium.

The myocardial cell is highly compartmented and the beat-by-beat control of myocardial contractility appears to involve regulation of calcium movements among several compartments (111, 112, 356). The following compartments or pools of calcium are thought to be involved in the excitation-contraction-relaxation sequence of the myocardial cell: a) extracellular free calcium; b) calcium bound to constituents (*e.g.*, mucopolysaccharides) located external to the sarcolemma; c) calcium localized to the sarcolemma *i.e.*, bound to the external and internal surfaces and/or contained within the membrane); d) intracellular free calcium; e) calcium bound to the membrane surfaces of the sarcoplasmic reticulum and/or that contained within the space of these intracellular tubules; f) perhaps, and under some conditions, mitochondrial calcium; and g) calcium in the sarcomere.

A current popular theory of the excitation-contraction-relaxation sequence is as follows. *Depolarization* of the sarcolemma brings about an increase in free intracellular calcium from perhaps 0.1 μM in the resting state to about 10 μM upon excitation. Presumably, much of this calcium may be released from an internal store of calcium, the sarcoplasmic reticulum, and this release is brought about by a decrease in the affinity of this system for calcium. There could be several ways in which this decrease in affinity is accomplished: a) Depolarization of the sarcolemma spreads into the invaginations of the cell membrane, the transverse tubule, and then into the cell itself to effect internal changes in depolarization (195). It is of interest in this regard that Endo *et al.* (164) in 1970 demonstrated a release of calcium from sarcoplasmic reticulum induced by "depolarization." b) Depolarization lowers intracellular pH to change the affinity (457); and/or c) a small amount of calcium enters during the plateau phase (phase 2) of the cardiac action potential and effects a "calcium-induced, calcium-release" from the sarcoplasmic reticulum through a series of cascading events (see below) (178). In dog ventricle, approximately 0.92 pmol of calcium per square centimeter moves into

the cell with each impulse (379a, 525a). It is possible also that calcium entering the cell during the plateau phase may be sufficiently high to activate contraction by releasing calcium from sarcolemmal stores (379). The sarcomere receptor for calcium is a component of troponin, and calcium interaction with this component alters the conformation of tropomyosin, exposing sites on actin to which the cross-bridges bind and *contraction* occurs.

Relaxation, or restoration of the sarcomere to its resting length reflects the reverse process. In some way, the affinity of the sarcoplasmic reticulum for calcium increases so that the intracellular free calcium concentration is reduced. The drop in free intracellular calcium causes a diffusion potential favoring movement of calcium out of the sarcomere, dissociation of calcium from troponin, and the resultant restoration of repression by tropomyosin. Finally, in order to maintain a steady state, the calcium that enters the myocardial cell during depolarization must be removed. This requires a calcium efflux system that must move calcium uphill (out of the cell) against its electrochemical gradient (ouabain-insensitive "pump" or a sodium-calcium "exchange system").

The sodium pump is believed to reside on the sarcolemma and possibly on its invaginations (T-system) into the cell. If the cardiac glycoside receptor associated with the pump is ultimately responsible for the positive inotropic effect of the drug, some link must exist between the cell membrane-associated receptor and the intracellular component directly involved in contraction, the sarcomere. This link is presumed to be calcium, so that the final effect of the drug-induced series of events may be to increase the intracellular concentration of free calcium around the sarcomere during and/or immediately after depolarization. A number of hypotheses have been offered as an explanation.

It has been stated that the positive inotropic action of digitalis must involve calcium (521). This arises from numerous studies clearly showing that relatively low concentrations of digitalis preparations alter calcium flux in intact tissues. The possible relationship between sodium and calcium with respect to the cardiac Na^+,K^+-ATPase was recognized first by Repke (521) who indicated that "the sodium/calcium antagonism possibly regulates the activity of the Na^+,K^+-ATPase in excitable tissues." Repke corroborated the Wilbrandt theory (702) that "digitalis increases calcium indirectly by inhibition of sodium-potassium transport" (522) and concluded that the digitalis effect is due indirectly to inhibition of Na^+,K^+-ATPase. Impairment of active transport by reducing outside sodium or outside potassium causes an increased calcium entry into the cell. This would be consistent with the well known contraction dependency of digitalis action (447). Baker, in a series of published studies, delineated a linked sodium-calcium exchange process, by using a well defined squid axon preparation which is a much simpler system than heart muscle. He showed that increasing intracellular sodium results in an increase of calcium influx and a decrease of calcium efflux; decreasing external sodium results in the same process (47, 48). With this type of experiment, Baker has suggested that the action of cardiac glycosides is due to an inhibition of the Na^+,K^+-ATPase. This results in an increase in internal sodium concentration which then causes an increase in calcium influx and a decrease of calcium efflux. Therefore, "ouabain seems to have no direct effect on calcium movements . . ." (47). This action of digitalis would also result in the well known increase of respiration induced by the drug that has been observed in brain slices and, many years ago, in heart slices (712). An alteration in calcium movements in heart muscle has been observed in experiments carried out by Langer (378, 379) with intact cardiac preparations, in particular, the interventricular perfused rab-

bit septum in which ^{45}Ca washout from various kinetically estimated compartments can be described along with changes in contraction. This attractive concept has been elaborated in this laboratory by Wood *et al.* (713). The results of Langer (378) are somewhat similar to those of Bailey and Dresel (45a) and Kim *et al.* (347) who used a gas-perfused cat heart preparation in which the extracellular space is "replaced" by gas. It appears from all these experiments that one compartment, presumably associated with superficial regions, possible anionic-containing sites, is directly involved with providing "activator calcium." According to Langer, the Baker suggestion that intra- and extracellular sodium control the influx and efflux of calcium in nerve tissue is also applicable to heart tissue. Langer (378, 379) and Langer and Serena (381) have proposed that digitalis causes an inhibition of the sodium-potassium coupled system which then results in an increase in coupled sodium-calcium transport, thus causing an increase in influx of calcium to the myofilaments. Thereafter, calcium procedes by a "one-way street" and is pumped out of the cell. According to this postulation, intracellular sodium in the region of the cell membrane should increase after addition of digitalis. It should also increase after a sudden lowering of temperature and after an increase in the frequency of contraction [the Bowditch or force-frequency phenomenon], which produces a "pump-lag" in glycoside-sensitive species, excluding the adult rat. [The neonatal rat, incidentally, behaves like a glycoside-sensitive species (Langer, personal communication).] Careful examination of the data, however attractive as the hypothesis is, reveals many complexities not the least of which is the inability to measure accurately intracellular sodium in the complex, heterogeneous cardiac muscle cell. Recognizing this important difficulty, Langer *et al.* (380) have developed a single layer cardiac cell preparation in which they should be able to study calcium kinetics accompanying "contraction." There are at least two contrary aspects to this generalized concept: a) The theory requires that inotropic responses must be either preceded or accompanied by a loss of potassium from the cells with a simultaneous gain of sodium [originally suggested by Hadju and Leonard (246) and by Glynn (223)]. Are these effects related or are they incidental? b) "Precise" *in vivo* correlative experiments on heart muscle are not yet available. This statement is made despite the many excellent studies showing, for example, the presence of a "slow inward calcium current" possibly associated with a portion of the plateau phase (phase 2) of the ventricular action potential (see for example, refs. 68–70). Reuter and his colleagues suggest that decreasing extracellular sodium or increasing intracellular sodium causes an increase in the slow inward calcium current (218). This fills intracellular calcium stores from which calcium can then be released by some unknown mechanism and presumably would represent at least part of "activator calcium." The investigators also suggest that the action of digitalis is to increase internal sodium. The methodology employed, namely, the voltage-clamp technique, is not without difficulty particularly when heterogeneous multicellular tissues such as the heart are used (213, 314). Morad and Goldman (445) have discussed in detail the complexities of interpretation of voltage-clamp experiments. Newer modifications such as the "guard-gap" have been designed in order to minimize leakage current. So-called "instantaneous ionic current" measurements can be made by the usual voltage-clamp techniques only from 5 to 10 mseconds after the clamp, clearly a grossly prolonged time to make meaningful interpretations with respect to excitable tissue or to cardiac muscle and ion movement. New and Trautwein (467) in 1972 and Tritthart *et al.* (674) in 1973 reexamined the concepts by using newer

techniques of clamping. Their results suggested that the amount of calcium that enters the cell (slow inward calcium current) is far too low to account for full activation of contraction. Accordingly, there may exist an intracellular release and reuptake system probably associated with the sarcoplasmic reticulum, a situation that has been described in detail for skeletal preparations. The amount of calcium required for full activation has been estimated at about 25 to 50 μmoles per kg of wet weight of heart muscle. The attractive "calcium-induced calcium release process," which is presumably a type of allosteric phenomenon, was first suggested for skinned skeletal muscle fibers by Endo *et al.* (164), later by Ford and Podolsky (179, 180), and by Taylor *et al.* (657). By this concept, a small amount of calcium can release a large amount of calcium.

A second hypothesis is that glycoside interaction with the pump increases the affinity of sites on the internal surface of the membrane for calcium (73). These sites may be the sodium activation sites of the pump since, as reviewed above, these sites do have affinity for calcium. In this case, depolarization followed by sodium influx may displace this membrane-bound calcium such that it contributes to the rise in the pool of intracellular free calcium available for contraction. There is some evidence against this suggestion. First, the dissociation constant of the sodium activation sites for calcium appears to be around 400 μM (397). The second objection to this hypothesis is that there appears to be too few sites per unit of tissue or cell to constitute a significant pool of bound calcium. Of course, the hypothesis may apply to sites other than the sodium activation sites, perhaps to sites on the internal surface of the membrane not directly on the pump which may only become available during digitalis treatment. This raises the interesting question of how the various states of the pump may, if at all, affect membrane structure around it and how far these hypothetical forces extend from the pump.

A third hypothesis is that the drug interacts with the pump in a manner to allow a calcium influx-potassium efflux exchange reaction (397). As outlined above, there is evidence that glycosides react with and stabilize a form of the pump with high affinity for potassium, and Glynn *et al.* (229) suggested that a component of potassium flux in the glycoside-treated red cell preparation may occur through the pump. There is evidence that calcium and potassium may have some relationship with respect to the potassium activation sites. For example, calcium was found to reduce the amount of potassium required to activate Na^+,K^+-ATPase by one-half of the maximum, and it was suggested that calcium may act as a substitute for potassium at one of the potassium activation sites (397). Thus, it may be that potassium and calcium fluxes take place through the glycoside-inhibited pump. If so, the direction of the chemical gradients favors calcium influx coupled to potassium efflux. Blum and Hoffman (80) observed a calcium-induced increase in the permeability of potassium in energy-depleted human erythrocytes.

The hypothesis that glycosides induce a potassium-calcium exchange is attractive, since exposure of myocardial preparations to cardiac glycosides results in an increase in exchangeable calcium in the myocardium (210–212, 238, 239, 240a, 350, 351, 381, 413) and ultimately, potassium, which may result in a loss of intracellular potassium. A carrier-mediated exchange process may explain these phenomena. Morad and Greenspan (446) proposed the operation of a carrier-mediated calcium-potassium exchange mechanism for the plateau phase of the cardiac action potential. Katz (338) pointed out that the duration of the plateau phase for a variety of species is an inverse function of the sensitivity of Na^+,K^+-ATPase to glycosides. Also, exposure to glycosides appears to be associated

with an altered duration of the plateau phase (158). These results suggest that a membrane constituent involved in the plateau phase may be associated in some manner with the sodium pump.

We emphasize that while this section is hopefully objective, it must reflect the bias of the authors which is that the Na^+,K^+-ATPase is an important pharmacological receptor for digitalis glycosides. We recognize and indeed emphasize that while the evidence in favor of this hypothesis is not completely secure, the evidence against the concept is equally, if not more, ephemeral. Nevertheless, the burden of proof is on the protagonist.

IX. Antibodies to Cardiac Glycosides

Substances with molecular weights of 1000 or less are not ordinarily antigenic. However, antibodies specific for small molecules may be produced by immunization with conjugates consisting of the low molecular weight substances, called haptens, covalently linked to proteins or synthetic polypeptides. There has been intense interest in using immunological techniques in pharmacology (*e.g.*, see refs. 71, 296). Methods have been described for the coupling of almost any small molecular hapten or carrier and thereby rendering it potentially antigenic. The possible clinical applications of such antibodies for diagnostic, prophylactic, therapeutic, and mechanistic purposes are great. However, there are other important aspects that may be of equal importance, namely, the study of the site and mechanism of action of digitalis.

A communication by Butler and Chen (100) contained the first known description of the experimental production of antibodies with specificities for cardiac glycosides, and it was hoped that information would prove useful in the development of an immunological assay for digoxin in plasma and other biological fluids. Prior to this procedure, the "best" assay for digoxin in serum employed the well known inhibition by digoxin of the Na^+,K^+-ATPase system associated with red blood cells or with other systems in which rubidium transport was usually measured (411). A number of publications describe the usefulness of the digoxin-specific antiserum (97–99). The concept of a radioimmunoassay for serum cardiac glycosides gained significant impetus through the work of Smith (622) and Smith *et al.* (625). Previously, Oliver *et al.* (475) showed the feasibility of a radioimmunoassay approach to determine the levels of digitoxin generally encountered clinically. They used rabbit antibodies to a 3-0-succinyl-digitoxigenin-serum albumin conjugate. Smith (623, 624) and Smith *et al.* (626), in a careful series of modifications, produced a relatively simple, workable procedure for the assay of serum cardiac glycosides. The concept, suggested by Smith and by Butler, of covalently linking a digitalis glycoside to a macromolecule promoted interest in using this procedure for examining the hypothesis that digitalis acts at a surface site rather than an intracellular site (see discussion above). Okarma *et al.* (472) prepared digoxin, dihydrodigoxin, and digitoxin derivatives of albumin by the method of Smith *et al.* (626). They used a photoelectric recording system and examined the actions of both parent glycosides and the covalently bound albumin conjugates on cultured rat heart cells obtained from 0- to 5-day-old rats. (It has been suggested that neonatal rats are very sensitive to cardiac glycosides; Dr. Glenn A. Langer, personal communication.) Covalently linked glycosides were shown to be free of unbound drug by gas-liquid chromatography (see below for criticism), and these compounds produced dose-related increases in contraction amplitude and contraction rate. Double-isotope-labeled experiments suggested that the covalent complex did not enter the cell. High concentrations of potassium prevented the increase in cellular contraction rate without altering the increase in contraction amplitude or the cellular uptake

of the glycosides. It is of interest that the covalently linked glycosides also specifically inhibited a Na^+,K^+-ATPase from rat heart. According to Okarma *et al.* (472), the data support the hypothesis that cardiac glycosides exert pharmacological actions at the cell surface. This would be consistent with the studies by Kuschinsky and Lullman (see above), with intact perfused heart preparations and, if the Na^+,K^+-ATPase enzyme is exclusively associated with the sarcolemma (the evidence on this point *is not secure*), the data would also be consistent with our own suggestion and that of Repke (521).

In a careful study by Smith *et al.* (627), ouabain and digoxin were joined covalently to several large protein molecules. It was assumed that the macromolecular conjugates were too large to enter intact cells, and, consequently, any pharmacological action must result from an interaction with a cell surface receptor. This important concept was derived from the initial immunological studies. The conjugates were applied to several physiological systems. These included the contractility response of isolated cardiac muscle, activity of radiolabeled rubidium uptake by red blood cells, enzymatic activity of isolated cardiac Na^+,K^+-ATPase and enzymatic activity of solubilized red cell Na^+,K^+-ATPase. In all of the systems, the conjugates were 100- to 1000-fold *less active* than the free glycosides, and *chromatographic examination of the various conjugates revealed a small but persistent amount of free cardiac glycoside in an amount ranging from 0.1 to 1% of the total covalently bound glycoside.* Smith *et al.* (627) felt that the "presence of this substance fully explains the levels of biological activity observed with the conjugates." In spite of the facts that the cardiac enzyme had a ouabain-sensitive component of only eight and that the Na^+,K^+-ATPase associated with the solubilized red blood cell was of the usual low activity (making it somewhat difficult to interpret inhibitor studies), the results reported in their paper were striking. It does appear that small amounts of active material might escape detection and, therefore, could enter the cell and produce an action. Smith (personal communication and presentation at the New York Academy of Sciences Na^+,K^+-ATPase Symposium, November, 1973) has indicated that characterization of the low molecular weight "free" material suggests that it is highly active in all of the systems tested. Also, it is not a native glycoside and it possesses a high content of amino acids of a composition different from that of a carrier protein. These data imply that digoxin or ouabain may be "clipped" from the carrier protein "as relatively polar oligopeptides." Smith and his colleagues synthesized and purified ala-gly-glydigoxin tripeptide and showed that such oligopeptides coupled at the terminal sugar are, indeed, highly active. It is possible, therefore, the Okarma *et al.* (472) were assaying organic solvent extracts of their conjugate which would possess the low molecular weight material and that this material, while evoking a significant tissue action, could have escaped detection by the methods employed. Butler (personal communication) agrees that it is difficult to eliminate the possibility that a small but significant amount of active material may penetrate the cell. It is clear that caution should be used in the interpretation of these experiments with respect to deciding whether a receptor is specifically and exclusively localized to a cell surface.

From a clinical and mechanistic point of view, studies have produced intriguing results. In 1971, Curd et *al.* (132) reported the isolation of a digoxin-specific antibody that bound 1.6 moles of digoxin per mole and had an association constant of $1.6 \times 10^8\ M^{-1}$, which is in the range of or perhaps even higher in some cases than that of the receptor for glycosides associated with the Na^+,K^+-ATPase. This aspect needs to be investigated thoroughly on a purified enzyme system. At almost stoi-

chiometric concentrations, the digoxin antibody reversed the digoxin-induced a) inhibition of ^{86}Rb transport in human erythrocytes, b) increase in tension developed in an isolated guinea pig atrial preparation, and c) ventricular tachycardia in intact dogs. The antibody also converted digoxin-induced rhythm changes to normal in isolated guinea pig atrial strips. Skelton *et al.* (608), with a papillary muscle preparation from cats, showed that digoxin antiserum was able to reverse the positive inotropic as well as the toxic electrophysiological effects of digoxin. It is of interest that the digoxin antiserum had *no significant effect on the contractile response to ouabain, thus indicating a high degree of specificity*. Butler stated that the reversal of the inotropic effects of digoxin could involve either an *effective* reduction in the extracellular fluid of the free digoxin level or direct interaction with the digoxin bound to its cellular receptor site. Simple removal of extracellular digoxin by repeated washing does not reverse the inotropic effect of digoxin on this preparation.

Reversal of digoxin inotropism by specific antibodies may not be simply related to a reduction of free digoxin in the bathing medium but may result from interaction of the antibody with digoxin bound to a receptor site. This is a rather attractive hypothesis, though difficult to prove, To clarify this point, Butler and his co-workers (99a, 608) administered digoxin and allowed the inotropic response to reach a maximum level. He then reversed the response by the addition of antidigoxin serum and exchanged the bathing medium to remove the digoxin-antibody complexes and any free antibody. At this point, another concentration of digoxin was added which resulted in the usual increase in the rate of tension development. Similar results were obtained when ouabain, rather than digoxin, was added at that point. This does suggest that antibodies may remove digoxin from its receptor site, which would allow the subsequent addition of glycoside to interact at that receptor site and result in a characteristic effect. This evidence is still indirect, and proof that antibodies actually remove digoxin from cardiac cells by a direct interaction requires further studies utilizing radioactive tracers and highly purified Na^+,K^+-ATPase preparations. Watson and Butler (688) showed that digoxin-specific antibodies are capable of removing essentially all intracellular digoxin from rat renal cortical slices or from human erythrocytes. In removing digoxin from the red blood cell, the antibodies are capable of reversing an effect of the drug on cellular potassium transport. This provides direct evidence that antibodies are capable of removing and thereby reversing biological effects of low molecular weight substances after they have been taken up by mammalian cells; it suggests, furthermore, that antidigoxin sera may prove useful in the reversal of digoxin toxicity. The rapid and essentially quantitative removal of digoxin from red cells by antibodies, however, was not accompanied by an immediate restoration of potassium influx to normal levels.

Evidence was also obtained which showed that antidigoxin antibodies were capable of reversing digoxin toxicity in the dog (562). Dogs were given digoxin until toxic arrhythmias developed. These persisted throughout a 6-hour study period. Six or eight dogs were given digoxin-specific antibodies in canine plasma and/or rabbit serum, and the arrhythmias returned to a sinus mechanism within 30 to 90 minutes after the start of the infusion. At the end of the 6-hour period of study, the six dogs were alive and had a normal sinus rhythm. These data provide evidence that digoxin-specific antibodies can reverse severe, established digoxin toxicity in the dog and may be useful in a clinical situation.

Mandel *et al.* (420) further evaluated the effect of digoxin-specific antibodies on

physiological parameters by using an isolated perfused canine Purkinje fiber and microelectrode techniques which recorded the resting potential, action potential, amplitude, rate of rise, over-shoot and duration (APD), membrane responsiveness, conduction velocity, and refractory period. The preparation, when exposed to 10^{-7} M digoxin, first revealed a prolonged APD and subsequently a marked shortening (a characteristic of the digoxin therapeutic effect followed by a toxic effect). At the time of toxicity, the fibers were perfused with serum containing digoxin-specific antibodies. Membrane characteristics were improved in 50 minutes and by 60 minutes were at control levels. It was concluded from these data that antibodies possess the ability to reverse pronounced toxic electrophysiological effects of digoxin in isolated cardiac tissue.

A most interesting study published by Smith (624) showed the effect of ouabain-specific antibodies on Na^+,K^+-ATPase exposed to ouabain. If the antibody possesses a very high affinity for the digitalis glycoside, but is still in the range of the affinity constant of the glycoside receptor associated with Na^+,K^+-ATPase, it would seem that the primary action of the antibody is to remove free cardiac glycoside rather than to remove glycosides directly bound to the receptor. On the other hand, even in glycoside-sensitive species, binding of digitalis to Na^+,K^+ATPase is not irreversible. The dissociation rate constant is very much lower than the association rate constant. In other words, dissociation from the receptor must proceed, albeit at a very slow rate. The antibody would simply serve as a "receptacle" or "sink" for digitalis and would enhance the removal. Another possibility is that, as indicated above, the antibody may interact directly with part of the receptor associated with the Na^+,K^+-ATPase and produce some type of configurational change that results in an enhanced removal of glycoside. If the latter were true, the kinetics of dissociation would be altered. Moreover, it is reasonable to suppose that the kinetics of association would also be altered if the receptor were pretreated with the antibody. In any event, by using the ouabain antibody, the intrinsic association constant which was about 1.5×10^9 M^{-1}, Smith found a blockade of ouabain-induced inhibition of canine cardiac Na^+,K^+-ATPase [prepared by the Matsui and Schwartz procedure, ref. 432; and assayed by a spectrophotometric assay (574, 580)]. Of greater interest was the fact that once inhibition of Na^+,K^+-ATPase was established (0.5 μM ouabain), addition of the ouabain-specific antibody produced a characteristically time-dependent, almost complete reversal of the inhibition. It would be of interest to use ^{3}H-ouabain in an attempt to correlate the removal of the drug from the enzyme with reversal of inhibition. (We have shown a direct relationship between drug binding and inhibition in a preparation containing magnesium plus ATP plus sodium plus potassium; see above.) Furthermore, the cardiac enzyme used was only 5 to 10% pure; it is possible that under the appropriate conditions, a more highly purified enzyme would possess an affinity for cardiac glycosides greater than the relatively impure preparation. If the effects of the antibody depend solely upon affinity constants, the antibody will have greater difficulty in removing tightly bound drug from its receptor. We have attempted to duplicate the experiments of Smith regarding the Na^+,K^+-ATPase, by using a digoxin-antibody (kindly supplied by V. P. Butler, Jr.) with an average affinity constant of about 1.7×10^{10} M^{-1} (626). We found that the antibody effectively blocks digoxin-induced inhibition when the drug was used in a concentration of about 0.5 to 1.0 μM and the antibody was present in a 4- to 10-fold excess. While we were able to show a complete blockade of

digoxin inhibition, we had considerable difficulty in reversing an already established inhibition. In four experiments, we produced about a 40 to 50% reversal after about 2 hours of treatment. This is in contrast to the almost complete reversal of inhibition obtained by Smith (624) with the ouabain-specific antibody. At a New York Academy of Sciences Symposium, (1973), Smith showed a nearly 75% reversal by a digoxin-antibody. Digoxin, being more nonpolar than ouabain, may bind either to more nonspecific hydrophobic sites than ouabain, or perhaps the apparent equilibrium constant or the maximum second-order rate constant of the Na^+,K^+-ATPase receptor for digoxin is higher than for ouabain. Alternatively, it may be that the dissociation rate constant for digoxin is lower than for ouabain. Yoda and Hokin (722) reported an I50 of 0.57 μM for digitoxin, another nonpolar glycoside, and 0.54 μM for ouabain. However, the I50 was 0.04 μM for digitoxin and was 0.23 μM for ouabagenin, suggesting that perhaps the dissociation rate constant for digoxin is lower than for ouabain if the association rate constants are the same. Simply stated, it may be that digoxin binds more "tightly" than ouabain; therefore, once having bound, the rate of dissociation is slower. Consequently, in the presence of excess digoxin antibody, a much greater time period and much more antibody would be required to effect complete dissociation.

The ouabain-Na^+,K^+-ATPase complex dissociates at measureable rates in a ouabain-free medium. Addition of the ouabain-specific antibody to this medium should not alter the dissociation rate constant. According to Yoda (721), the dissociation rate constant depends exclusively on the sugar portion of the cardiac monoglycoside and is independent of the aglycone. This could account for the differences between digoxin and ouabain, discussed above. If, on the other hand, the antibody to the hapten is in some way not only specific for the small molecules but also has some effect on the enzyme itself, changes in the rate of dissociation and probably in the rate of association should occur.

The time for half-maximal binding in the presence of ATP plus magnesium plus sodium and 1 μM ^{3}H-ouabain to a purified kidney Na^+,K^+-ATPase is 18 seconds. Taking into consideration the time for half maximal dissociation of about 3 hours, the dissociation constant for the reaction is calculated to be about 1.4×10^{-9} M for this binding ligand condition. It does appear, therefore, that the digoxin antibody has about the same affinity or perhaps even a little higher one for its respective glycoside, as is the case with the ouabain-specific antibody.

A very recent and interesting study involves a test of the hypothesis that the digitalis antibody acts solely as a sink. Gardner *et al.* (198) concluded that two processes are involved: a) digoxin binds to the membrane; and b) digoxin accumulates inside the cell. Ouabain, on the other hand, binds only to the membrane. This is consistent with the studies of Kuschinsky and Lullman on isolated perfused hearts (see above). A single class of saturable binding sites was involved in digoxin binding, and this binding was reversible, temperature sensitive, and correlated with inhibition of potassium flux. Digoxin uptake, however, was a linear function of the digoxin concentration and did not correlate with inhibition of potassium flux. Digoxin antibodies prevented and reversed the digoxin effect on potassium flux by *decreasing the amounts of digoxin* that were bound to the erythrocyte membrane. The antibodies also reduced uptake, but this component was unrelated to the component causing inhibition of potassium flux, which again substantiated the concept that the primary pharmacological action of digitalis glycosides may be associated with

a membrane event. These authors (198) concluded that the digitalis antibodies function only as a "sink" for "free" digoxin molecules, thereby decreasing the "effective" concentration of the drug.

Our recent (unpublished, 1974) experiments with digoxin antisera and a purified Na^+,K^+-ATPase confirm the suggestion of Gardner *et al.* (198) that the antibodies *probably* do not interact with the receptor but function merely as a sink. We cannot find changes in the rate constant for dissociation or association. However, the caveat is that this experiment involved enzyme activity and not direct studies on the binding and dissociation of labeled drug. Attempts at the latter type of study were unsuccessful, since it is extremely difficult to remove antibody from the enzyme by Millipore filtration or centrifugation, techniques which are employed to separate ^{3}H-digitalis glycoside from the enzyme.

X. Antibodies Which Affect Na^+,K^+-ATPase and the Sodium Pump

It is attractive to consider that at least some membrane surface antigens are membrane components that participate in the control of ion transport. Antibodies against these antigens should prove to be powerful tools in investigating the molecular aspect of transport.

With respect to the sodium pump, antibodies have been used for this procedure. Harris *et al.* (251) reported a procedure for preparing a ouabain-binding membrane fraction from brain in which rough estimates of "pump sites" could be made. Dunham and Hoffman (152), with erythrocyte preparations from high potassium (HK) and low potassium (LK) lambs and using ^{3}H-ouabain, estimated the number of total pump sites, pump fluxes, and mean cell volume and from these values calculated a turnover number (ions pumped per minute per site). The number of pump sites per cell is 40 for HK and 7.6 for LK cells. LK cells are four times more permeable to potassium than HK cells and probably more permeable to sodium as well. The difference in transport rates may be explained by a difference in the number of transport sites measured by simultaneous determinations of ^{3}H-ouabain binding and inhibition of potassium transport. The overall number of the sodium pump in HK adult erythrocytes was about 6000 and in LK adult cells was about 4250 per second.

Evidence reported in 1969 indicates that treatment of LK erythrocytes with antiserum prepared against other LK cells caused a dramatic increase in the potassium pump flux (161). It has also been shown that the antiserum caused an increase in the number of ouabain binding sites per cell (387). In terms of the Dunham-Hoffman hypothesis (152), the antibody could act by converting "leak" sites back to effective pump units. Consistent with this interpretation is the fact that a decrease in leak flux accompanies the increase in the pump caused by the antibody. Also, reticulocytes from LK sheep apparently have a relatively high number of ouabain binding sites (162). The ratio of bound ouabain molecules to erythrocytes at 100% inhibition was 5.5, HK:LK, and the ratio of the normal potassium pump fluxes was 7.2, HK:LK. The similarities in these ratios suggest that the differences in pump fluxes between LK and HK erythrocytes are probably the consequence of factors other than the number of pump sites. The turnover time was 6000 and 4800 for HK and LK cells, respectively, and the results indicate a high specificity of binding of ouabain to pump sites (153). With erythrocyte preparations and ^{3}H-ouabain binding, Hoffman determined that the total number of ouabain molecules bound to a single cell when the pump was inhibited by 100% averaged about 200 molecules per cell. In terms of surface area, this suggests the presence of a little less than one ouabain binding site per square micron of surface. If there is a one

to one correspondence between glycoside binding sites and pumps, that is, one site per pump, then the turnover rate would be approximately 150 ions pumped per site per second. Hoffman (277) reported that binding of glycoside to the membrane was supported by ITP, CTP, and ADP, and these were just as effective as ATP. UTP, GTP, dATP, and dADP also promote glycoside binding, essentially to the same extent. These results are the same as those reported from this laboratory, namely, that "any nucleotide triphosphate and most nucleotide diphosphates will do the job." As Hoffman points out, this is very odd, since the pump as well as the Na^+,K^+-ATPase has an exclusive dependence on purine nucleotides, greatly preferring ATP over ITP. Hoffman reported the inability to bind ^3H-ouabain to his red cell preparation in the presence of nucleotides other than ATP, but now, as indicated above, agrees with our observations.

Lauf and his colleagues (78, 386, 387) carried out an interesting study with anti-L serum prepared by immunization of HK animals with blood from an LK sheep. The serum contained an antibody which stimulated a 4- to 6-fold potassium pump influx in LK sheep red cells. Exposure to anti-L serum produced an almost 2-fold increase in the number of pump sites of the LK cells, as measured by the binding of ^3H-ouabain to LK sheep red cells. This suggests that the formation of the complex between L-antigen and its antibody *stimulates* active transport in LK sheep red cell, both by altering the kinetics of the pump and by increasing the number of pump sites. The action of the antibody was accompanied by an increase in the number of ouabain binding sites on the surface of the red cells, as though the antibody were unmasking latent pump sites. It is also suggested that the antibody might be acting by increasing the selective affinity of the pump sites facing toward the inside of the cell for sodium ions. However, one can argue that the increase in selective affinities for sodium may not be correct, but rather, the ability of the sites to discriminate between sodium and potassium may be altered. It is not necessary to assume that the affinity for sodium or for potassium is changed by the antibody; it is possible that V_{max} is altered.

As indicated in the section above, Glynn and Ellory (224) approached the problem by using fragmented erythrocyte ghosts and measuring ouabain-sensitive ATPase activity instead of ouabain-sensitive potassium influx. They concluded that the antiserum apparently does increase the affinity of the sodium-loading sites of the pump. The increase in affinity for sodium is not accompanied by a change in the affinity of the outward-facing pump sites for potassium. Consequently, the sensitivity to potassium present in the medium is not appreciably affected by treatment with the antibody.

How can antibodies stimulate enzymes? The neutralization of the effect of an inhibitory protein is one way; a direct effect of the antibody on the enzyme is also possible in that the combination of a macromolecule with antibody may induce conformational changes (108). One of the following effects then can be produced by antibody binding: a) hindrance of an active site or part of it, in which substrate or a cofactor or activator is prevented from binding; b) modification (for example, by shielding of charged groups) of some portion of the protein that is critical for determining the correct folding or spatial organization of the macromolecule; or c) modification by a similar mechanism in the environment of the active site, with a consequent change in either direction of the affinity and/or the rate of catalysis for various substrates. These effects can be compared to those induced, with some amino acid residues being altered, by a genetic mutation or by a chemical reaction. Antibody-mediated changes of conformation are easier to visualize when the antibody interacts with a "conformational"

determinant. Enzymes or cofactors or substrates which have been studied with regard to these postulations are lysozyme, ribonuclease, penicillinase, heme proteins (such as cytochrome c, myoglobin and hemoglobin), β-D-galactosidase, and insulin. It is attractive to suggest that the increased enzymatic activity might be due to a configurational change. There is, however, no direct evidence for this. If the same situation with respect to antibody effect on the red blood cell system can be duplicated in a highly purified fragmented preparation, ^{3}H-ouabain binding experiments should be revealing. Ellory and Tucker (161) pointed out that "studies with labeled cardiac glycosides should give further information to elucidate the mechanism of the antiserum-membrane interaction." A study published in 1974 on the mechanism of anti-L-antiserum (554b) revealed: a) in HK cells intracellular potassium acts both as an inhibitor of the pump and at low concentrations actually as a stimulator, and b) anti-L stimulation is probably due to a reduction of the affinity of the intracellular site of the pump for potassium at the site at which potassium acts as an inhibitor (sodium?).

In 1969, Åverdunk *et al.* (37a) reported the isolation of an antiserum against Na^+,K^+-ATPase which inhibited catalytic activity. Askari and Rao (34) in 1972 developed an antiserum to a Na^+,K^+-ATPase derived from rat brain. Interaction of the antibody with the complex caused almost complete inhibition of the Na^+,K^+-ATPase activity but had little or no effect on potassium-dependent *p*-nitrophenylphosphatase activity. In other words, sodium-dependent phosphorylation of the complex by ATP was blocked in the presence of the antibody, but the potassium-dependent breakdown of the phosphoprotein was not affected. These interesting data suggest that the two potassium-dependent activities (*viz.*, *p*-nitrophenylphosphatase activity and potassium-dependent hydrolysis of phosphoenzyme complex) are functions of a component of a complex that is antigenically distinct from a second component involved in the sodium-dependent phosphorylation of the complex by ATP. It would be most attractive to study ^{3}H-ouabain binding in such a system. Jørgensen *et al.* (318) in 1973 provided some interesting data which confirm the studies by Askari and Rao. Antiserum to a Na^+,K^+-ATPase was prepared by immunizing rabbit with a highly active (Jørgensen-Skou medullary preparation) Na^+,K^+-ATPase. The antiserum to the whole enzyme inhibited Na^+,K^+-ATPase, cross-reacted and was incorporated into human erythrocyte preparations. The ouabain-sensitive efflux of sodium into both 15 mM potassium and a potassium-free, high sodium medium was completely abolished. Nonimmune serum had no effect. If the antiserum was applied only to the outer surface, no effect was noted, suggesting, but not proving, that an antibody to a "catalytic" or inner surface "site" was prepared. These investigators stated that "the binding of ^{3}H-ouabain to an ox brain preparation in the presence of ATP plus magnesium plus sodium was also depressed by the immune serum." Fifty milliliters of serum inhibited an amount of enzyme yielding 0.4 μmoles of inorganic phosphate per minute. If the "molecular activity" of this enzyme was as stated, 9000 moles of inorganic phosphate per minute, this amount of enzyme equaled 2.7×10^{13} pump sites. If each red cell has 300 pump sites, 5.6×10^{-16} liters of serum would be necessary to inhibit all of the "catalytic" sites.

In 1973, Smith *et al.* (628) presented some preliminary results of interest in this regard. Antibodies were obtained which specifically interacted with the Na^+,K^+-ATPase isolated from canine renal medulla (prepared by the Kyte procedure) and purified. *Gamma*-globulin from immunized animals, but not from control animals or preimmune serum, inhibited the Na^+,K^+-ATPase activity in a concentration-de-

pendent manner, with maximum inhibition occurring within 5 minutes at 37°C. The magnesium-dependent, nonsodium, potassium-activated and nonouabain-inhibited component of activity (in other words, the Mg^{2+}-ATPase) was unaltered. The antibodies also produced a concentration-dependent inhibition of canine myocardial microsomal and human erythrocyte ghost Na^+,K^+-ATPase activities up to about 50 to 98%, respectively. Control and preimmune *gamma*-globulin fractions had no effect on canine myocardial or human erythrocyte Na^+,K^+-ATPase. It is of interest that the magnesium-dependent ATPase was unaltered by all antisera preparations, including those that had marked Na^+,K^+-ATPase inhibitory activity. Despite the marked inhibition of Na^+,K^+-ATPase activity in these preparations, the experiments with canine renal slices and human red cells showed no specific effect of the antibody on ouabain-inhibitable radiolabeled rubidium uptake, indicating a lack of inhibition of active monovalent cation transport in the intact cell (Smith, personal communication). The experiments, according to these investigators, demonstrated immunological cross-reactivity among Na^+,K^+-ATPases from different organs and different species. In addition, the data from the experiments indicate that the antibody response is directed against an antigenic determinant inaccessible to macromolecules at the outer cell surface. Again, this intriguing study, along with the above information, suggests the usefulness of continuing investigations with purified enzyme preparations and ^{3}H-ouabain.

In a study reported in 1974 (441) rabbits were immunized with a highly purified Na^+,K^+-ATPase prepared from canine renal outer medulla by the method developed by Lane *et al.* (377). This enzyme preparation consists of two polypeptides in a molar ratio of one and contains "supporting" lipids. Some weeks later the animals were bled, and the serum was fractionated by ammonium sulfate precipitation, yielding an immune globulin fraction. A concentrated suspension of Na^+,K^+-ATPase (in excess) was added to the immune globulin. After incubation, the enzyme-antibody complexes were recovered by centrifugation, and the antibodies specifically bound to the Na^+,K^+-ATPase preparation were recovered by incubation of the preparation at pH 2.8. The latter treatment solubilized the antibodies and allowed separation by centrifugation of the antibodies from the insoluble Na^+,K^+-ATPase. Upon dialysis of the antibodies at pH 7.4, the antibodies were once again exposed to the Na^+,K^+-ATPase, but this time the enzyme had been reacted previously with a high concentration of ouabain. Enzyme-^{3}H-ouabain complex and bound antibody were separated from unbound antibody by centrifugation, and the supernatant containing unbound antibody was decanted and saved. The bound antibody was eluted from the enzyme-^{3}H-ouabain complex by incubation at pH 2.8 and centrifugation, as described above. The antibody eluted from enzyme-^{3}H-ouabain complex was designated as "anticatalytic antibody" (anti-cat) and that which did not bind to the complex was designated as "anti-digitalis receptor antibody" (anti-DR), for reasons which will become apparent.

Antiserum from rabbits immunized against Na^+,K^+-ATPase inhibited catalytic activity of the enzyme up to 80%. This effect of antiserum to Na^+,K^+-ATPase is in agreement with those reported by Askari and Rao (34), by Jørgensen *et al.* (318), and by Smith *et al.* (628). The control serum had no effect. When the Na^+,K^+-ATPase antibody was added to enzyme that was actively turning over (using the linked-enzyme assay method), the inhibition produced by the antibody was time-dependent, reaching a maximum within 8 to 10 minutes. As judged by the amount of antiserum protein required to reduce catalytic rate by 50%, these procedures yielded a 58-fold purification of the anti-

catalytic antibody. Similar levels of inhibition were obtained by antisera and anticatalytic antibody, but complete inhibition of catalysis was not observed under these experimental conditions. In more recent experiments, 90% inhibition has been found.

When the antibody eluted from Na^+,K^+-ATPase was incubated with the enzyme-^{3}H-ouabain complex, approximately one-half of the antibody protein did not bind to the complex and was recovered in the supernatant, as described above. This fraction, antidigitalis receptor antibody, had little inhibitory effect upon catalytic activity of Na^+,K^+-ATPase when incubated with enzyme under turnover conditions. The theory involved in these experiments is simple. The antibody to the holoenzyme consists of various components, one of which is specific to the digitalis "site." Incubation of the antibody with a completely blocked enzyme should reveal the glycoside receptor component. The latter should not bind to the inhibited enzyme. Antidigitalis receptor antibody did inhibit ^{3}H-ouabain binding to the enzyme in the presence of magnesium plus inorganic phosphate. The antibody which bound to the enzyme-^{3}H-ouabain complex (anticatalytic antibody) did not inhibit ^{3}H-ouabain binding despite its inhibitory effects on catalysis. Similarly, whole antiserum, immune globulin, and antibody eluted from the enzyme (*i.e.*, not previously reacted with ouabain) did not inhibit ^{3}H-ouabain binding.

Antidigitalis receptor antibody inhibited ^{3}H-ouabain binding supported by magnesium plus ATP plus sodium or by magnesium plus inorganic phosphate but more so in the latter case. It should be noted that the enzyme-ouabain complex used to separate anticatalytic antibody and antidigitalis receptor antibody in these experiments was formed in the presence of magnesium plus inorganic phosphate. Anticatalytic antibody had no effect upon magnesium plus inorganic phosphate-induced binding but appeared to *increase* the binding supported by magnesium plus ATP plus sodium above that obtained in the control. However, the level of control binding obtained with magnesium plus ATP plus sodium was lower than that with magnesium plus inorganic phosphate, and the apparent increase produced by anticatalytic antibody was only to the level obtained in the presence of magnesium plus inorganic phosphate. This suggested that the anticatalytic antibodies may act to produce a conformation more conducive to ^{3}H-ouabain binding than was present with magnesium plus ATP plus sodium as ligands. It also indicates that the lack of an inhibitory effect of anticatalytic antibodies upon binding was not due to the inability of the antibody to bind to the enzyme under the ligand conditions described.

The results of these studies indicate that the antiserum produced by immunization with a highly purified Na^+,K^+-ATPase contains a complex mixture of antibodies which differ with respect to the effects produced upon the enzyme. Presumably, such differences reflect the fact that individual "families" of antibodies are specific for different regions of the enzyme molecule(s) and/or for different conformations. We and others have shown that the Na^+,K^+-ATPase is a complex, conformationally altered enzyme, and that ouabain is an allosteric-type inhibitor (see part V, section E, and ref. 577). The results with the antibodies reinforce this concept. These results also indicate the feasibility of separating the individual antibodies by absorbing an antibody fraction onto enzyme (antigen) which has been altered such that certain determinants are no longer available to the antibody (either because the ligands cover the site or induce an alteration in the conformation of the determinant). These data provide the first direct evidence that the digitalis binding site or conformation is different from the "catalytic" center (577, fig. 13).

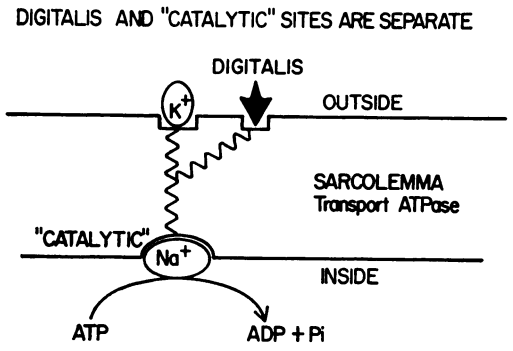

FIG. 13. Diagrammatic representation of differences in digitalis and "catalytic" sites on the sodium pump.

In 1974, Kyte (371a) reported the preparation of a ferritin conjugated immunoglobulin to the catalytic subunit of the Na^+,K^+-ATPase. By using influenza virus which reacts only with the cell surface, Kyte showed that the antibody to the catalytic polypeptide bound only to the inner surface and did not affect enzyme activity. These results, Kyte feels, eliminate any mechanism for enzyme action that involves relatively large movements of the protein through the bilayer and consequently are inconsistent with a diffusional carrier model for active transport of sodium and potassium.

XI. Relationship between the Na^+,K^+-ATPase and Adenylate Cyclase

Some years ago, turnover of phosphorylserine in phosphoproteins associated with membranes was observed in intact tissues (256). In particular, it was found that electrical pulses of respiring cerebral cortical slices caused an increase in the turnover of the protein-bound phosphorylserine of a fraction located in the neuronal membrane (257, 673). This suggested the concept that a membrane phosphoprotein is directly involved as a carrier in the active transport of sodium, in a manner that was previously suggested for phosphatidic acid 284, 287, 288). Further studies of phosphoprotein as well as phospholipids have not substantiated this hypothesis (17). The increased turnover on electrical stimulation may, however, be related to the increased level of tissue cyclic-AMP, and, therefore, somehow be related indirectly to the Na^+,K^+-ATPase. Both the adenylate cyclase and Na^+,K^+-ATPase are believed to be associated with the cell membrane. In 1969, Moszik (448) compared the Na^+,K^+-ATPase and adenylate cyclase isolated from rat heart, rat stomach and human gastric mucosa with respect to the effects of a variety of drugs. In general, he found opposing effects of some drugs and specific effects of others and concluded the two enzyme systems are separate but that stimulation of the cyclase is usually associated with blockade of the ATPase. A direct inhibitory effect by cyclic-AMP and 5′-AMP on Na^+,K^+-ATPase from human mucosa was observed. An antagonistic relationship between the cyclase and ATPase was suggested.

There appear to be three general mechanisms by which the adenylate cyclase and Na^+,K^+-ATPase activities might be related. The first is that they are the function of a common membrane-bound protein catalyzing the two reactions, one at fast rates (Na^+,K^+-ATPase) and one at slow rates (adenylate cyclase). This seems to be a remote possibility but cannot be completely eliminated on the basis of existing evidence. It is clear that both activities are functions of ligands in the incubation medium and the lipid composition of the membrane, and both activities can be modified by association with other proteins in or on the membrane. Thus, one activity might be activated at the expense of the other depending upon the microenvironment.

A second means of interrelationship may be through the intramembranal transfer of information between two different and physically separated proteins in the membrane which catalyze the two reactions. A possibility in this respect might be a

change in the state of the lipids around one or the other of the enzymes upon activation or inhibition.

The third possibility is that a product of one of the reactions modulates the activity of the other system. Specifically, cyclic-AMP, formed from ATP by adenylate cyclase, could activate protein kinase such that phosphorylation and, consequently, modulation of the Na^+,K^+-ATPase might be achieved.

It is well known that calcium affects both the ATPase and the cyclase. Goodman *et al.* (235) showed that the neurotubular proteins subunit is a natural substrate for a cyclic AMP-dependent protein kinase in brain. It was suggested that phosphorylation of microtubules is followed by activation of the phosphoprotein by calcium in a manner similar to what is thought to occur in heart, *i.e.*, calcium and cyclic-AMP may both be "messengers" (516). It is of interest that one of us (573) observed some time ago that ouabain stimulated oxygen consumption of isolated cerebral cortical slices concomitant with an inhibition of active sodium-potassium transport but only in the presence of calcium. A link, albeit indirect, between cAMP-dependent protein kinase-catalyzed phosphorylation, adenylate cyclase, and the Na^+,K^+-ATPase is intriguing. In this regard, Himms-Hagen (270) in 1970, with adipose tissue, proposed that inhibition of the Na^+,K^+-ATPase by ouabain or digitoxin or by potassium deficiency may be associated with a loss of sensitivity of the cyclase system to stimulation by epinephrine or cAMP, and with an increased sensitivity to inhibition by prostaglandin (PGE_1). Structural changes may modify the properties of the cyclase system. Inhibition of the cyclase by stimulation of *alpha*-receptors (by epinephrine) may be associated with inhibition of the Na^+,K^+-ATPase, at least in fat cells.

Shimizer *et al.* (593) found a marked stimulation of cAMP when cerebral slices were incubated with depolarizing agents such as batrachotoxin, veratridine, potassium, or ouabain. The process required calcium and was inhibited by theophylline. Forte (184) isolated a plasma membrane fraction from rat kidney that contained adenylate cyclase and Na^+,K^+-ATPase activities, but Rodnight and Weller (545) found that cAMP had no effect by itself on Na^+,K^+-ATPase, and that a serine hydroxyl group is probably not involved in the hydrolysis of ATP by the Na^+,K^+-ATPase. Hegyvary and Post (259) and Post *et al.* (495) found that a nucleotide binding site associated with the Na^+,K^+-ATPase had an extremely low affinity for cAMP. Moreover, Weller and Rodnight (690) found that the turnover of any phosphoprotein in the membrane is too slow to account for the rapid changes in permeability in sodium that must accompany the depolarization event. The latter suggested, however, that an intrinsic protein kinase (inside of membrane) may control phosphorylation of a membrane protein involved in the "passive permeability" of the membrane to various cations.

A relationship between calcium, Na^+,K^+-ATPase and adenylate cyclase is consistent with the data of Shlatz and Marinetti (596, 597), Weiss *et al.* (689), Wolff and Jones (711), Guthrow *et al.* (244), Weller and Rodnight (691), Roses and Appel (549), Forte *et al.* (185), Kant and Steck (335), and with the suggestions of Barnabei *et al.* (60) who in 1972 reported an inhibition of cAMP on Na^+,K^+-ATPase isolated from rat liver.

The reports published nearest to the time of this writing with respect to the heart Na^+,K^+-ATPase are those by Krause *et al.* (360) and Will *et al.* (704) in 1973 from the laboratory of Wollenberger. With a Na^+,K^+-ATPase isolated from pig heart by the method of Matsui and Schwartz (432), these investigtors found an endogenous protein kinase that phosphorylates a 20,000 dalton component. Moreover, they found that this ATPase preparation bound calcium to two classes of sites, one class

possessing "high affinity" (6.4×10^{-8} M) and one "low affinity" (1.1×10^{-6} M). After phosphorylation, the affinity of the "high affinity" site for calcium increased 4-fold. The endogenous protein kinase did not phosphorylate histone until the membranes were solubilized by treatment of the Na^+,K^+-ATPase preparation with 0.2% Triton X-100. We stress caution in interpretation since a 20,000 dalton component of isolated cardiac SR (see ref. 339) and cardiac troponin has been reported to be phosphorylated by protein kinase and both can be contaminants of the Na^+,K^+-ATPase preparation. From our experience with this ATPase preparation, it is difficult, if not impossible, to draw definitive conclusions about these types of data. The preparation at best is 5 to 7% pure. The results are, however, very provocative, particularly after reviewing the authors' calculations that the number of "binding sites" for calcium affected by phosphorylation is similar to the calculated number of "sodium binding sites," *viz.*, 0.3 nmol/mg protein. Unfortunately, the preparation is too impure to be certain that the authors were not measuring nonspecific sites not associated at all with the Na^+,K^+-ATPase.

We have, independently, carried out a detailed study of protein kinase-catalyzed phosophorylation of Na^+,K^+-ATPase preparations ranging in purity from low to very high. Beef brain and heart and dog kidney and submaxillary gland were the sources. Endogenous protein kinase activity was associated with all the preparations except with the highly purified enzyme from the outer medulla of the canine kidney. Although we found that cAMP stimulated phosphorylation of endogenous substrates, we could not confirm the specificity of a 20,000 dalton component, *i.e.*, multiple components were phosphorylated. The highly purified preparation contained no endogenous protein kinase, but substrates were present, associated in part with the 93,000 dalton "catalytic" component (see section IV). It is of further interest that our semipurified ATPase preparations bound specifically 600 pmol of phosphate derived from ATP [hydroxylamine-insensitive], catalyzed by cAMP-dependent protein kinase, whether the ATPase was active, heat-inactivated, or inhibited with high concentrations of ouabain. This is in contrast to the report by Krause *et al.* (360) which indicated that only 8 pmol of phosphoprotein were formed. It is possible that the "impure" nature of the ATPase employed by these investigators explains the low activity. A word of caution in interpretation of phosphorylation: Membrane fragments from heart muscle are particularly heterogeneous (which may be a euphemism for impure). Although it is attractive to think of a specific relationship between the Na^+,K^+-ATPase and other membrane-bound enzymes such as adenylate cyclase and protein kinase, vigorous criteria must be adhered to before specificity is assigned.

Alexander and Rodnight (21) in 1974 re-examined the possible relationship between a cAMP-dependent endogenous protein kinase and Na^+,K^+-ATPase in a membrane preparation from ox brain. In the absence of sodium, a hydroxylamine-stable labeling that was stimulated by cAMP was found, but not in the 102,000 dalton "catalytic" subunit. The authors suggest that the intrinsic protein kinase and the substrate (a serine residue) are in protein(s) ". . . unconnected with the Na^+,K^+-ATPase." Their conclusion is based on the fact that 3 mM di-isopropyl phosphorofluoridate, a serine-reacting reagent of limited specificity at best, inhibited the Na^+,K^+-ATPase by 70%, whereas the reagent had no effect on the protein kinase activity, although 1 mM $MnCl_2$ inhibited the kinase by 25% and yet had no effect on the Na^+,K^+-ATPase. cAMP had no effect on the Na^+,K^+-ATPase preparation. However, the authors did state that a serine residue is involved at the active center of the enzyme (because di-

isopropyl[^{32}p]-phosphorofluoridate was present in the 102,000 subunit, and the labeling was diminished by 3 mM ATP), and that it may be involved in the binding of ATP to the enzyme before phosphorylation. However, most of the *labeling* of the reagent was found in proteins "unassociated with the Na^+,K^+-ATPase . . .". Although these experiments are interesting, the conclusion that the cAMP-dependent protein kinase is not involved in the modulation of Na^+,K^+-ATPase is not based upon conclusive data. The reagent employed is quite nonspecific. One of the binding "conformations" for digitalis glycosides may be a phosphoenzyme of low energy. It is worth examining the possible relationship between protein kinase-catalyzed phosphorylation and digitalis-enzyme interaction.

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