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Interaction of Cardiac Glycosides with ($Na^+ + K^+$)-Activated ATPase. A Biochemical Link to Digitalis-Induced Inotropy

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I. Introduction

A. Cardiac Glycosides and the Sodium Pump

THE NOW classic description by William Withering (174) of the use of foxglove in the treatment of several symptoms originating from chronic congestive heart failure appeared in 1785. Nearly two centuries would pass before the first indication emerged about the mechanism of action of digitalis on mammalian tissues. A loss of intracellular potassium was known to take place from cardiac as well as skeletal muscles during treatment with cardiac glycosides, but usually an effect on energy metabolism was sought [for references, see Schatzmann (138) and Glynn (51)]. A real breakthrough came in 1953 when Schatzmann (138) published his observations on the effect of the drugs on red cells. Neither respiration nor glycolysis of erythrocytes was markedly affected by the concentrations of cardiac glycosides which efficiently inhibited the active transport of potassium and sodium. Schatzmann thus concluded that the glycosides probably were bound to a putative carrier-enzyme which was assumed to be responsible for potassium and sodium transport against electrochemical gradients. Glynn (51) in 1957 described that in human red cells some downhill fluxes of monovalent cations were inhibited by cardiac glycosides, e.g. Na^+-influx in the absence of external K^+, suggesting that they acted on the transport mechanism itself rather than on the energy supply to the pump. He was even able to give an estimation of the number of binding sites for the glycoside, scillaren, per red cell. The number of a few hundreds per cell seemed remarkably low at that time, but was later proved to be correct.

With the technique of reversible hemolysis Gardos (48) observed in 1954 that only Na^+, Mg^{2+} and ATP needed to be introduced in resealed erythrocyte ghosts for K^+-accumulation to take place. The uptake of K^+ against an electrochemical gradient was thus energized by ATP in the absence of glycolysis. A new and quite different approach to the study of the membrane transport mechanism was initiated with the discovery by Skou (152) of the ($Na^+ + K^+$)-activated ATPase associated with plasma membrane fragments. An essential argument that the microsomal ATPase-activity in the presence of Na^+ as well as K^+ was the enzymatic expression of the

sodium pump of intact cells, turned out to be that both were specifically inhibited by G-strophanthin (ouabain) (34, 153).

The present review deals with some of the information on the Na,K-ATPase and the specific transport system for monovalent cations which can be obtained from studies with digitalis and especially ouabain. Some new observations with the less specific but equally potent inhibitor, vanadate, are considered as well since they appear to give much useful information about the digitalis-ATPase complex.

Most investigators engaged in Na,K-ATPase research now agree that the positive inotropic action of cardiotonic drugs as well as their toxic effects are intimately related to their binding to the sodium pump. This view is based on circumstantial evidence ranging from a long list of factors that influence digitalis binding to Na,K-ATPase and the development of positive inotropy in the same direction [review by Akera (6)] to the tight correlation between ouabain binding and increase in force of contraction of the heart [review by Erdmann (41)]. Smith and Barry in their overview (160) of unitary versus pluralistic views on digitalis-induced inotropy tentatively concluded that inhibition of monovalent cation transport is directly and causally related to the positive inotropic effect of cardiac glycosides. Observations of lack of correlation between pump inhibition and increased force of contraction have, however, been collected by Noble (115). A possible insight into some of the apparent contradictions has been gained recently and will be dealt with later on.

It is widely thought that the direct link between sodium pump inhibition and the increase in contractile force of heart muscle is an increase in the intracellular calcium concentration transient following membrane excitation, the sequence being an increased intracellular Na^+-concentration leading to increased Na^+/Ca^{2+} exchange or, alternatively, a displacement of Ca^{2+} from membrane-associated Ca^{2+}-pools. Direct experimental evidence for an augmented intracellular calcium concentration transient in frog heart muscle occurring just prior to the mechanical contraction in response to the cardiac steroid acetylstrophanthidin was provided by Allen and Blinks (11) with the photoactive calcium-sensitive protein, aequorin. A similarly increased Na^+-concentration transient has not been demonstrated, but this is possibly due to the insenisitivity or the latency of the present Na^+ sensitive electrodes (5).

Whether the relationship between glycoside binding to Na,K-ATPase and inotropic action is causal or coincidental, it remains valid that this enzyme is the only known receptor of the cardiac glycosides and factors that influence their interaction are fundamental for the subsequent cardiotonic action. This review deals with the reactive state of the Na,K-ATPase seen from the point of view of the cardiac glycosides, i.e. their accessibility to their receptors. The still speculative steps by which an inhibition of the sodium pump eventually leads to positive inotropy is outside the scope of this review.

B. Ouabain as a Tool in Na,K-ATPase Studies

Ouabain at a low concentration does not just bind to an Na,K-ATPase enzyme preparation suspended in a suitable buffer. Some ligands and substrates necessary for enzyme activity and pump function or products of such processes have to be present. They facilitate or promote ouabain binding. Other ligands should preferably be absent since they inhibit binding. This does not rule out the use of ouabain in experimental situations without the addition of these factors, e.g. to define hydrolysis not dependent on $Na^+ + K^+$. Maximum pump inhibition will nevertheless be achieved at rather higher ouabain concentrations, probably due to minor contaminations with facilitating factors and the law of mass action.

If specified requirements are met, ouabain binding takes place with very high affinity and with most enzyme preparations stable enzyme-ouabain complexes are obtained. The complexes, although generally stable and thus suitable for handling, can be characterized according to their rate of decay.

The dependence on a number of factors for the apparent affinity of ouabain for Na,K-ATPase and relatively stable enzyme-ouabain complexes are prerequisites for using ouabain to deduce ligand-enzyme interaction and to characterize some intermediary steps in $(Na^+ + K^+)$-dependent ATP hydrolysis. The ouabain binding capacity can be used for quantification of the Na,K-ATPase based on the stochiometric relationship between binding capacity and hydrolytic activity, number of α-peptides per weight unit or other parameters of the enzyme.

The object of the following account will be to describe the use of ouabain in studies on: 1) ligand interaction with Na,K-ATPase as deduced from changes in ouabain binding; 2) intermediary steps in ATP hydrolysis based on enzyme-ouabain stability; 3) homogeneity or heterogeneity of a given enzyme harvest and, related to this problem, subunit-subunit interaction; 4) quantification of a given enzyme preparation or the number of sodium pumps in intact tissues.

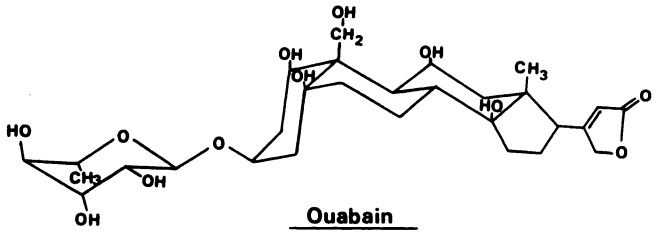
C. Other Aspects of the Na,K-ATPase and the Digitalis Glycosides

The present review deals primarily with the information on Na,K-ATPase that can be obtained from binding studies of cardiac glycosides. Readers interested in a more extensive and general treatment of the literature on Na,K-ATPase and the sodium pump are referred to earlier reviews (22, 53, 54, 89, 91, 92, 117, 131, 143, 145, 154, 156).

The term Na,K-ATPase is usually applied to more or less purified preparations where the enzyme is still attached to a fragment of the plasma membrane. In most

of the studies quoted the more hydrophilic cardiac glycoside ouabain has been used; in some, however, the more lipophilic glycosides were used. Unspecific binding of ouabain to membrane fragments is negligible whereas such binding of the more lipophilic glycosides may complicate the analysis (181). On the other hand, with intact cells internalization of ouabain does take place during prolonged incubation with sublethal doses, probably in parallel with the normal turnover of Na,K-ATPase (124).

The very interesting aspects of the stereochemistry of cardiac glycosides (see the structural formula of ouabain), the special steroid backbone of the aglycones with cis-fusion at two of the ring junctions where most other steroids have transconfiguration, the attached lactone ring at C_{17} and the attached sugar components at C_3 characterizing the various cardioactive drugs is likewise outside the scope of this presentation [see e.g. Henderson (78); Repke and Portius (130)]. From the structure-activity relationship of aglycones and cardiac glycosides and their dissociation rates from the receptors, models of the spatial arrangement of the receptor binding sites for several components of the drugs have been proposed (2, 38, 129, 173, 176–178).



Ouabain

II. Requirements for Ouabain Binding to Take Place

During the decade after the discovery that cardiac glycosides were specific inhibitors of the sodium pump (138) several controversial observations on ATPase-glycoside interaction, as deduced from inhibition of cation transport and ATP-hydrolysis, were made. Even after the introduction of isotopically labeled glycosides, the interpretation of a number of observations on ATPase-glycoside interaction remain controversial.

In his early, comprehensive work on the action of cardiac glycosides on cation movements in red cells, Glynn (51) suggested that a reversible sodium pump-glycoside interaction took place since only submaximal inhibition was demonstrated at low inhibitor concentration. By increasing the external potassium concentration, inhibition was completely reversed at low glycoside concentration thereby suggesting competitive K^+ and glycoside interaction with the pump. Simple competitive interaction of potassium and strophanthin was later denied (34) since the reversal by K^+ of the ATPase inhibition of red cell ghosts caused by higher strophanthin concentrations was incomplete. At increasing K^+-concentration the ATPase activity levelled off far below full reactivity. Also Schatzmann (139) noticed that high external K^+-concentration was unable to overcome the ouabain effect on enzymic activity of resealed ghosts.

These and other early observations raised some questions on the nature of ouabain binding to the enzyme i.e.: 1) Is enzyme phosphorylation necessary for ouabain binding? 2) Do ouabain and K^+ compete for the same site? 3) Is enzyme-ouabain interaction reversible?

A. The Na,K-ATPase Conformation Necessary for Ouabain Binding

The first indication that ouabain was preferentially bound to the phosphorylated Na,K-ATPase appeared when Charnock and Post (24) showed that phosphorylation of kidney enzyme from $AT^{32}P$ in the presence of Na^+ took place independently of the presence of ouabain, whereas dephosphorylation by K^+ was blocked by the drug. The observation was confirmed by Gibbs et al. (50) with a beef brain preparation except that a partial inhibition of phosphorylation was also seen.

With the availability of labelled cardiac glycosides Matsui and Schwartz (110) noted the similarity of the increase and decrease of both the digoxin binding and the phosphorylated intermediate of the ATPase under various conditions. They, therefore, suggested that digoxin was bound to the phosphorylated conformation of the enzyme. The binding required ATP or another nucleotide and Mg^{2+}, was stimulated by Na^+, and depressed by K^+. Also acetyl phosphate could facilitate digoxin binding. In a subsequent publication the same group (144) reported that inorganic phosphate (P_i) could also act as a cofactor for digoxin binding in the presence of Mg^{2+}, but in this case Na^+ as well as K^+ inhibited binding. They also noticed that some binding with the same characteristics took place in the presence of Mn^{2+} or Mg^{2+} only, and to a minor degree with a few other divalent cations. They, therefore, suggested that the conformational state of the enzyme rather than the phospho- or dephosphoform of the enzyme was of significance for glycoside interaction. The observations were confirmed with ouabain by Albers et al. (8), who were able to demonstrate directly an incorporation of $^{32}P_i$ into ouabain treated microsomal ATPase from Electrophorus electric organ. A remarkably good glycoside binding was observed in the presence of Mg^{2+}, Na^+ and ADP by various authors (8, 110, 144) and presumably binding occurred to the pump whether phosphorylated or not (8). In reporting a study on ouabain inhibition of Na,K-ATPase Sen et al. (149) emphasized that the enzyme reacted with ouabain either before or after phosphorylation by $AT^{32}P$, in the first case preferentially at low ionic strength, the absence of monovalent cations, and accelerated by $Mg^{2+} + P_i$. The nucleotide-phosphorylated enzyme was ouabain sensitive even in the presence of Na^+, but gradually lost phosphate with no decrease in the extent of inhibition. This also means that the ouabain-bound dephosphoenzyme was resistant to rephosphorylation by $AT^{32}P$, but, remarkably, it might be phos-

phorylated by $^{32}P_i$. Tobin and Sen (164) suggested that the Na^+-stimulated pathway for ouabain binding in the presence of ATP was dependent on phosphorylation and that the apparent ADP facilitation of ouabain binding could be explained by an adenylate kinase activity also exhibited by most membrane preparations. A similar explanation for the promotion of ouabain binding by ADP was discussed by Skou et al. (155) although they also maintained that the ATP-bound form of the enzyme might react with ouabain. A similar conclusion was reached by Schönfeld et al. (148) based on the very low Mg^{2+}-requirement for ouabain binding in the presence of Na^+ + ATP, which could even be replaced by ATP dialdehyde which is thought not to be hydrolyzed by Na,K-ATPase.

In a study on ATP, ADP, and ouabain binding to Na,K-ATPase from ox brain, Hansen et al. (62) found equal binding capacities for the nucleotides and the glycoside and, moreover, that ouabain binding as facilitated by Mg^{2+} and P_i, prevented subsequent nucleotide binding. The opposite was also shown, i.e. that nucleotide binding prevented ouabain binding under conditions where phosphorylation scarcely took place. If adenylate kinase activity was minimized by omission of Mg^{2+} some phosphorylation and ouabain binding still occurred in the presence of Na^+ + ATP but to a much smaller extent in the presence of Na^+ + ADP. With ouabain binding at equilibrium achieved in the presence of Mg^{2+} + P_i addition of ATP or ADP could be shown to diminish glycoside binding, probably via occupation of non-ouabain-bound enzyme. Siegel and Josephson (151) also observed inhibition of ouabain binding by an excess of free ATP and ADP. They suggested that MgATP was the substrate necessary for glycoside binding and that ATP inhibited by competing with MgATP or by decreasing free Mg^{2+}. It was, however, also shown (62) that addition of ethylene diamine tetraacetate (EDTA) in concentrations that simulated the chelation of Mg^{2+} by ATP did not promote ouabain dissociation.

The question whether nucleotide binding per se or phosphorylation is the prerequisite for high affinity ouabain binding seemed solved when Tobin et al. (165, 166) showed that β,γ-methylene ATP, which is resistant to hydrolysis would not facilitate ouabain binding and even inhibited the binding obtained with Mg^{2+} or Mg^{2+} + P_i.

In their pioneer contribution on conformational states of Na,K-ATPase, Post et al. (125) observed that ouabain was preferentially bound to the phosphorylated E_2 conformation of the enzyme. In the so-called Albers-Post Scheme [Albers (7); Post et al. (125)] E_1 designates the form which binds ATP and/or Na^+ and which in the presence of nucleotide, Mg^{2+} + Na^+ is phosphorylated to E_1P. The latter form is dephosphorylated by ADP. The E_1 or E_1P forms are converted to another conformational state, E_2 or E_2P, in the continued presence of Mg^{2+}. E_2P is dephosphorylated by K^+. In a later version the principal phosphorylated conformations of Na,K-ATPase are designated $E_1 \sim P$ and E_2-P of which the first form can donate its phosphate reversibly to ADP and the second one exchanges P_i with water (128). Confirmation of the reactive states was later obtained in studies on graded trypsin digestion of the catalytic protein of Na,K-ATPase in the presence of different ligands (90) and by the use of extrinsic fluorescence probes and intrinsic tryptophan fluorescence as tools (74, 94, 95, 157, 158). Whether they are relevant to transport models for monovalent cations is another question. The classical "consecutive" model for the hydrolysis of ATP by Na,K-ATPase implicating a sequence through the reactive states mentioned above is based upon the results of extreme and simplified experimental situations. Serious doubt has been raised as to whether the model can account for the rate of hydrolysis under more physiological conditions. It is probable that an alternative route of hydrolysis has to be added (100, 117) and that the main flux does not involve an acid-stable phosphorylated intermediate (123). The consequences of such a model for the binding of ouabain under in vivo conditions has not been worked out.

By using a gelfiltration method for trapping unbound ouabain Yoda and Yoda (182) confirmed that ouabain is preferentially bound to the K^+-sensitive phosphoprotein (E_2P) of electric eel Na,K-ATPase. At Na^+-concentrations where the ADP-sensitive phosphoprotein (E_1P) predominated, ouabain binding was much reduced. The authors suggested that this binding was due to conversion of E_1P to E_2P by ouabain and that E_1P did not accept ouabain.

Seen in the light of the $E_1 \rightleftharpoons E_2$ conception of Na,K-ATPase mutual exclusion of ATP and ouabain binding should be expected if ouabain is bound to the E_2-conformation (62). Later experiments have shown that ouabain binding does not exclude low affinity ATP-binding, since release of ouabain from the enzyme-ouabain complex was accelerated by ATP, especially in combination with Na^+ (167, 179). Low-affinity ATP binding to the E_2 conformation is also in keeping with a supposed accelerating role of nucleotide in potassium dissociation (126), the sequence being K^+-acceleration of dephosphorylation and ATP acceleration of K^+ dissociation from the resulting E_2K. Jensen and Ottolenghi (83) provided experimental evidence for low-affinity nucleotide binding to K^+-complexed enzyme and Ottolenghi and Jensen (119) showed that ouabain-complexed enzyme did bind ATP though with much reduced affinity.

The question as to whether the phosphorylated E_2-P form is needed for ouabain binding is unsolved so far, since some contamination with P_i and other substrates is inevitable. It is safe to conclude, however, that the apparent ouabain affinity in the presence of Mg^{2+} only is rather low compared to the situation with Mg^2 + P_i or Mg^{2+} + Na^+ + ATP (36, 63). Congeners of phosphate,

e.g. arsenate and vanadate, may substitute for P_i in promotion of glycoside binding (67, 144) and in a later section dealing with vanadate the question whether Mg^{2+} will suffice for ouabain binding to take place is examined.

B. *The Potassium-Ouabain Antagonism*

As to the question of the role of K^+ in ouabain binding to Na,K-ATPase it is quite clear that it does not fit into the $E_1 \rightleftharpoons E_2$ conception in a simple way. K^+ as well as ouabain binding take place to an E_2-conformation in spite of the K^+-ouabain antagonism. One explanation of the paradox could be the role of K^+ for dephosphorylation, at least in the $Mg^{2+} + Na^+ + ATP$ promoted pathway for ouabain binding. The role of K^+ with respect to P_i-phosphorylation is not so clear. In $^{32}P_i$-phosphorylation experiments with Na,K-ATPase from guinea pig kidney it was shown that the steady level of phosphorylation was lower in the presence of K^+ and that the rate of dephosphorylation was more rapid (128). With Na,K-ATPase from electric eel organ an increase in degree of phosphorylation was seen in the presence of K^+ (103). A rapid Na,K-ATPase catalyzed exchange of water oxygen with P_i was observed in the presence of ($Mg^{2+} + K^+$) which was interpreted as equilibration of the K^+-phosphoenzyme with P_i (32).

A steady state level of phosphoenzyme thus seems to exist in the presence of P_i, Mg^{2+}, and K^+; and ouabain binding could be expected to take place if the phosphorylated E_2-conformation is the only requirement for binding. Vanadate, which has some similarity to phosphate, is preferentially bound to Na,K-ATPase in the presence of K^+ as well as Mg^{2+} (85). The vanadate-complexed enzyme will accept ouabain with high affinity, but this binding is counteracted by K^+ (67). In section V the aspect of the membrane from which K^+ counteracts ouabain and promotes vanadate binding is discussed.

The K^+-ouabain antagonism is thus not simply due to K^+-induced dephosphorylation, but can be explained by a reduced affinity of the K^+-complexed phosphoenzyme or vanadate bound enzyme for ouabain. On the other hand, the K^+-complexed enzyme does bind ouabain, but with reduced affinity. In ouabain binding experiments it can be shown that the equilibrium binding at increasing K^+-concentration first decreases and then levels off (63). The final binding level reached above a certain K^+-concentration is dependent on the ouabain concentration. At high glycoside concentration hardly any inhibition of ouabain binding by K^+ is seen.

These considerations on the effect of K^+ on ouabain binding refer to the equilibrium situation, whereas the rate of binding is definitely reduced by K^+ (3, 105, 148). Since the ouabain binding affinity is decreased, but the binding capacity is unchanged by the presence of K^+ (36, 63) it appears that the crucial point as to whether the extent of ouabain binding is reduced or not by K^+ is simply dependent on the ouabain concentration used. At a high ouabain concentration one may have the impression that K^+ does not interfere with ouabain binding (16). It does, however, interfere at nonsaturating ouabain concentrations and this can be shown by addition of K^+, after having established binding equilibrium in the absence of K^+, when release of bound ouabain is then seen (26, 61, 63). The stabilization of the enzyme-ouabain complex by K^+ seen under some conditions (section IV) clearly shows simultaneous K^+ and ouabain binding. On the other hand, as already mentioned, there are indications that ouabain binds to the K^+-enzyme complex with reduced affinity, i.e. the inhibition is not simply competitive and cannot be for the same site. Han et al. (60) arrived at a similar conclusion, since K^+ was much more effective in reducing the steady state level of phosphoenzyme than the equilibrium level of ouabain binding.

C. *Reversibility of Ouabain Binding*

Some of the observations discussed above imply that ouabain binding to Na,K-ATPase is reversible, e.g. the nucleotide effect on an established equilibrium binding level of ouabain in the absence of Mg^{2+} or the K^+-effect on the equilibrium binding level. Since only submaximal inhibition of Na,K-ATPase activity was seen at low glycoside concentration Glynn's data (51) suggested that the binding should be reversible. In studies with different cardiac glycosides (175) and with ^3H-labeled ouabain (8), it was noticed that the interaction of glycosides with Na,K-ATPase from calf brain and Electrophorus electric organ, respectively, was essentially irreversible. In the former case the time of incubation was too short, especially in the presence of K^+, and in the latter case the temperature used was 24° to 26°C, and these appeared to be the explanations. Sen et al. (149) and Tobin and Sen (164) found that while the ouabain binding was readily reversible at 37°C the glycoside was essentially irreversibly bound at 0°C. The dissociation rate constant increased exponentially with increasing temperature, the half life of an enzyme-ouabain complex obtained with guinea pig kidney Na,K-ATPase being 9 h at 0°C and 3 min at 37°C. The activation energy calculated from an Arrhenius plot and the free energy of the interaction of enzyme and ouabain indicated a high entropy change and a large conformational change for ouabain binding to take place (164). A similar conclusion was made by Kuriki et al. (103) from calorimetric studies on Na,K-ATPase from electric eel organ revealing extraordinarily large enthalpy changes associated with the binding of Mg^{2+} and P_i to the enzyme.

Dissociation of cardiac glycoside from its complex with Na,K-ATPase implies the regeneration of the enzyme's activity. The fulfillment of this demand for true reversibility was nicely demonstrated by Huang and Askari (80) with red cell Na,K-ATPase. The regeneration of enzyme activity previously inhibited by ouabain, digoxin, and digitoxin clearly took place at 37° C. The regeneration could be accelerated considerably by the presence of $Na^+ + ATP$ in the absence of Mg^{2+}; see section IV. This

behavior of enzyme-glycoside complexes formed the basis of a method for the estimation of pretreatment values of enzyme activity in patients exposed to cardiac glycosides (80).

The relative stability of the enzyme-ouabain complex at low temperature is exploited in experiments where it is desired to get rid of free ligands. After centrifugation at 0° to 2°C a nearly 100% recovery of ouabain-bound enzyme can be obtained. Several studies taking advantage of this fact are dealt with in later sections.

Summary. Ouabain binds preferentially to the phosphorylated (or vanadate bound) E_2-form of the Na,K-ATPase by a reversible process. K^+-binding to Na,K-ATPase antagonizes ouabain binding, but does not exclude the glycoside interaction, i.e. K^+ and ouabain do not compete for the same site. On the other hand, the K^+-induced reduction in ouabain affinity is not simply explainable by the increase in dephosphorylation rate by K^+.

III. Descriptive Models for Ouabain Interaction with Na,K-ATPase and Ligand Modulation of the Reaction

A. Conceptions of a Simple Bimolecular Reaction

As described in section II the interaction between Na,K-ATPase and ouabain is reversible. Several factors facilitate the process or are even indispensable for ouabain binding to take place. The latter fact is partly disregarded in the following considerations of ouabain binding models, since prevalence of the ouabain binding conformation is implied.

Barnett (16) found that the inhibition of Na,K-ATPase from lamb brain by ouabain was first order (i.e. pseudo-first order) in both enzyme and ouabain concentrations. With intact mammalian cells Baker and Willis (14) observed that the rate of ouabain binding was first order with respect to the glycoside concentration. Graphs of ouabain binding data presenting bound ligand as a function of the free ligand concentration at equilibrium of binding seem hyperbolic and apparently exhibit saturation kinetics, but a more exact treatment of the data requires a transformation to one of several possible reciprocal graphs (for details, see APPENDIX). Manipulation of Eq. (7) giving the relationship between bound ouabain at equilibrium and the total ouabain binding capacity, nonbound ouabain, and the dissociation constant for the process to reciprocal plots describing straight lines are useful for testing binding models and for calculation of binding constants. Analysis of ouabain-binding data originally indicated one single type of ouabain receptor. Data on [^{3}H]ouabain binding to Na,K-ATPase from ox brain obtained under steady-state conditions in the presence of Mg^{2+}, Na^+ and ATP seemed adequately described by a simple bimolecular reaction (61). Binding isotherms measured at different initial ouabain concentrations and plotted as bound/free versus free ouabain (see APPENDIX) could be shown to fall on a straight line. The data thus fitted a second order reaction according to the mass law equation

$$E + O \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EO$$

in which E represents receptor sites available for ouabain binding (i.e. $E = EO_{max} - EO$), O free (nonbound) ouabain, EO the enzyme ouabain complex with the maximum value EO_{max}, and k_1 and k_{-1} the forward and backward rate constants for the process.

The ouabain-binding model was confirmed by Schönfeld et al. (148) by application of a double reciprocal plot on binding data obtained with a preparation from pig heart. Erdmann and Schoner (35, 36) verified the binding model with Na,K-ATPase preparations obtained from ox brain, kidney, and heart and from guinea pig kidney. Also, in the presence of K^+ as well as Mg^{2+}, Na^+, and ATP the ouabain binding data could be fitted to straight lines (148). The steeper slopes in the presence of K^+ indicated reduced ouabain affinities (higher apparent dissociation constants), but the same number of receptor sites. The model implies the existence of a single ouabain receptor type (exception, vide infra), but inspection of the plots of Schönfeld et al. (148) seems to disclose a tendency towards higher slopes and thus reduced affinity at high ouabain concentration, i.e. near saturation. Also, Whittam and Chipperfield (172) interpreted their Scatchard plots of ouabain binding data to Na,K-ATPase from ox brain as straight lines and binding to a single class of receptor sites. However, their binding isotherms seemed better fitted to a curved line. Taniguchi and Iida (162, 163) reported on two kinds of ouabain binding sites in Na,K-ATPase from ox brain. Since the sum of high and low affinity sites were roughly the double of maximum phosphorylation and since the number of low affinity sites varied with the K^+ concentration, their observations seem to be at variance with those of most other investigators.

The steady-state conditions for ouabain binding in the presence of (Mg^{2+} + Na^+ + ATP) are unsuitable for further analysis due to ATP hydrolysis with generation of ADP and P_i. True equilibrium conditions for ouabain binding are more likely to exist in the presence of (Mg^{2+} + P_i). Scatchard plots of ouabain binding data obtained with (Mg^{2+} + P_i) and ox brain Na,K-ATPase were claimed to be linear (36, 63) and a single class of ouabain receptors thus seemed to exist under most experimental conditions. Thus, it was found by both groups of investigators that K^+ (or its congeners) increased the dissociation constant of the ouabain-receptor complex and that the total number of ouabain binding sites was not changed in the presence of monovalent cations. Some deficiencies in the simple one ouabain receptor model and the simple K^+-ouabain competition model were, however, noted. Erdmann and Schoner (36) noticed that

Scatchard plots of ouabain binding data obtained in the presence of Mg^{2+} and the absence of P_i were concave upward. Hansen and Skou (63) made the reservation that since K^+ was unable to prevent ouabain binding, a K^+-bound form of Na,K-ATPase should also be able to bind ouabain, though with reduced affinity. A reduction in ouabain binding was observed at low K^+-concentrations, but finally a constant, K^+-independent ouabain binding level was obtained by increasing the K^+-concentration.

Erdmann and Schoner (36) proposed that the curvilinear Scatchard plots could be explained by the existence of the ouabain receptor in two different conformational states. In the presence of P_i and Mg^{2+} all receptors were in the high affinity state. In the presence of Mg^{2+} only, an equilibrium between high and low affinity receptors was set up, whereas the total number of binding sites was unchanged. This explanation does not hold, however, provided an instantaneous equilibrium between two ouabain binding conformers is assumed to exist. Also in this case a Scatchard plot of the binding isotherms should describe a straight line as shown with another example in section B of the APPENDIX. Assuming that K^+-bound and non-K^+-bound enzyme forms are in instantaneous equilibrium with one another, both accepting ouabain, though with different affinities, it is seen that binding data plotted as bound versus bound/free ouabain according to Eq. (22) should fall on a straight line. The observations by Hansen and Skou (63) that binding data independent of the presence of monovalent cations could be fitted to straight lines, are thus compatible with a model consisting of two ouabain-binding species in equilibrium with one another.

Real doubt about the view of a single class of receptors or instantaneously interconvertible receptor sites was raised when Nørby and Jensen (116) showed that ATP binding to an ATPase preparation from ox brain in the presence of K^+ resulted in curvilinear Scatchard plots. In the absence of K^+ linear plots were obtained. They proposed that the nonlinearity could be explained by the existence of two types of ATP binding sites differing in their sensitivity to K^+. In this context it seems to be relevant to stress that both nucleotide binding sites are those that have a high affinity for both ATP and ADP and are present in every enzyme preparation in exactly the same number as ouabain binding sites. In spite of the complex kinetics of ATP hydrolysis as a function of the substrate concentration and the complex inactivation kinetics exhibited by a number of inhibitors there seems to be no experimental evidence for a "regulatory low-affinity ATP site" but most observations seem compatible with a model assuming the existence of one flexible substrate site per molecule (117).

B. One Homogeneous, Two or More Nonuniform Populations of Ouabain Receptors, or Anticooperativity among Primary Identical Sites?

The nonlinear Scatchard plot of ouabain-binding data obtained with Mg^{2+} in the absence of P_i (36), the unsatisfactory linear fits to data obtained with low P_i-concentrations (63), and the observations of Nørby and Jensen (116) on the effect of K^+ on nucleotide binding to Na,K-ATPase made a critical reinvestigation necessary. In a new study on ouabain binding to Na,K-ATPase from ox brain, it was concluded (64) that binding was adequately described under special conditions only by a simple bimolecular reaction and thus binding to a homogeneous population of sites or, alternatively, to instantaneously interconvertible sites. Due to the high apparent ouabain affinity under optimum conditions for binding (i.e. the shallow slope of the Scatchard plot equal to the low dissociation constant), a deviation from linearity can hardly be seen. By decreasing the concentration of some of the facilitating ligands, e.g. P_i, the curvature is accentuated. However, in the presence of K^+ in combination with ($Mg^{2+} + P_i$) a steeper and apparently straight graph is obtained.

These observations cannot be due to experimental errors for the following reasons: 1) The tritiated ouabain used was purified by a method which takes advantage of the specific binding of the glycoside to Na,K-ATPase, i.e. by chromatography on the enzyme (64). An impurity, on the other hand, was more likely to give an underestimation of binding with especially striking consequences at low ouabain concentrations leading to downward concave curves. 2) By a similar reasoning a downward concave curve was expected if equilibrium of binding was not attained. 3) The rate of ouabain binding is delayed by K^+ and equilibrium of binding may not be reached in this situation. This should straighten the nonlinear (upward concave) curve obtained in the absence of K^+. Having reached a binding equilibrium without K^+, the addition of K^+, however, always lowers binding at nonsaturating concentrations of ouabain.

Other investigators concluded that ouabain interaction with at least some Na,K-ATPase preparations was not adequately described by binding models based on one homogeneous receptor type (39, 140). The meaning of the nonlinear Scatchard plot seemed to them rather obscure and several theoretical possibilities were mentioned, among them artifacts, multiple classes of cooperatively interacting sites, and multiple classes of independent binding sites.

By using a fluorescent derivative of ouabain, anthroylouabain, Moczydlowski and Fortes (112) observed pseudo-first order binding kinetics to Na,K-ATPase from rabbit kidney and eel electroplax under some conditions. A deviation from pseudo-first order kinetics was observed for the eel enzyme with anthroylouabain binding in the presence of $Mg^{2+} + P_i$, however. This was assumed to be due to the existence of two or more conformations that bind anthroylouabain at different rates and interconvert slowly compared to the rate of anthroylouabain binding. After addition of a few milli-

molar K^+ the anthroylouabain binding kinetics were linear.

A great many of the observations on ouabain binding were made with Na,K-ATPase isolated from brain tissue. In a study published in 1979 Sweadner (161) emphasized that brain contains two distinct molecular forms of Na,K-ATPase, whereas only one form is found in non-neuronal tissues. The two molecular forms differ in the composition of the large peptide and, important for the present considerations, in their sensitivity to strophanthidin and perhaps to ouabain.

In our studies (63, 64) we used grey matter from ox brain, not whole brains. Nevertheless, it is likely that our preparation would contain both forms of ATPases. Two or more nonuniform populations of ouabain-binding sites due to different sources of origin were discussed (64) as one of the possible reasons for the curvilinear Scatchard plots of ouabain-binding data and apparent ouabain dissociation constants were determined for this situation. However, the two dissociation constants for ouabain differed by only a factor of 20 whereas the two half inhibition constants differed by a factor of more than 300 for calf brain and 10^3 for rat brain (161). Even more controversial is the apparent conversion to uniformity of the ouabain binding populations by low concentrations of K^+ and the complex binding kinetics also observed with Na,K-ATPase from other sources than brain (112). With kidney enzyme preparations it is more difficult to show deviation from the simple kinetics expected with one homogeneous population of ouabain-binding sites. It can, however, be seen from figure 1 that the ouabain-binding isotherms with kidney Na,K-ATPase are better described by curvilinear plots in the absence of P_i.

In a study of nucleotide binding to pig kidney Na,K-ATPase, Ottolenghi and Jensen (119) observed heterogeneity induced by K^+ among an otherwise homogeneous population of nucleotide-binding sites. In the presence of Na^+, ouabain binding was compatible with nonhomogeneous binding populations. Clear evidence for a dimeric structure of the Na,K-ATPase with interaction (anticooperativity) between subunits, each containing a nucleotide and a ouabain-binding site, was presented. Also radiation inactivation of pig kidney Na,K-ATPase points to a dimeric structure of the enzyme (120). The target size seems to vary with the parameter measured and integrity of a domain varying from 38% to 55% of the apparent molecular weight of the dimer was needed for ouabain binding, depending on the factors used for facilitation. This observation seems compatible with the potential accommodation of two ouabain molecules per dimer. For more details on the subunit structure see section IV.

In case of a dimeric structure of the enzyme and anticooperativity among primary, identical binding sites the mathematical description and the resolution of the

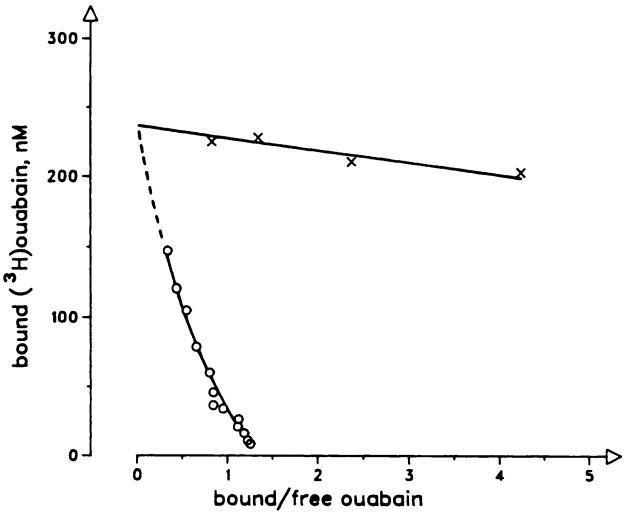


FIG. 1. Curvilinear Scatchard plot of ouabain-binding data obtained with purified pig kidney enzyme in the absence of added P_i. Purified Na,K-ATPase was prepared from red outer medulla of pig kidney by incubation of a microsomal fraction with SDS and ATP followed by an isopycnic-zonal centrifugation [Jorgensen (87)]. Final ouabain binding capacity, 2.77 nmoles/mg of protein; K^+-pNPPase activity, 245 μmoles·mg^{-1}·h^{-1}. Enzyme corresponding to 0.085 mg·ml^{-1} was incubated with 3 mM Mg^{2+}, 40 mM Tris (pH 7.25), ○, or with 3 mM Mg^{2+}, 3 mM P_i, 40 mM Tris, ×, and 1.5 ·10^{-8}–6·10^{-7} M [^{3}H]ouabain for 120 min at 37°C. Ouabain binding was determined as described [Hansen (66)].

curvilinear plots into two components is similar to the procedure in case of two equal-sized and independent populations of sites. The meaning of the calculated constants is, however, different (101, 119), and the microscopic or intrinsic binding constants are different from those based on a model assuming two independent ouabain-binding sites (64).

Other pieces of evidence for a dimeric structure of the Na,K-ATPase is scanty, especially from a functional point of view. Substituting Ca^{2+} for Mg^{2+}, Askari et al. (13) found an unaltered ouabain-binding capacity (the affinity being much reduced) of dog kidney Na,K-ATPase, but the incorporation of $^{32}P_i$ was halved. They interpreted the observation as evidence for a dimeric structure and a half-of-the-sites reactivity mechanism. Hansen (71), however, was unable to confirm their observation using [^{48}V]vanadate together with ouabain instead of inorganic phosphate. An accelerated release of bound ouabain at saturation binding was expected compared to the situation at low occupancy in case of a polymeric structure and site-site interaction (33). The rate of ouabain dissociation, however, was the same at saturation and at low occupancy of ouabain binding whether achieved by complexation of Mg^{2+} (addition of EDTA) or by addition of a surplus of unlabelled ouabain (figure 2; unpublished data).

It was mentioned previously that most authors find a close correlation between ouabain binding and positive inotropic effect on the heart. A discrepancy may exist for some rodent species, especially the rat whose heart is

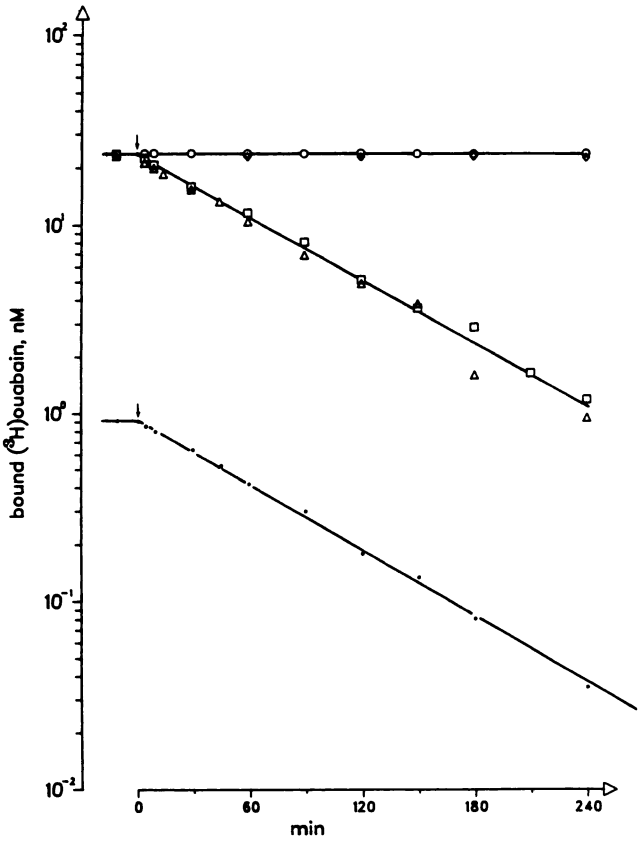


FIG. 2. [^{3}H]ouabain dissociation after addition of unlabelled ouabain or Mg^{2+} complexation. Pig kidney enzyme equivalent to a ouabain binding capacity of 75 nM was incubated with 3 mM Mg^{2+}, 3 mM P_i, 40 mM Tris (Δ, ◇) or 1 mM Mg^{2+}, 3 mM P_i, 40 mM Tris (○, □, •) and 10^{-9} –$2.6 \cdot 10^{-8}$ M [^{3}H]ouabain at 45°C for 90 min. Then unlabelled ouabain 10^{-4} M (Δ) or 12 mM EDTA (□, •) or 12 mM EDTA + 12 mM Mg^{2+} (○) or, for control of heat damage, nothing was added (◇). Binding of [^{3}H]ouabain was measured with the filtration technique.

rather insensitive to cardiac glycosides. Rat heart cell membranes (but not ventricular strips) exhibit two classes of ouabain binding sites with dissociation constants that differ by two orders of magnitude. Occupation of the minor, high-affinity component appears to be associated with the increase in force of contraction of the strips, whereas occupation of the major, low-affinity component appears to be associated with the inhibition of hydrolysis and the rate of uptake of K^+ (40). Adams et al. (1) confirmed the discrepancy between the ouabain concentration needed for half inhibition (IC50) of isolated Na,K-ATPase from rat heart and the high-affinity dose-response curve of ouabain on contractile force of ventricular muscle. On the other hand, their dose-response curve also exhibited a low-affinity component that was compatible with the low-affinity site for enzyme inhibition. The very low recovery of Na,K-ATPase activity with most methods for the preparation of enzyme-rich membrane fractions from skeletal and heart muscle, however, makes such correlations hazardous. The real possibility of a preferential isolation of neural tissue ATPase by these same methods has been discussed (29).

The question, as to whether diversity of the site population could be explained by a fixed ratio of the two conformational states of the enzyme set up by the concentration of auxiliary ligands present, is often raised. This would imply nonequilibrium among two ouabain-binding species with different ouabain affinities as described previously. Since most binding experiments were carried out at 37°C and binding equilibrium is attained after hours of incubation the explanation seems less likely. At lower temperatures the hypothesis seems more acceptable and curvilinear vanadate binding isotherms were obtained with Na,K-ATPase at 25°C and 30°C but not at 37°C (unpublished data).

C. Ligand Modulation of Ouabain Binding and Derived Ligand Affinities

Ouabain binding is intimately related to Na,K-ATPase cycling. Some factors are indispensable for ouabain binding, others promote or inhibit rate and extent of binding. If equilibrium binding is modulated by a specific ligand, information about the enzyme-ligand interaction may be deduced from the equilibrium binding data. An expanded ouabain binding model compatible with binding data is the prerequisite for such studies.

In a study of (Mg^{2+} + P_i)-promoted ouabain binding to ox brain Na,K-ATPase, the modulation by monovalent cations of the equilibrium binding level was studied (63). Since no ouabain binding took place in the presence of P_i and the absence of Mg^{2+}, whereas P_i increased binding in the presence of Mg^{2+}, a sequential model with Mg^{2+}-interaction prior to P_i-phosphorylation was proposed. Binding data and the calculated apparent dissociation constants seemed compatible with a 1:1:1 ratio model for Mg^{2+}, P_i, and ouabain interaction with the enzyme. A model that could accommodate binding data in the presence of K^+, Na^+, or $Tris^+$ required at least two binding sites for monovalent cations per ouabain binding unit and complex enzyme-ligand dissociation constants, Whittam and Chipperfield (172) made similar studies on Na,K-ATPase from ox brain, but did not observe multiple sites for K^+ or Na^+. Neither did Lindenmayer et al. (106) adapt a multiple site model to their initial rate studies on ouabain binding as modulated by Na^+ and K^+.

These considerations were based on a model assuming one single population of ouabain-binding sites. In a later publication Hansen (64) presented data that required at least two different ouabain receptors or, more likely, two basic P_i-receptors. Even assuming a two-component model, the apparent dissociation constants for each ouabain-binding component as a function of the inverse P_i-concentration fitted the model (unpublished data). Likewise, since K^+ seems to eliminate the difference between the two binding components, the statements for the extended ouabain-binding model (64) may still hold, but, of course, not the absolute value of the calculated dissociation constants.

No attention was paid to a possible interconversion

between an orthophosphate bound form of the enzyme (the Michaelis complex) and a phosphoprotein in which phosphate is covalently bound to the enzyme as described for Ca^{2+}-ATPase (102, 109) and for Na,K-ATPase (13, 103). Covalently bound phosphate may explain the acid resistance (128, 150). The interconversion was neglected in our studies as was the direct P_i-pathway prior to Mg^{2+} interaction. Our P_i-dissociation constant(s) may thus be complex compared to others based on the scheme

$$P_i + M^{2+} + E \rightleftharpoons EM^{2+} + P_i \rightleftharpoons EP_iM \rightleftharpoons EM - P$$
$$\rightleftharpoons EP_i + M^{2+}$$

where M^{2+} stands for a divalent cation, e.g. Mg^{2+} or Mn^{2+}.

It is likely that EM-P is the preferred ouabain-binding species (103, 104, 149) but also EM^{2+} may bind ouabain. Two independent pathways for P_i-phosphorylation, two E conformations slowly interconvertible or noninterconvertible or a dimeric structure of E that can accommodate two interacting phosphate molecules are needed to explain the curvilinear Scatchard plots of ouabain-binding isotherms.

Studies of the interacting ligands modulating acid precipitable P_i-phosphorylation as depicted in the scheme are difficult due to the low P_i-affinity. For that reason the high affinity ligand ouabain is often coupled to the process to stimulate phosphorylation in such studies. Such equilibrium binding studies have recently become possible in the absence of ouabain due to the discovery of the high affinity analogue of phosphate, vanadate, which is supposed to be a transition state analogue of phosphate (see section V).

Summary. With most conventional ATPase preparations ouabain interaction seems poorly described by a simple bimolecular reaction between homogeneous ouabain binding sites and the ligand. Two or more distinct classes of noninterconvertible sites, possibly originating from different cell types, may be part of the explanation, but hardly the only one. The apparent uniformity of ouabain binding sites from ox brain in the presence of K^+ has to be explained as has the apparent heterogeneity induced by Na^+ even in relatively pure kidney enzyme preparations. Slow interconversion between two ouabain binding species with different affinities seem less likely during hours of incubation at 37°C. Anticooperativity among two primary identical sites is an attractive hypothesis, though the meaning from a functional point of view is obscure.

The ratio of Mg^{2+} to P_i to ouabain in (Mg^{2+} + P_i)-promoted ouabain binding seems to be 1:1:1, whereas more than one monovalent cation modulates each ouabain binding site.

IV. Reactive States of Ouabain-bound Na,K-ATPase

In a previous section the conventional definitions of the main conformations of Na,K-ATPase, E_1 and E_2, were discussed. The ligand conditions that will promote ouabain binding, e.g. (Mg^{2+} + P_i) or (Mg^{2+} + Na^+ + ATP), are such that the preferred ouabain-binding species is the E_2-conformation as also shown directly with (Mg^{2+} + P_i)-induced ouabain binding to fluorescein-complexed Na,K-ATPase (77). Studies with the specific fluorescent probe anthroylouabain suggested that the same enzyme conformation binds anthroylouabain under both ligand conditions (47). Studies of the dissociation rates of enzyme-ouabain complexes with different origin and the influence of ligands on the decay rates have shown, however, that several ouabain-complexed subconformations may exist.

From kinetic experiments Yoda (180) arrived at the conclusion that Na,K-ATPase has at least two specific sites for the attachment of cardiac glycosides, one for the steroid portion and the lactone ring and another one for the sugar moiety. The association rate constant depends primarily on the steroid moiety whereas the dissociation seems more dependent on the sugar moiety of the glycoside. Direct information on the digitalis binding areas of Na,K-ATPase has been obtained from experiments with derivatives of cardiac glycosides that contain photosensitive reactive groups that can be activated to form covalent bonds with the enzyme protein.

Membrane-bound Na,K-ATPase from a variety of sources has been shown to have an equimolar composition of two peptides, a so-called α-peptide of M_r ~100,000 and a β-peptide of M_r ~40,000 (92). Whether the protomer $\alpha\beta$ or an oligomer, e.g. $\alpha_2\beta_2$, is the functional unit is still not settled (82, 92, 120) as discussed in a previous section. Some investigators have also recognized a small proteolipid, the γ-peptide of M_r ~12,000 (31, 43), copurifying with the above-mentioned α- and β-peptides. The membrane-spanning α-peptide contains the residues necessary for all the investigated partial reactions and for the complete hydrolytic cycle whereas no functional role has been attributed to the glycosylated β-peptide (92) or the γ-peptide (45). The derivatives of cardiac glycosides that photoaffinity label the Na,K-ATPase do so on the catalytic α-peptide (42, 43, 132, 134, 136). This holds true for cardiac glycosides having the photosensitive group on the steroid moiety or on the sugar moiety of monoglycosides (45, 55). Labelling of the β-peptide, on the other hand, takes place when digitoxin derivatives having the photolabel beyond the third sugar moiety are used (58). The interpretation is probably that the α- and β-peptides are in intimate contact, at least in the region of the third digitoxose of the sugar-specific binding area (45, 58).

In contrast to the β-peptide the proteolipid or γ-peptide is labelled by some of the monoglycoside derivatives that have the reactive group attached to rhamnose (31, 45). On the other hand, by using a *p*-aminobenzenediazonium derivative of ouabain (reactive group on rhamnose), in which case photodecomposition and pho-

tosuicide inactivation of the enzyme can be induced by energy transfer through a tryptophan residue, Goeldner et al. (55) found no labelling of the proteolipid but solely of the α-peptide. With this procedure, as well as with an alkylating ouabain derivative (133), it could be shown after trypsinolysis that ouabain is bound to a fragment of the α-peptide with $M_r \sim 41{,}000$.

The catalytic peptide thus seems to be the primary receptor for cardiac glycosides as well as for other ligands. A preliminary mapping of various reactive groups on the α-subunit for import ligands and substrates has been proposed (23, 92, 134). Though the ouabain-binding site is on the extracellular aspect (19, 122) and the substrate and phosphorylation site on the intracellular aspect of the cell membrane it is perhaps not surprising that a number of factors may modulate the reactive state of the ouabain-binding species.

In the early studies it was pointed out that the phosphoprotein formed from inorganic phosphate in the presence of ouabain was indistinguishable from that formed from ATP (25, 150). Whether enzyme-ouabain complex formation was induced by ($Mg^{2+} + Na^+ + ATP$) or by ($Mg^{2+} + P_i$) the association and dissociation kinetics of ouabain suggested that the two complexes, usually named complex I and II, respectively, were identical (146). However, this appeared to be due to the continued presence of the facilitating ligands and these affected the dissociation rate as visualized by the addition of a surplus of unlabelled ouabain. Akera and Brody (4) and Allen et al. (10) discovered that after isolation of the two complexes they behaved differently. At 0°C the enzyme-ouabain complexes are stable (149, 164) and the ATPase-containing membranes may be precipitated and washed free of nonbound ligands. After resuspension at 37°C the rate of ouabain dissociation for the ($Mg^{2+} + Na^+ + ATP$)-promoted complex appeared to be fast in the absence of K^+ but slow after addition of K^+ at low concentration. The ($Mg^{2+} + P_i$)-promoted complex had a slow rate of decay which was unaffected by the presence or absence of the ligands that promoted binding or the addition of K^+. The phenomenon was thought to be due to the phosphorylating substrate. With the artificial substrate *p*-nitrophenyl-P an intermediate rate of decay of the enzyme-ouabain complex was observed (10).

The real difference between complex I promoted by ($Mg^{2+} + Na^+ + ATP$) and those obtained with other phosphorylating substrates, e.g. complex II promoted by ($Mg^{2+} + P_i$), appeared to be determined by the monovalent cation present during binding (65, 66). Na^+ is inhibitory to ouabain binding in the presence of ($Mg^{2+} + P_i$). That complex formation which does take place, has a relatively fast rate of decay after isolation, but the complex is stabilized by the addition of K^+. The concentration dependent effect of Na^+ during binding on the stability of the enzyme-ouabain complex formed is much more pronounced when *p*-nitrophenyl-P is used for promotion of ouabain fixation (66). Moreover, in this case, it certainly affects all ATPase units since the ouabain affinity to brain enzyme is less sensitive to Na^+ in combination with *p*-nitrophenyl-P. Na,K-ATPase from kidney may differ in this respect (unpublished data).

The main thing is that a fast-dissociating enzyme-ouabain complex that is K^+-sensitive is obtained in the presence of Na^+ during complex formation irrespective of the phosphorylating substrate. The reactive state is probably determined prior to ouabain binding which apparently merely freezes the enzyme in that state. On the other hand, the stabilization by K^+ during dissociation, i.e. the apparent conversion of complex I to complex II, clearly shows that the enzyme-ouabain complex after formation is accessible to cation interaction.

The sliding transition from a slowly dissociating complex formed in the absence of Na^+ to a fast-dissociating complex in the presence of Na^+ at high concentration means that the borderline between complex I and II is more blurred than previously supposed. The two originally defined complexes were, however, shown to differ in the number of reactive sulfhydryl groups (75). It would be interesting to know how many reactive groups were exposed in the presence of (Na^+ + *p*-nitrophenyl-P).

Originally, an explanation for the difference of the enzyme-ouabain complexes was proposed to be the stability of the phosphorylated intermediate whether achieved from ATP or P_i in the presence and absence of Na^+ (68). Though the phosphoenzyme is stabilized by ouabain it is known that the phosphate from ATP is lost before ouabain dissociation (147, 149) and that rephosphorylation from P_i may take place. Since vanadate was retained in the enzyme-vanadate-ouabain complex, P_i was assumed to behave similarly in the ($Mg^{2+} + P_i$)-promoted complex. The latter complex is, however, available for vanadate binding which probably means that P_i is also lost prior to ouabain (71). The stability of the ($Mg^{2+} + P_i$)-facilitated enzyme-ouabain complex after separation from the ligands that promoted binding can thus not be due to the stability of the phosphorylation but should be explained by the reactive state induced, mainly by Na^+, prior to ouabain binding. All ouabain-binding species probably still have to be classified as E_2-conformations. If those obtained in the presence of Na^+ are more towards the E_1-conformation, a relaxation to a real E_1-conformation that does not retain ouabain may easily take place. K^+, on the other hand, stabilizes the E_2-conformation and ouabain binding is therefore also stabilized. It is a paradox that Na^+ present at high concentrations during the dissociation abolishes the fast dissociation rate, since Na^+-bound nonphosphorylated enzyme is classified as an E_1-conformation.

It may be relevant to mention that the enzyme-ouabain complexes obtained with Mg^{2+} plus P_i or *p*-nitrophenyl-P in the absence of Na^+ have some similarity to the K^+-insensitive, relatively slowly dephosphorylating phos-

phoenzyme obtained under similar conditions (128). On the other hand, the dephosphorylation rate constant of the non-ouabain-bound enzyme is several orders of magnitude faster than that of ouabain release.

Attempts to change complex II to complex I after its creation, i.e. the reverse of the apparent transition mentioned above, have been unsuccessful unless the observation by Tobin et al. (167) and Yoda and Yoda (179), that addition of ATP in combination with Na^+ and the absence of Mg^{2+} will accelerate ouabain release, is interpreted in this direction. Explanations that emphasize the role of Na^+ and in accordance with the traditionally accepted conformation definitions may be offered as well. The combined effect of (Na^+ + ATP) on the (Mg^{2+} + P_i)-promoted complex under nonphosphorylating conditions fits with the idea that ouabain release is accelerated by E_1-drawing forces. Interestingly, the putative ATP-analogues, suramin and eosin (46, 157), in combination with Na^+ have similar effects on ouabain dissociation (fig. 3). These observations will be discussed in more detail in section V.

Finally, it can be mentioned that at least the (Mg^{2+} + P_i)-promoted enzyme-ouabain complex may represent a unique E_2-conformation of the enzyme. An indication in this direction is the observations on fluorescence change upon ouabain binding (77) and observations from calorimetric studies (59). Control enzyme underwent a major endothermic transition associated with enzyme denaturation at 55.3°C while ouabain-bound enzyme did at 59.5°C.

Conclusion. The role of the monovalent cation present during enzyme-ouabain complex formation for the character of the complex created is emphasized at the expense of the role of the phosphorylating substrate. After formation, the complex may be modified by monovalent cations and, in combination with Na^+, by ATP.

V. Information on Enzyme-Ouabain Complexes Obtained with Vanadate

The transition element vanadium is characterized by its occurrence at variable and alternating valencies, among them the oxygen compounds in the +4 and +5 oxidation state, vanadyl and vanadate, respectively. These states of the element appear to be very efficient inhibitors of phosphotransferases and phosphohydrolases, probably due to the chemical similarity of vanadium to phosphorous. For a review on this topic and references see Hansen (72).

Vanadium in the +5 oxidation state (vanadate) also appeared to be a powerful inhibitor of Na,K-ATPase and a useful tool in studies of this enzyme (20, 21, 85, 159). Evidence for the proposed role of vanadate as a transition state analogue of phosphate towards phosphohydrolases (108) was added when it was shown that vanadate at very low concentrations promoted ouabain binding to Na,K-ATPase (67, 68, 114, 169). The aspects of high affinity binding of vanadate previously to or simultaneously with ouabain is dealt with in this section.

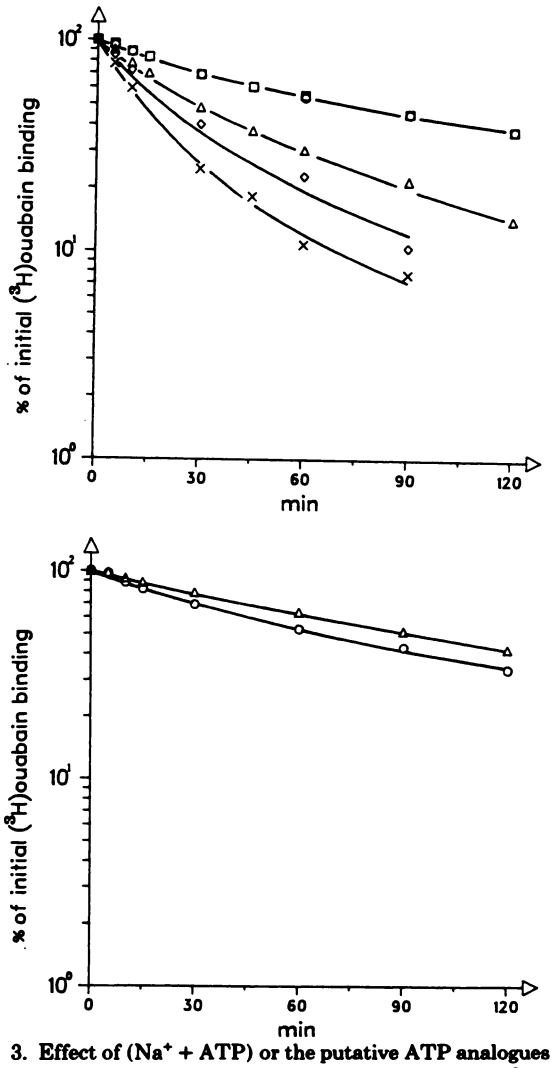


FIG. 3. Effect of (Na^+ + ATP) or the putative ATP analogues eosin and suramin on ouabain dissociation after (A, top) (Mg^{2+} + P_i)-facilitated or (B, bottom) (Mg^{2+} + V)-facilitated binding. [^{3}H]ouabain was incubated with ox brain enzyme (specific activity 200 μmoles·mg^{-1}·h^{-1}) for 60 min at 37°C in the presence of (A) 3 mM Mg^{2+}, 3 mM P_i, and 40 mM Tris or (B) 3 mM Mg^{2+}, 70 μM vanadate, and 40 mM Tris. After cooling to 0°C the enzyme-ouabain complexes were washed twice by centrifugation in 4 mM Tris in the cold. Resuspension took place in 4 mM Tris, 10^{-3} M ouabain (○) *plus* 1 mM suramin (□), or 1 mM suramin + 120 mM Na^+ (△) or 100 μM eosin + 120 mM Na^+ (◇) or 1 mM EDTA + 1 mM ATP + 100 mM Na^+ (×). [^{3}H]Ouabain binding was determined with the filtration technique. Before wash the binding was near saturation and the loss during the washing procedure was insignificant.

For vanadate to bind to the Na,K-ATPase a divalent cation such as Mg^{2+} or Mn^{2+} is required, but K^+ has an additional role in facilitation of the binding (21, 71). Despite the synergistic role of Mg^{2+} and K^+ in vanadate binding, the antagonism between ouabain and K^+ is retained in (Mg^{2+} + V)-facilitated ouabain binding (67, 114). K^+ even seems to be able to antagonize ouabain binding completely in the vanadate-induced pathway contrary to what is seen in (Mg^{2+} + P_i)-facilitation (63). The explanation is possibly that the observed effects of

K^+ on ouabain and vanadate binding are from the extracellular and intracellular aspect of the cell membrane, respectively. Such information is only obtained from studies on intact systems, e.g. red blood cells or squid giant axons, where separation of the ionic milieu on the two sides is possible.

The K^+-facilitation of vanadate binding under equilibrium conditions (i.e. the absence of ATP) is from the cytoplasmic aspect of the membrane (97). Extracellular K^+ is known to inhibit the rate and probably also to decrease the steady state level of ouabain binding to squid giant axons and to red cells which means that the (Mg^{2+} + Na^+ + ATP)-facilitated pathway of ouabain binding is antagonized by extracellular K^+ (14, 15, 17, 137). Intracellular K^+ on the other hand probably also modulates ouabain binding directly or indirectly via the steady state level of phosphorylation or the turnover number (84). However, the inhibitory effect of K^+ on ouabain binding in the (Mg^{2+} + Na^+ + ATP), as well as in the (Mg^{2+} + P_i)-stimulated pathway, seems mainly to be extracellular (18, 107). By comparison it is supposed that K^+ antagonizes vanadate-promoted ouabain binding extracellularly and thus not necessarily from the intracellular K^+-site that promotes vanadate binding.

Vanadate appeared to be retained in the enzyme-ouabain complex much more firmly than phosphate (68, 71). Vanadate itself also has a slower release from the enzyme than phosphate even in the absence of ouabain, but the dissociation rate is decreased considerably after ouabain complexation. It is known that the phosphorylation that accompanies ouabain binding facilitated by (Mg^{2+} + Na^+ + ATP) is lost before ouabain is released and that rephosphorylation by P_i of the enzyme-ouabain complex is possible (147, 149). The $^{32}P_i$ incorporation that parallels ouabain binding in the presence of (Mg^{2+} + P_i) is, like the ATP phosphorylation, acid precipitable (8, 142) and thus probably represents a covalent attachment to a β-aspartyl carboxylate of the hydrolytic peptide in both cases (127, 150). The observed medium $P_i \rightleftharpoons HOH$ exchange catalyzed by the Na,K-ATPase is slowed considerably but not totally by ouabain, and this is also consistent with an enzyme-acyl-phosphate intermediate, though other interpretations are possible (32, 121). Vanadium is usually supposed to interfere with enzymatic phosphoryl transfer reactions by mimicking a postulated metastable pentacovalent phosphorous intermediate with a trigonal bipyramidal structure (108, 121), i.e. a step not identical to the true phosphoenzyme. Irrespective of the mode of action, vanadate is much more firmly bound than phosphate.

The Mg^{2+}-facilitated enzyme-ouabain complex obtained in the absence of added P_i is also able to incorporate vanadate after its formation. Since the (Mg^{2+} + Na^+ + ATP)-facilitated enzyme-ouabain complex can be rephosphorylated by P_i it is perhaps not surprising that this complex too will accept vanadate after separation from the ligands that promoted ouabain binding. More surprisingly, the (Mg^{2+} + P_i)-facilitated complex is also able to take up vanadate, which probably means that P_i-phosphorylation of ouabain-bound enzyme is also easily lost (71). Together these observations may imply a repetitive role for traces of P_i (but not of vanadate) in the apparent Mg^{2+}-facilitation of ouabain binding. Vanadate binding to preformed enzyme-ouabain complexes occurs at a slower rate than to enzyme not occupied by ouabain, but apparently with an even higher affinity. Mg^{2+} (or Mn^{2+}) is necessary for vanadate binding to ouabain-bound enzyme which may indicate a transitory role of this ligand during the phosphorylation and ouabain binding.

As mentioned in a previous section there are indications that ATP is able to react with ouabain-bound enzyme (167, 179), especially when Na^+ is also present and Mg^{2+} is absent, i.e. under conditions where phosphorylation (from contaminating P_i ?) does not take place. After isolation of the (Mg^{2+} + P_i)-facilitated enzyme-ouabain complex an acceleration of ouabain release by ATP + Na^+ is seen. This should not be confused with the inhibitory action of nucleotides or nucleotide analogues on ouabain binding noticed under equilibrium or steady-state conditions (62, 165) which indicates a much reduced ouabain affinity to nucleotide-occupied enzyme (and vice versa). The latter observations were interpreted as suggestive of a two-subunit mechanism working out of phase, i.e. phosphorylation of one subunit accompanied by ATP-binding of the other one (52, 53). This seemed an attractive hypothesis assuming a dimeric structure of the hydrolytic peptide, a view which is no longer favoured [for references, see review by Jørgensen (92)]. The former observations (167, 179), made under isolated dissociation conditions, on the other hand, would certainly be suggestive of a half-of-the-sites-reactivity mechanism provided P_i remained bound to the enzyme-ouabain complex at the time of nucleotide interaction. As seen in the previous paragraph the assumption seems not to be fulfilled and is thus not indicative of such a mechanism. The recent observation by Askari and Huang (12) of a decellerated P_i-release by nucleotides under isolated dissociation conditions does show that simultaneous ATP- and P_i-binding may take place.

In experiments with the (Mg^{2+}+ vanadate)-promoted enzyme-ouabain complex it was shown that the dissociation of vanadate as well as of ouabain was decellerated by ATP + Na^+ (71). As seen from figure 3 this effect could be mimicked by the putative nucleotide analogues suramin and eosin in combination with Na^+. They also both accelerate ouabain release from the (Mg^{2+} + P_i)-promoted enzyme-ouabain complex in a similar manner to ATP. Whether the former observations should be interpreted as suggestive of the coexistence of two ATP sites on the functional unit of the enzyme, as proposed by Askari and Huang (12), or merely that the substrate

site is able to accommodate a nucleotide triphosphate simultaneously with P_i or vanadate, as known for the diphosphate and P_i, remains to be unravelled.

VI. The Use of Ouabain Binding Capacity for Characterization of Na,K-ATPase Preparations or the Pump Density of Tissues

A. Native or Detergent-Treated Enzyme

For the function of the Na,K-ATPase the enzyme has to be attached to the plasma membrane or embedded into reconstituted phospholipid vesicles. Under such conditions a characterization and quantification of the enzyme with rigorous protein-chemical methods is difficult and binding capacities for specific ligands may be used as substitute. It thus becomes an important question whether or not the binding capacity is a reliable measure of active enzyme.

The ligands available for quantification after measurements under equilibrium conditions are ADP or ATP, vanadate, and ouabain. With detergent-treated ATPase preparations from pig kidney and well-defined binding conditions the binding capacities for the three parameters are equal (69). Obviously, this is not the case with impure preparations since nucleotides and vanadate are bound to several other enzymes. Equal binding capacities for nucleotides and ouabain have been found with ox brain Na,K-ATPase (62) and with pig kidney Na,K-ATPase (88), and also for ouabain and vanadate to pig kidney Na,K-ATPase (71). Cantley et al. (21) originally assumed the existence of two vanadate binding sites per ouabain binding site, but the additional vanadate binding was later supposed to be nonspecific (159).

A prerequisite for equal binding capacities of the three ligands is that they, as well as the factors that may be necessary for binding promotion, have access to their binding sites. The membrane fragments containing the Na,K-ATPase derived from tissue homogenates have to be treated by detergent to achieve this. The detergent demasking of latent Na,K-ATPase activity and the increased availability of one or more of the three parameters used for quantification is probably due to disruption of closed vesicles (86, 135). Since the receptors for nucleotides and vanadate are localized on the intracellular aspect and the ouabain receptor on the extracellular aspect of the plasma membrane, vesicle formation does not affect binding of the three ligands equally. Most vesicles are orientated right-side-out and (Mg^{2+} + P_i)-supported ouabain binding was almost maximal even in the absence of detergent, whereas (Mg^{2+} + Na^+ + ATP)-promoted ouabain binding only took place to a fraction of the potential binding sites and this was probably due to broken or leaky membrane vesicles (44). Apparently enough Mg^{2+} and P_i gained access to the intravesicular volume or were trapped there during homogenization, whereas the ATP permeability is too slow compared to the inevitable hydrolysis. Na^+ would then under these conditions counteract any P_i-supported ouabain binding. Walter (170) reached a similar conclusion about ouabain availability to untreated vesicles. A study of Wellsmith and Lindenmayer (171) on ouabain binding to a sarcolemmal preparation from heart muscle indicated two ouabain-binding components prior to detergent treatment. The vesicular structure was discussed as one of the possible explanations. The behaviour of crude microsomal Na,K-ATPase with the striking ratio of 2:1 between (Mg^{2+} + P_i)-supported ouabain binding on one hand and vanadate, nucleotide, and (Mg^{2+} + Na^+ + ATP)-supported ouabain-binding capacity on the other hand gave rise to the proposal by Hansen et al. (69) of a subunit structure of the enzyme with negatively cooperating sites. This anticooperativity seemed to be disturbed by detergents. A more likely explanation appeared to be the vesicular structure and the relative impermeability of the vesicles as indicated by the differential trypsin inactivation (44). This interpretation was supported by an experiment with the ionophor nystatin which doubled the vanadate binding of microsomal Na,K-ATPase without activation of the ATPase activity, figure 4 (unpublished observation).

Careful demasking of latent ATPase activity under optimum conditions is thus the prerequisite for the finding of equal binding capacities of nucleotides, vanadate, and ouabain and for maximum catalytic center activity (turnover number or molecular activity). Enzyme prep-

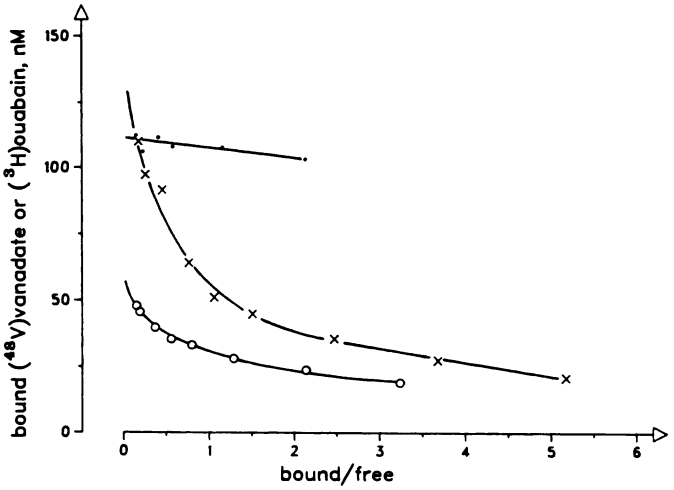


FIG. 4. Effect of nystatin (Mycostatin) on [^{48}V]vanadate binding to microsomal Na,K-ATPase from pig kidney. Binding data are plotted according to Scatchard as bound ligand versus bound/free. Microsomal Na,K-ATPase was prepared as described (69, 82) and incubated with 3 mM Mg^{2+}, 10 mM K^+, 40 mM Tris (pH 7.25) and 1 vol % dimethylformamide containing 10 mg nystatin (Mycostatin) per ml (final concentration of nystatin 0.1 mg·ml^{-1} or 450 units·ml^{-1}). After 30 min [^{48}V]vanadate was added. Vanadate binding was measured with the filtration technique described [Hansen (71)] after 60 min of incubation at 25°C (×). Control enzyme was treated in the same way except that nystatin was absent (○). The ouabain-binding capacity of control enzyme was determined after 120 min at 37°C in the presence of 3 mM Mg^{2+}, 3 mM P_i, and 40 mM Tris (●). Ouabain binding supported by (Mg^{2+} + Na^+ + ATP) was very similar to vanadate binding in the absence of nystatin (not shown).

arations treated in this way were examined for the relationship between ($Na^+ + K^+$)-activated ATPase activity and ouabain-binding capacity. A linear relationship between the two parameters was found for ox brain Na,K-ATPase isolated from grey matter (61) and for a much broader spectrum of activities of kidney enzyme isolated from outer medulla (86, 88). Likewise a linear correlation between ouabain occupancy and inhibition of Na,K-ATPase or the associated K^+-phosphatase was found (38, 62, 119). A fixed stochiometric relationship between ATPase activity and ouabain-binding capacity and between fractional ouabain occupancy and fractional inhibition of the hydrolytic activity thus exists.

The occasional finding of an activation of the Na,K-ATPase or stimulation of the sodium pumping by very low concentrations of cardiac glycosides [for references, see Ghysel-Burton and Godfraind (49); Grupp et al. (57)] have never been verified with purified preparations. Hougen et al. (79) have presented convincing evidence that the stimulation seen in intact tissues or homogenates is mediated via β-adrenergic receptors. Catecholamines (and insulin) are known to stimulate pump-mediated ^{42}K influx without affecting the number of pumps (28, 113), the underlying mechanism remaining obscure. The overall stimulation of sodium/potassium pumping at low ouabain concentration can apparently be ascribed to high-affinity ouabain binding to neural tissue triggering a release of catecholamines. These in turn stimulate sarcolemmal Na,K-ATPase which, because of low-affinity, is not occupied by ouabain. The stimulation of some pumps by catecholamines in intact tissue may thus overshadow the inhibition by ouabain of others. Should we then find that purified preparations of Na,K-ATPase contained ouabain binding sites of different affinities? The number of high-affinity sites copurified with the low-affinity ouabain sites during Na,K-ATPase purification is probably insignificant and not easily detected. However, the peculiar shape of curves of ATPase-activity as a function of log ouabain concentration of "pure" kidney enzyme preparations may reflect just such a phenomenon (see ref. 91; figure 4). In Na,K-ATPase isolated from muscle tissue neural enzyme may even predominate (29).

In intact cells the rate of pumping and the hydrolytic activity is far from maximum. Small changes in the concentration of, for example, monovalent cations or the action of some hormones may accelerate the pumping rate. For that reason, a fractional occupancy by ouabain is unlikely to inhibit ion translocation or ATP splitting proportionally. Rate of ouabain binding on the other hand apparently parallels rate of pumping (28, 84) whether induced by an increasing intracellular Na^+ concentration or a hormonal acceleration of the pump leading to decreased Na^+ concentration.

Though influenced by several unknown factors, ouabain binding to intact cells shows the same dependence on monovalent cations, substrate, etc. as the purified, membrane bound Na,K-ATPase (14, 15, 27). The ouabain-binding capacity is a characteristic and regulated feature of different tissues. Thus, an age-dependent change in ouabain-binding capacity is seen in skeletal muscles (98, 30). A change was also observed by exposure to an excess of thyroxine or withdrawal of thyroxine (99). It is difficult to measure the Na,K-ATPase of muscle homogenates, but the binding capacity seems to be related to the K^+-dependent methylfluorescein phosphatase activity (118), which indicates that the binding capacity is a reliable measure of potential pump activity.

B. Modulation of the Na,K-ATPase and the Consequences for Ouabain Binding

After demasking of latent hydrolytic activity/binding sites by mild detergent treatment the ouabain-binding capacity of membrane-bound Na,K-ATPase is a measure of the enzyme units present. The ouabain-binding capacity of tissues also indicates the potential Na,K-pump activity. But, the enzyme may be modified by several reagents such that one or more of the partial reactions supposed to be integrated in the overall hydrolysis or an associated reaction is changed without affecting ouabain binding qualitatively or quantitatively. The ouabain-binding capacity is thus less vulnerable than the overall hydrolysis and does not guarantee native enzyme, although the phosphorylation capacity probably has to be retained [but see Erdmann and Schoner (37)].

Phospholipase A and C treatment of Na,K-ATPase from ox brain did not change ouabain-binding rate or capacity significantly (37, 73, 163) though the overall hydrolysis was changed considerably. Treatment of electroplax Na,K-ATPase with phospholipase A destroyed ouabain binding along with the hydrolytic ability and this was probably due to production of lysophosphatides and fatty acids. In contrast, phospholipase C treatment did not alter the binding of ouabain but the hydrolytic activity was markedly reduced (56). Na,K-ATPase from shark rectal glands solubilized with deoxycholate and freed of the lipids by gel filtration retained the nucleotide binding ability when devoid of hydrolytic activity (81). Ouabain binding obtained with ($Mg^{2+} + P_i$) was reduced, however (Ottolenghi and Hansen, unpublished observation).

Graded treatment of Na,K-ATPase by the mercurial thimerosal induced a decrease in ouabain-binding capacity (whether induced by $Mg^{2+} + P_i$ or by $Mg^{2+} + Na^+ +$ ATP) that was much less than the reduction in overall hydrolytic activity (70). Remarkably, the associated K^+-activated phosphatase activity (pNPPase) was doubled relative to the ouabain-binding capacity. Ouabain binding also appeared to be more resistant to treatment with the SH-reagents, ethacrynic acid and N-ethylmaleimide, than the Na,K-ATPase activity. The remaining phosphorylation by ATP and, to some degree, the K-pNPPase activity seemed more closely related to the remaining

ouabain-binding capacity (37, 76, 168). In contrast, Schoot et al. (141) observed a strictly parallel inhibition of all partial reactions and Na,K-ATPase activity by N-ethylmaleimide.

In a previous section it was mentioned that experiments with radiation inactivation of pig kidney Na,K-ATPase had shown that a domain varying from 38% to 55% of the apparent molecular weight of the dimer ($\alpha_2\beta_2$) was needed for ouabain binding, depending on the factors used for facilitation (120). The disappearance of hydrolytic activity was much faster and the enzymatic activity was thus dependent on the integrity of a bigger part of the dimer.

Addition of relatively high concentrations of dimethylsulfoxide or glycerol to the incubation medium reversibly inhibit the Na,K-ATPase activity concurrent with an activation of the pNPPase activity of enzyme from ox brain (111) and electric organ (9) while the nucleotide- and ouabain-binding capacity seems unchanged (93).

Covalent attachment of fluorescein isothiocyanate to Na,K-ATPase, probably near or at the nucleotide binding site, blocks nucleotide binding, ATP-phosphorylation, and overall hydrolysis; but to a lesser extent the K-pNPPase activity, P_i-phosphorylation and (Mg^{2+} + P_i)-induced ouabain binding (77, 96). Ouabain binding to fluorescein-bound enzyme promoted by (Mg^{2+} + Na^+ + ATP) did not take place or the affinity was reduced dramatically (unpublished observation).

Conclusion. As a rule, the ouabain-binding capacity of a certain enzyme preparation or tissue will express the number of Na,K-ATPase units present and will indicate the potential hydrolytic activity on ATP or Na,K-transport capacity. It is a prerequisite that no physical obstruction exist for the accessibility of ouabain or the ligands that promote its binding. The hydrolytic activity seems to be more vulnerable than the ouabain binding ability and some chemical reagents or physical treatments will not destroy the two manifestations of Na,K-ATPase in parallel.

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Appendix

Ouabain-Binding Models and Reciprocal Plots for Testing the Models

In sections A and B it is ignored that the ouabain-binding species is probably never the enzyme as such, but more likely a Mg^{2+}-induced phosphorylated form of the enzyme (e.g. $EMgP_i$).

A. Provided the binding model is

$$E + O \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EO \tag{1}$$

in which E means receptor sites not occupied by ouabain, O free (non-bound) ouabain, EO the enzyme-ouabain complex, and k_1 and k_{-1} the forward and backward velocity constants, respectively, the equilibrium situation may be expressed as

$$k_1 \cdot [E] \cdot [O] = k_{-1} [EO] \tag{2}$$

or

$$[EO] = \frac{k_1}{k_{-1}} \cdot [E] \cdot [O]\,. \tag{3}$$

Since the maximum number of ouabain-binding sites $[EO_{max}] = [E] + [EO]$ and thus

$$[E] = [EO_{max}] - [EO]\,, \tag{4}$$

substitution of [E] in Eq. (3) may give

$$[EO] = \frac{k_1}{k_{-1}} \cdot ([EO_{max}] - [EO]) \cdot [O]\,. \tag{5}$$

Multiplication with $\frac{k_{-1}}{k_1}$ and isolation of EO will give

$$\begin{gathered} \frac{k_{-1}}{k_1} [EO] = ([EO_{max}] - [EO]) \cdot [O] \\ [EO] = -\frac{k_{-1}}{k_1} \cdot \frac{[EO]}{[O]} + [EO_{max}]\,. \end{gathered} \tag{6}$$

Eq. (6) is the so-called Scatchard plot applied on equilibrium-binding data. Plots of the binding isotherms as bound ligand ([EO]) against bound ligand/free ligand ([EO]/[O]) should, according to this model, fit a straight line with the ordinate intercept $[EO_{max}]$ and the slope $-\frac{k_{-1}}{k_1}$, the numerical value of which is defined as the dissociation constant K_d, from Eq. (2).

Rearrangement of Eq. (5) may also give

$$[EO] = \frac{\frac{k_1}{k_{-1}} \cdot [EO_{max}] \cdot [O]}{1 + \frac{k_1}{k_{-1}} \cdot [O]}\,. \tag{7}$$

Expressed reciprocally Eq. (7) becomes

$$\begin{gathered} \frac{1}{[EO]} = \frac{1 + \frac{k_1}{k_{-1}} \cdot [O]}{\frac{k_1}{k_{-1}} [EO_{max}] \cdot [O]} \\ \frac{1}{[EO]} = \frac{k_{-1}}{k_1} \cdot \frac{1}{[EO_{max}]} \cdot \frac{1}{[O]} + \frac{1}{[EO_{max}]}\,. \end{gathered} \tag{8}$$

Eq. (8) is equivalent to the so-called Lineweaver-Burk plot. Instead of reaction rates, equilibrium-binding data is used. Data points plotted according to Eq. (8) are clustered in one end of the scale resulting in unbalanced weight put on some of the data. This inconvenience is overcome by multiplication with [O]

$$\frac{[O]}{[EO]} = \frac{1}{[EO_{max}]} \cdot [O] + \frac{k_{-1}}{k_1} \cdot \frac{1}{[EO_{max}]}\,. \tag{9}$$

Plots of $\frac{[O]}{[EO]}$ against [O] are used in Ref. 61.

B. More Than One Ouabain-Binding Species Exist.

1. Two Interconvertible Ouabain-Binding Species. As an example, if two enzyme species, K^+-bound enzyme and enzyme not occupied by K^+, will bind ouabain with different affinities and if instantaneous equilibrium exists between the distinct enzyme-ligand forms, the following three equations express the situation

$$E + K \rightleftharpoons EK \quad (10)$$

$$E + O \rightleftharpoons EO \quad (11)$$

$$EK + O \rightleftharpoons EKO\,. \quad (12)$$

At equilibrium the concentration of the ligand-complexed species may be expressed by using the respective association constants of Eqs. (10) to (12)

$$[EK] = k_{EK}\cdot[K]\cdot[E] \quad (13)$$

$$[EO] = k_{EO}\cdot[O]\cdot[E] \quad (14)$$

$$[EKO] = k_{EKO}\cdot[O]\cdot[EK]\,. \quad (15)$$

Substitution in Eq. (15) with Eq. (13) gives

$$[EKO] = k_{EKO}\cdot k_{EK}\,[O]\cdot[K]\cdot[E]\,. \quad (16)$$

Since the total number of ouabain-binding receptors

$$[EO_{max}] = [E] + [EK] + [EO] + [EKO] \quad (17)$$

and since the actual number of sites occupied by ouabain is [B] = [EO] + [EKO], Eq. (17) may be expressed as

$$[EO_{max}] = [E] + [EK] + [B]\,. \quad (18)$$

Substitution of EK with Eq. (13) will give

$$[EO_{max}] = [E]\cdot(1 + k_{EK}[K]) + [B]\,. \quad (19)$$

Bound ouabain, [B], is also obtained from Eq. (14) plus Eq. (16)

$$[B] = [EO] + [EKO] = [E]\cdot[O]\cdot(k_{EO} + k_{EKO}\cdot k_{EO}\cdot[K])\,. \quad (20)$$

Isolation of [E] will give

$$[E] = \frac{[B]}{[O]}\cdot\frac{1}{k_{EO} + k_{EKO}\cdot k_{EO}\cdot[K]}\,. \quad (21)$$

Substitution of [E] in Eq. (19) with Eq. (21) may give

$$[EO_{max}] = \frac{1 + k_{EK}\cdot[K]}{k_{EO} + k_{EKO}\cdot k_{EK}\cdot[K]}\cdot\frac{[B]}{[O]} + B\,. \quad (22)$$

Eq. (22) is equivalent to Eq. (6), except that the constant is more complex. The equation thus describes a straight line with a complex slope, but the ordinate intercept [EO_{max}].

2. Two Non-interconvertible Ouabain-Binding Species. Under a given set of conditions, e.g. with a given concentration of ligands, two non-interconvertible enzyme species, *E′* and *E″*, will bind ouabain with different affinities

$$E' + O \rightleftharpoons E'O \quad (23)$$

$$E'' + O \rightleftharpoons E''O \quad (24)$$

Eq. (6) can be applied on each ouabain-binding species:

$$[E'O] = -K'_{dis}\cdot\frac{[E'O]}{[O]} + [E'O_{max}] \quad (25)$$

$$[E''O] = -K''_{dis}\cdot\frac{[E''O]}{[O]} + [E''O_{max}] \quad (26)$$

in which $K'_{dis} \neq K''_{dis}$

Calling $E'O + E''O = EO$ and $E'O_{max} + E''O_{max} = EO_{max}$, Eq. (25) + Eq. (26) equals

$$[EO] = -(K'_{dis}\cdot[E'O] + K''_{dis}\cdot[E''O])\cdot\frac{1}{[O]} + [EO_{max}]\,. \quad (27)$$

Eq. (27) may also be expressed as

$$[EO] = -\left(K'_{dis}\cdot\frac{[E'O]}{[EO]} + K''_{dis}\frac{[E''O]}{[EO]}\right)\cdot\frac{[EO]}{[O]} + [EO_{max}]\,. \quad (28)$$

Plotting observed binding, [EO], versus $\frac{[EO]}{[O]}$ the binding isotherms cannot be fitted to a straight line since ths slope according to Eq. (28) is not a constant.

Eq. (28) is not suitable for a computer program and therefore the sum of the two components is expressed according to Eq. (7) for such programs e.g. as

$$[EO] = \frac{[E'O_{max}]\cdot[O]}{[O] + K'_{dis}} + \frac{[E''O_{max}]\cdot[O]}{[O] + K''_{dis}}\,. \quad (29)$$

The numerical value of the slopes of the two components directly indicate K'_{dis} and K''_{dis}.

3. Anticooperativity Among Two Primary Identical Ouabain-Binding Species. The binding isotherms plotted according to Scatchard will give upward concave curves as was the case in section B2. Resolution of the curves may take place according to eq. (29) by setting $E'O_{max} = E''O_{max}$ which is equivalent to substitution of a complex expression containing polynomials by the sum of two first order terms (82, 101). The calculated ouabain dissociation constants have no meaning but may be used for calculation of the intrinsic dissociation constants, dissociation constant 1 and dissociation constant 2.

REFERENCES

1. ADAMS, R. J., SCHWARTZ, A., GRUPP, G., GRUPP, I., LEE, S., WALLICK, E. T., POWELL, T., TWIST, V. W., AND GATHIRAM, P.: High-affinity ouabain binding site and low-dose positive inotropic effect in rat myocardium. Nature (Lond.) **296:** 167–169, 1982.
2. AHMED, K., ROHRER, D. C., FULLERTON, D. S., DEFFO, T., KITATSUJI, E., AND FROM, A. H. L.: Interaction of (Na^+,K^+)-ATPases and digitalis genins. A general model for inhibitory activity. J. Biol. Chem. **258:** 8092–8097, 1983.
3. AKERA, T.: Quantitative aspects of the interaction between ouabain and ($Na^+ + K^+$)-activated ATPase in vitro. Biochim. Biophys. Acta **249:** 53–62, 1971.
4. AKERA, T., AND BRODY, T. M.: Membrane adenosine triphosphatase. The effect of potassium on the formation and dissociation of the ouabain-enzyme complex. J. Pharmacol. Exp. Ther. **176:** 545–557, 1971.
5. AKERA, T.: Membrane adenosinetriphosphatase: A digitalis receptor? Science **198:** 569–574, 1977.
6. AKERA, T.: Effects of cardiac glycosides on Na^+,K^+-ATPase. *In* Handbook of Experimental Pharmacology, ed. by K. Greeff, vol. 56, pp. 287–336, Springer-Verlag, Berlin, 1981.
7. ALBERS, R. W.: Biochemical aspects of active transport. Annu. Rev. Biochem. **36:** 727–756, 1967.
8. ALBERS, R. W., KOVAL, G. J., AND SIEGEL, G. J.: Studies on the interaction of ouabain and other cardioactive steroids with sodium-potassium-activated adenosine triphosphatase. Mol. Pharmacol. **4:** 324–336, 1968.
9. ALBERS, R. W., AND KOVAL, G. J.: Sodium-potassium-activated adenosine triphosphatase of electrophorus electric organ. VIII. Monovalent cation sites regulating phosphatase activity. J. Biol. Chem. **248:** 777–784, 1973.
10. ALLEN, J. C., HARRIS, R. A., AND SCHWARTZ, A.: The nature of the transport ATPase-digitalis complex. I. Formation and reversibility in the presence and absence of a phosphorylated enzyme. Biochem. Biophys. Res. Commun. **42:** 366–370, 1971.
11. ALLEN, D. G., AND BLINKS, J. R.: Calcium transients in aequorin-injected frog cardiac muscle. Nature (Lond.) **273:** 509–513, 1978.
12. ASKARI, A., AND HUANG, W.: Na^+,K^+-ATPase: Evidence for the binding of ATP to the phospho-enzyme. Biochem. Biophys. Res. Commun. **104:** 1447–1453, 1982.
13. ASKARI, A., HUANG, W.-H., AND MCCORMICK, P. W.: ($Na^+ + K^+$)-dependent adenosine triphosphatase. Regulation of inorganic phosphate, magnesium ion, and calcium ion interactions with the enzyme by ouabain. J. Biol. Chem. **258:** 3453–3460, 1983.
14. BAKER, P. F., AND WILLIS, J. S.: Potassium ions and the binding of cardiac glycosides to mammalian cells. Nature (Lond.) **226:** 521–523, 1970.
15. BAKER, P. F., AND WILLIS, J. S.: Inhibition of the sodium pump in squid giant axons by cardiac glycosides: Dependence on extracellular ions and metabolism. J. Physiol (Lond.) **224:** 463–475, 1972.
16. BARNETT, R. E.: Effect of monovalent cations on the ouabain inhibition of the sodium and potassium ion activated adenosine triphosphatase. Biochemistry **9:** 4644–4648, 1970.
17. BODEMANN, H. H., AND HOFFMAN, J. F.: Side-dependent effects of internal versus external Na and K on ouabain binding to reconstituted human red blood cell ghosts. J. Gen. Physiol. **67:** 497–525, 1976a.
18. BODEMANN, H. H., AND HOFFMAN, J.: Comparison of the side-dependent

effects of Na and K on orthophosphate, UTP- and ATP-promoted ouabain binding to reconstitued human red blood cell ghosts. J. Gen. Physiol. **67:** 527–545, 1976b.
19. CALDWELL, P. C., AND KEYNES, R. D.: The effect of ouabain on the efflux of sodium from squid giant axon. J. Physiol. (Lond.) **148:** 8P–9P, 1959.
20. CANTLEY, L. C., JR., JOSEPHSON, L., WARNER, R., YANAGISAWA, M., LECHENE, C., AND GUIDOTTI, G.: Vanadate is a potent (Na,K)-ATPase inhibitor found in ATP derived from muscle. J. Biol. Chem. **252:** 7421–7423, 1977.
21. CANTLEY, L. C., JR., CANTLEY, L. G., AND JOSEPHSON, L.: A characterization of vanadate interactions with the (Na,K)-ATPase. Mechanistic and regulatory implications. J. Biol. Chem. **253:** 7361–7368, 1978.
22. CANTLEY, L. C.: Structure and mechanism of the (Na,K)-ATPase. Current Top. Bioenerg. **11:** 201–237, 1981.
23. CASTRO, J., AND FARLEY, R. A.; Proteolytic fragmentation of the catalytic subunit of the sodium and potassium adenosine triphosphatase. J. Biol Chem. **254:** 2221–2228, 1979.
24. CHARNOCK, J. S., AND POST, R. L.: Evidence of the mechanism of ouabain inhibition of cation activated adenosine triphosphatase. Nature (Lond.) **199:** 910–911, 1963.
25. CHIGNELL, C. F., AND TITUS, E.: Identification of components of (Na^+ + K^+)-adenosine triphosphatase by double isotopic labelling and electrophoresis. Proc. Natl. Acad. Sci. **64:** 324–329, 1969.
26. CHOI, Y. R., AND AKERA, T.: Kinetics studies on the interaction between ouabain and (Na^+ + K^+)-ATPase. Biochim. Biophys. Acta **481:** 648–659, 1977.
27. CLAUSEN, T., AND HANSEN, O.: Ouabain binding and Na^+-K^+ transport in rat muscle cells and adipocytes. Biochim. Biophys. Acta **345:** 387–404, 1974.
28. CLAUSEN, T., AND HANSEN, O.: Active Na-K transport and the rate of ouabain binding. The effect of insulin and other stimuli on skeletal muscle and adipocytes. J. Physiol. (Lond.) **270:** 415–430, 1977.
29. CLAUSEN, T., HANSEN, O., AND LARSSON, L. -I.: Sympathetic nerve terminal destruction has no effect on specific [^{3}H]ouabain binding to intact mouse and rat skeletal muscle. Eur. J. Pharmacol. **72:** 331–335, 1981.
30. CLAUSEN, T., HANSEN, O., KJELDSEN, K., AND NØRGAARD, AA.: Effect of age, potassium depletion and denervation on specific displaceable [^{3}H] ouabain binding in rat skeletal muscle in vivo. J. Physiol. (Lond.) **333:** 367–381, 1982.
31. COLLINS, J. H., FORBUSH, B., III, LANE, L. K., LING, E., SCHWARTZ, A., AND ZOT, A.: Purification and characterization of an (Na^+ + K^+)-ATPase proteolipid labeled with a photoaffinity derivative of ouabain. Biochim. Biophys. Acta **686:** 7–12, 1982.
32. DAHMS, A. S., AND BOYER, P. D.: Occurrence and characteristics of ^{18}O exchange reactions catalyzed by sodium- and potassium-dependent adenosine triphosphatase. J. Biol Chem. **248:** 3155–3162, 1973.
33. DE MEYTS, P., BIANCO, A. R., AND ROTH, J.: Site-site interactions among insulin receptors. Characterization of the negative cooperativity. J. Biol. Chem. **251:** 1877–1888, 1976.
34. DUNHAM, E. T., AND GLYNN, J. M.: Adenosine-triphosphatase activity and the active movements of alkali metal ions. J. Physiol. (Lond.) **156:** 274–293, 1961.
35. ERDMANN, E., AND SCHONER, W.: Ouabain-receptor interactions in (Na^+ + K^+)-ATPase preparations from different tissues and species. Determination of kinetic constants and dissociation constants. Biochim. Biophys. Acta **307:** 386–398, 1973.
36. ERDMANN, E., AND SCHONER, W.: Ouabain-receptor interactions in (Na^+ + K^+)-ATPase preparations. II. Effect of cations and nucleotides on rate constants and dissociation constants. Biochim. Biophys. Acta **330:** 302–315, 1973.
37. ERDMANN, E., AND SCHONER, W.: Ouabain-receptor interactions in (Na^+ + K^+)-ATPase preparations. III. On the stability of the ouabain receptor against physical treatment, hydrolases and SH reagents. Biochim. Biophys. Acta **330:** 316–324, 1973c.
38. ERDMANN, E., AND SCHONER, W.: Ouabain-receptor interactions in (Na^+ + K^+)-ATPase preparations. IV. The molecular structure of different cardioactive steroids and other substances and their affinity to the glycoside receptor. Naunyn-Schmiedeberg's Arch. Pharmacol. **283:** 335–356, 1974.
39. ERDMANN, E., KRAWITZ, W., AND PRESEK, P.: Receptor for cardiac glycosides. *In* Myocardial Failure, ed. by G. Riecker, A. Weber, and J. Goodwin, pp. 120–131, Springer-Verlag, Berlin, 1977.
40. ERDMANN, E., PHILIPP, G., AND SCHOLZ, H.: Cardiac glycoside receptor, (Na^+ + K^+)-ATPase activity and force of contraction in rat heart. Biochem. Pharmacol. **29:** 3219–3229, 1980.
41. ERDMANN, E.: Influence of cardiac glycosides on their receptor. In Handbook of Experimental Pharmacology, ed. by. K. Greeff, vol. 56, pp. 337–380, Springer-Verlag, Berlin, 1981.
42. FORBUSH, B., III, AND HOFFMAN, J. F.: Evidence that ouabain binds to the same polypeptide chain of dimeric Na,K-ATPase that is phosphorylated from P_i. Biochemistry **18:** 2308–2315, 1979.
43. FORBUSH, B., III, AND HOFFMAN, J. F.: Direct photoaffinity labelling of the primary region of the ouabain binding site of (Na^+ + K^+)-ATPase with [^{3}H]ouabain, [^{3}H]digitoxin and [^{3}H]digitoxigenin. Biochim. Biophys. Acta **555:** 299–306, 1979.
44. FORBUSH, B., III: Characterization of right-side-out membrane vesicles rich in (Na,K)-ATPase and isolated from dog kidney outer medulla. J. Biol. Chem. **257:** 12678–12684, 1982.
45. FORBUSH, B., III: Cardiotonic steroid binding to Na,K-ATPase. *In* Current Topics in Membranes and Transport, ed. by J. F. Hoffman and B. Forbush III, vol. 19, pp. 167–201, Academic Press, New York, 1983.
46. FORTES, P. A. G., ELLORY, J. C., AND LEW, V. L.: Suramin: A potent ATPase inhibitor which acts on the inside surface of the sodium pump. Biochim. Biophys. Acta **318:** 262–272, 1973.
47. FORTES, P. A. G.: Anthroylouabain: A specific fluorescent probe for the cardiac glycoside receptor of the Na-K-ATPase. Biochemistry **16:** 531–540, 1977.
48. GARDOS, G.: Akkumulation der Kaliumionen durch menschliche Blutkörperchen. Acta Physiol. Acad. Sci. Hung. **6:** 191–199, 1954.
49. GHYSEL-BURTON, J., AND GODFRAIND, T.: Stimulation and inhibition of the sodium pump by cardioactive steroids in relation to their binding sites and their inotropic effect on guinea-pig isolated atria. Br. J. Pharmacol. **66:** 175–184, 1979.
50. GIBBS, R., RODDY, P. M., AND TITUS, E.: Preparation, assay and properties of an Na^+- and K^+-requiring adenosine triphosphatase from beef brain. J. Biol. Chem. **240:** 2181–2187, 1965.
51. GLYNN, I. M.: The action of cardiac glycosides on sodium and potassium movements in human red cells. J. Physiol. (Lond.) **136:** 148–173, 1957.
52. GLYNN, I. M., AND KARLISH, S. J. D.: Different approaches to the mechanism of the sodium pump. *In* Energy Transformation in Biological Systems, pp. 205–223, Ciba Foundation Symposium 31, ASP, (Elsevier. Excerpta Medica, North Holland), Amsterdam 1975.
53. GLYNN, I. M., AND KARLISH, S. J. D.: The sodium pump. Annu. Rev. Physiol. **37:** 13–55, 1975.
54. GLYNN, I. M.: The Na^+,K^+-transporting adenosine triphosphatase. *In* Enzymes of Biological Membranes, 2nd ed., ed. by A. Martinosi, vol. 3, Plenum Publishing, New York, in press, 1984.
55. GOELDNER, M. P., HIRTH, C. G., ROSSI, B., PONZIO, G., AND LAZDUNSKI, M.: Specific photoaffinity labeling of the digitalis binding site of the sodium and potassium ion activated adenosinetriphosphatase induced by energy transfer. Biochemistry **22:** 4685–4690, 1983.
56. GOLDMAN, S. S., AND ALBERS, R. W.: Sodium-potassium-activated adenosine triphosphatase. IX. The role of phospholipids. J. Biol. Chem. **248:** 867–874, 1973.
57. GRUPP, G., GRUPP, J. L., GHYSEL-BURTON, J., GODFRAIND, T., AND SCHWARTZ, A.: Effects of very low concentrations of ouabain on contractile force of isolated guinea-pig, rabbit and cat atria and right ventricular papillary muscles: An interinstitutional study. J. Pharmacol. Exp. Ther. **220:** 145–151, 1982.
58. HALL, C., AND RUOHO, A.: Ouabain-binding-site photoaffinity probes that label both subunits of Na^+,K^+-ATPase. Proc. Natl. Acad. Sci. U.S.A. **77:** 4529–4533, 1980.
59. HALSEY, J. F., MOUNTCASTLE, D. B., TAKEGUCHI, C. A., BILTONEN, R. L., AND LINDENMAYER, G. E.: Detection of a ouabain-induced structural change in the sodium, potassium-adenosine triphosphatase. Biochemistry**16:** 432–435, 1977.
60. HAN, C. S., TOBIN, T., AKERA, T., AND BRODY, T. M.: Effects of alkali metal cations on phosphoenzyme levels and [^{3}H]ouabain binding to (Na^+ + K^+)-ATPase. Biochim. Biophys. Acta **429:** 993–1005, 1976.
61. HANSEN, O.: The relationship between g-strophanthin-binding capacity and ATPase activity in plasma membrane fragments from ox brain. Biochim. Biophys. Acta **233:** 122–132, 1971.
62. HANSEN, O., JENSEN, J., AND NØRBY, J. G.: Mutual exclusion of ATP, ADP and g-strophanthin binding to Na,K-ATPase. Nature New Biol. **234:** 122–124, 1971.
63. HANSEN, O., AND SKOU, J. C.: A study on the influence of the concentration of Mg^{2+}, P_i, K^+, Na^+ and Tris on (Mg^{2+} + P_i)-supported g-strophanthin binding to (Na^+ + K^+)-activated ATPase from ox brain. Biochim. Biophys. Acta **311:** 51–66, 1973.
64. HANSEN, O.: Non-uniform populations of g-strophanthin binding sites of (Na^+ + K^+)-activated ATPase. Apparent conversion to uniformity by K^+. Biochim. Biophys. Acta **433:** 383–392, 1976.
65. HANSEN, O.: Ouabain used as a tool for trapping and characterizing phosphorylation products of Na,K-ATPase. *In* Membrane Proteins, ed. by P. Nicholls, J. V. Møller, P. L. Jorgensen, and A. L. Moody, vol. 45, pp. 191–197, Pergamon Press, Oxford, 1978.
66. HANSEN, O.: The effect of sodium on inorganic phosphate- and p-nitrophenyl phosphate-facilitated ouabain binding to (Na^+ + K^+)-activated ATPase. Biochim. Biophys. Acta **511:** 10–22, 1978.
67. HANSEN, O.: Facilitation of ouabain binding to (Na^+ + K^+)-ATPase by vanadate at in vivo concentrations. Biochim. Biophys. Acta **568:** 265–269, 1979.
68. HANSEN, O.: Reactive states of the Na,K-ATPase demonstrated by the stability of the enzyme-ouabain complex. *In* Na,K-ATPase. Structure and Kinetics, ed. by J. C. Skou and J. G. Nørby, pp. 169–180, Academic Press, New York, 1979.
69. HANSEN, O., JENSEN, J., NØRBY, J. G., AND OTTOLENGHI, P.: A new proposal regarding the subunit composition of (Na^+ + K^+)-ATPase. Nature (Lond.) **280:** 410–412, 1979.
70. HANSEN, O., JENSEN, J., AND OTTOLENGHI, P.: Na,K-ATPase: The uncoupling of its ATPase and p-nitrophenyl phosphatase activities by thime-

rosal. *In* Na,K-ATPase. Structure and Kinetics, ed. by J. C. Skou, and J. G. Nørby, pp. 217–226, Academic Press, New York, 1979.
71. HANSEN, O.: Studies on ouabain-complexed ($Na^+ + K^+$)-ATPase carried out with vanadate. Biochim. Biophys. Acta **692:** 187–195, 1982.
72. HANSEN, O.: Vanadate and phosphotransferases with special emphasis on ouabain/Na,K-ATPase interaction. Acta Pharmacol. Toxicol. **52:** suppl. I, 1–19, 1983.
73. HARRIS, W. E., SWANSON, P. D., AND STAHL, W. L.: Ouabain binding sites and the ($Na^+ + K^+$)-ATPase of brain microsomal membranes. Biochim. Biophys. Acta **298:** 680–689, 1973.
74. HARRIS, W. E., AND STAHL, W. L.: Conformational changes of purified ($Na^+ + K^+$)-ATPase detected by a sulfhydryl fluorescence probe. Biochim. Biophys. Acta **485:** 203–214, 1977.
75. HART, W. M., JR., AND TITUS, E. O.: Sulfhydryl groups of sodium-potassium transport adenosine triphosphatase. Protection by physiological ligands and exposure by phosphorylation. J. Biol. Chem. **248:** 4674–4681, 1973.
76. HEGYVARY, C.: Ouabain-binding and phosphorylation of ($Na^+ + K^+$)ATPase treated with N-ethylmaleimide or oligomycin. Biochim. Biophys. Acta **422:** 365–379, 1976.
77. HEGYVARY, C., AND JØRGENSEN, P. L.: Conformational changes of renal sodium plus potassium ion-transport adenosine triphosphatase labelled with fluorescein. J. Biol. Chem. **256:** 6296–6303, 1981.
78. HENDERSON, F. G.: Chemistry and biological activity of the cardiac glycosides. *In* Digitalis, ed. by C. Fisch, and B. Surawicz, pp. 3–12, Grune & Stratton, New York, 1969.
79. HOUGEN, T. J., SPICER, N., AND SMITH, T. W.: Stimulation of monovalent cation active transport by low concentrations of cardiac glycosides. Role of catecholamines. J. Clin. Invest. **68:** 1207–1214, 1981.
80. HUANG, W. -H., AND ASKARI, A.: Red cell Na^+,K^+-ATPase: A method for estimating the extent of inhibition of an enzyme sample containing an unknown amount of bound cardiac glycoside. Life Sci. **16:** 1253–1262, 1975.
81. JENSEN, J., AND OTTOLENGHI, P.: Adenosine diphosphate binding to sodium-plus-potassium ion-dependent adenosine triphosphatase. The role of lipid in the nucleotide-potassium ion interplay. Biochem. J. **159:** 815–817, 1976.
82. JENSEN, J., NØRBY, J. G., AND OTTOLENGHI, P.: Sodium and potassium binding to the sodium pump: Stoichiometry and affinities evaluated from nucleotide-binding behaviour. J. Physiol. (Lond.) **346:** 219–241, 1984.
83. JENSEN, J., AND OTTOLENGHI, P.: Binding of Rb^+ and ADP to a potassium-like form of Na,K-ATPase. *In* Current Topics in Membranes and Transport, ed. by J. F. Hoffmann, and B. Forbush, III, vol. 19, pp. 223–227, Academic Press, New York, 1983.
84. JOINER, C. H., AND LAUF, P. K.: Modulation of ouabain binding and potassium pump fluxes by cellular sodium and potassium in human and sheep erythrocytes. J. Physiol. (Lond.) **283:** 177–196, 1978.
85. JOSEPHSON, L., AND CANTLEY, L. C., JR.: Isolation of a potent (Na-K)ATPase inhibitor from striated muscle. Biochemistry **21:** 4572–4578, 1977.
86. JØRGENSEN, P. L., AND SKOU, J. C.: Purification and characterization of ($Na^+ + K^+$)-ATPase. I. The influence of detergents on the activity of ($Na^+ + K^+$)ATPase in preparations from the outer medulla of rabbit kidney. Biochim. Biophys. Acta **233:** 366–380, 1971.
87. JØRGENSEN, P. L.: Techniques for the study of steroid effects on membraneous Na,K-ATPase. Methods Enzymol. **36A:** 434–439, 1974.
88. JØRGENSEN, P. L.: Purification and characterization of ($Na^+ + K^+$)-ATPase. IV. Estimation of the purity and of the molecular weight and polypeptide content per enzyme unit in preparations from the outer medulla of rabbit kidney. Biochim. Biophys. Acta **356:** 53–67, 1974b.
89. JØRGENSEN, P. L.: Isolation and characterization of the components of the sodium pump. Q. Rev. Biophys. **7:** 239–274, 1975.
90. JØRGENSEN, P. L., AND PETERSEN, J.: Purification and characterization of ($Na^+ + K^+$)-ATPase. V. Conformational changes in the enzyme. Transitions between the Na-form and the K-form studied with tryptic digestion as a tool. Biochim. Biophys. Acta **401:** 399–415, 1975.
91. JØRGENSEN, P. L.: Sodium and potassium ion pump in kidney tubules. Physiol. Rev. **60:** 864–917, 1980.
92. JØRGENSEN, P. L.: Mechanism of the Na^+,K^+ pump. Protein structure and conformations of the pure ($Na^+ + K^+$)-ATPase. Biochim. Biophys. Acta **694:** 27–68, 1982.
93. KANIIKE, K., ERDMANN, E., AND SCHONER, W.: Study on the differential modifications of ($Na^+ + K^+$)-ATPase and its partial reactions by dimethylsulfoxide. Biochim. Biophys. Acta **352:** 275–286, 1974.
94. KARLISH, S. J. D., YATES, D. W., AND GLYNN, J. M.: Conformational transitions between Na^+-bound and K^+-bound forms of ($Na^+ + K^+$)-ATPase, studied with formycin nucleotides. Biochim. Biophys. Acta **525:** 252–264, 1978.
95. KARLISH, S. J. D., AND YATES, D. W.: Tryptophan fluorescence of ($Na^+ + K^+$)-ATPase as a tool for study of the enzyme mechanism. Biochim. Biophys. Acta **527:** 115–130, 1978.
96. KARLISH, S. J. D.: Characterization of conformational changes in [Na,K] ATPase labelled with fluorescein at the active site. J. Bioenerg. Biomembranes **12:** 111–136, 1980.
97. KARLISH, S. J. D., AND PICK, U.: Sidedness of the effects of sodium and potassium ions on the conformational state of the sodium-potassium pump. J. Physiol. (Lond.) **312:** 505–529, 1981.
98. KJELDSEN, K., NØRGAARD, AA., AND CLAUSEN, T.: Age-dependent changes in the number of [^{3}H]ouabain binding sites in rat soleus muscle. Biochim. Biophys. Acta **686:** 253–256, 1982.
99. KJELDSEN, K., AND NØRGAARD, AA.: Regulation of Na-K-pumps in skeletal muscle and the implication for the Na-K-homeostasis during K-depletion. Institute of Physiology, University of Aarhus, 1982.
100. KLODOS, I., NØRBY, J. G., AND PLESNER, I. W.: The steady-state kinetic mechanism of ATP hydrolysis catalyzed by membrane-bound ($Na^+ + K^+$)-ATPase from ox brain. II. Kinetic characterization of phosphointermediates. Biochim. Biophys. Acta **643:** 463–482, 1981.
101. KLOTZ, I. M., AND HUNSTON, D. L.: Protein affinities for small molecules: Conceptions and misconceptions. Arch. Biochem. Biophys. **193:** 314–328, 1979.
102. KOLASSA, N., PUNZENGRUBER, C., SUKO, J., AND MAKINOSE, M.: Mechanism of calcium-independent phosphorylation of sarcoplasmic reticulum ATPase by orthophosphate. Evidence of magnesium-phosphoprotein formation. F.E.B.S. Lett. **108:** 495–500, 1979.
103. KURIKI, Y., HALSEY, J., BILTONEN, R., AND RACKER, E.: Calorimetric studies of the interaction of magnesium and phosphate with (Na^+,K^+)ATPase: Evidence for a ligand-induced conformational change in the enzyme. Biochemistry **15:** 4956–4961, 1976.
104. LINDENMAYER, G. E., LAUGHTER, A. H., AND SCHWARTZ, A.: Incorporation of inorganic phosphate-32 into a Na^+,K^+-ATPase preparation: Stimulation by ouabain. Arch. Biochem. Biophys. **127:** 187–192, 1968.
105. LINDENMAYER, G. E., AND SCHWARTZ, A.: Conformational changes induced in ($Na^+ + K^+$)-ATPase by ouabain through a K^+-sensitive reaction: Kinetic and spectroscopic studies. Arch. Biochem. Biophys. **140:** 371–378, 1970.
106. LINDENMAYER, G. E., SCHWARTZ, A., LEWIS, J. M., ANDERSON, L. D., AND HARRIS, R. A.: Nature of the transport adenosine triphosphatase digitalis complex. J. Biol. Chem. **248:** 1291–1300, 1973.
107. LISHKO, V. K., MALYSHEVA, M. K., AND GREVIZIRSKAYA, T. J.: The interaction of the ($Na^+ + K^+$)-ATPase of erythrocyte ghosts with ouabain. Biochim. Biophys. Acta **288:** 103–106, 1972.
108. LOPEZ, V., STEVENS, T., AND LINDQUIST, R. N.: Vanadium ion inhibition of alkaline phosphatase-catalysed phosphate ester hydrolysis. Arch. Biochem. Biophys. **175:** 31–38, 1976.
109. MARTIN, D. W., AND TANFORD, C.: Phosphorylation of calcium adenosinetriphosphatase by inorganic phosphate: van't Hoff analysis of enthalpy changes. Biochemistry **20:** 4597–4602, 1981.
110. MATSUI, H., AND SCHWARTZ, A.: Mechanism of cardiac glycoside inhibition of the ($Na^+ + K^+$)-dependent ATPase from cardiac tissue. Biochim. Biophys. Acta **151:** 655–663, 1968.
111. MAYER, M., AND AVI-DOR, Y.: Interaction of solvents with membranal and soluble potassium ion dependent enzymes. Biochem. J. **116:** 49–54, 1970.
112. MOCZYDLOWSKI, E. G., AND FORTES, P. A. G.: Kinetics of cardiac glycoside binding to sodium, potassium adenosine triphosphatase studied with a fluorescent derivative of ouabain. Biochemistry **19:** 969–977, 1980.
113. MOORE, R. D.: Effects of insulin upon ion transport. Biochim. Biophys. Acta **737:** 1–49, 1983.
114. MYERS, T. D., BOERTH, R. C., AND POST, R. L.: Effects of vanadate on ouabain binding and inhibition of ($Na^+ + K^+$)-ATPase. Biochim. Biophys. Acta **558:** 99–107, 1979.
115. NOBLE, D.: Mechanism of action of therapeutic levels of cardiac glycosides. Cardiovasc. Res. **14:** 495–514, 1980.
116. NØRBY, J. G., AND JENSEN, J.: Binding of ATP to Na^+,K^+-ATPase. Ann. N. Y. Acad. Sci. **242:** 158–167, 1974.
117. NØRBY, J. G.: Ligand interactions with the substrate site of Na,K-ATPase: Nucleotides, vanadate, and phosphorylation. *In* Current Topics in Membranes and Transport, ed. by J. F. Hoffman and B. Forbush, III, vol. 19, pp. 281–314, Academic Press, New York, 1983.
118. NØRGAARD, A., KJELDSEN, K., AND HANSEN, O.: ($Na^+ + K^+$)-ATPase activity of crude homogenates of rat skeletal muscle as estimated from their K^+-dependent 3-O-methylfluorescein phosphatase activity. Biochim. Biophys. Acta **770:** 203–209, 1983.
119. OTTOLENGHI, P., AND JENSEN, J.: The K^+-induced apparent heterogeneity of high-affinity nucleotide-binding sites in ($Na^+ + K^+$)-ATPase can only be due to the oligomeric structure of the enzyme. Biochim. Biophys. Acta **727:** 89–100, 1983.
120. OTTOLENGHI, P., AND ELLORY, J. C.: Radiation inactivation of (Na,K)-ATPase, an enzyme showing multiple radiation-sensitive domains. J. Biol. Chem. **258:** 14895–14907, 1983.
121. PEREZ, B., MIARA, J., AND DAHMS, A. S.: Probes at the medium and intermediate water oxygen exchange reactions of the Na,K-ATPase. *In* Na,K-ATPase. Structure and Kinetics, ed. by J. C. Skou and J. G. Nørby, pp. 343–358, Academic Press, New York, 1979.
122. PERRONE, J. R., AND BLOSTEIN, R.: Asymmetric interaction of inside-out and right-side-out erythrocyte membrane vesicles with ouabain. Biochim. Biophys. Acta **291:** 680–689, 1973.
123. PLESNER, I. W., PLESNER, L., NØRBY, J. G., AND KLODOS, I.: The steady-state kinetic mechanism of ATP hydrolysis catalyzed by membrane-bound ($Na^+ + K^+$)-ATPase from ox brain. III. A minimal model. Biochim. Biophys. Acta **643:** 483–494, 1981.
124. POLLACK, L. R., TATE, E. H., AND COOK, J. S.: Na^+,K^+-ATPase in HeLa cells after prolonged growth in low K^+ or ouabain. J. Cell. Physiol. **106:**

85–97, 1981.
125. POST, R. L., KUME, S., TOBIN, T., ORCUTT, B., AND SEN, A. K.: Flexibility of an active center in sodium-plus-potassium adenosine triphosphatase. J. Gen. Physiol. **54:** 306s–326s, 1969.
126. POST, R. L., HEGYVARY, C., AND KUME, S.: Activation by adenosine triphosphate in the phosphorylation kinetics of sodium and potassium ion transport adenosine triphosphatase. J. Biol. Chem. **247:** 6530–6540, 1972.
127. POST, R. L., AND KUME, S.: Evidence for an aspartyl phosphate residue at the active site of sodium and potassium ion transport adenosine triphosphatase. J. Biol. Chem. **248:** 6993–7000, 1973.
128. POST, R. L., TODA, G., AND ROGERS, F. N.: Phosphorylation by inorganic phosphate of sodium plus potassium ion transport adenosine triphosphatase. J. Biol. Chem. **250:** 691–701, 1975.
129. REPKE, K., EST, M., AND PORTIUS, H. J.: Über die Ursache der Speciesunterschiede in der Digitalisempfindlichkeit. Biochem. Pharmacol. **14:** 1785–1802, 1965.
130. REPKE, K. R. H., AND PORTIUS, H. J.: Molekularbiologische Wertbestimmung von Verbindungen des Digitalistyps. Planta Med. suppl. **4:** 66–78, 1971.
131. ROBINSON, J. D., AND FLASHNER, M. S.: The ($Na^+ + K^+$)-activated ATPase. Enzymatic and transport properties. Biochim. Biophys. Acta **549:** 145–176, 1979.
132. ROGERS, T. B., AND LAZDUNSKI, M.: Photoaffinity labelling of the digitalis receptor in the (sodium + potassium)-activated adenosine triphosphatase. Biochemistry **18:** 135–140, 1979.
133. ROSSI, B., VUILLEUMIER, P., GACHE, C., BALERNA, M., AND LAZDUNSKI, M.: Affinity labeling of the digitalis receptor with *p* -nitrophenyltriazene-ouabain, a highly specific alkylating agent. J. Biol. Chem. **255:** 9936–9941, 1980.
134. ROSSI, B., PONZIO, G., AND LAZDUNSKI, M.: Identification of the segment of the catalytic subunit of (Na^+,K^+)ATPase containing the digitalis binding site. EMBO **1:** 859–861, 1982.
135. ROSTGAARD, J., AND MØLLER, O. J.: Electron microscopy of a microsomal fraction rich in ($Na^+ + K^+$)-ATPase and isolated from kidney cortex. Exp. Cell. Res. **68:** 356–371, 1971.
136. RUOHO, A., AND KYTE, J.: Photoaffinity labelling of the ouabain-binding site on ($Na^+ + K^+$)adenosine triphosphatase. Proc. Natl. Acad. Sci. U.S.A. **71:** 2352–2356, 1974.
137. SACHS, J. R.: Interaction of external K,Na and cardioactive steroids with the Na-K pump of the human red blood cell. J. Gen. Physiol **63:** 123–143, 1974.
138. SCHATZMANN, H. -J.: Herzglykoside als Hemmstoffe für den aktiven Kalium- und Natriumtransport durch die Erythrocytenmembran. Helv. Physiol. Acta **11:** 346–354, 1953.
139. SCHATZMANN, H. J.: The role of Na^+ and K^+ in the ouabain-inhibition of the $Na^+ + K^+$-activated membrane adenosine triphosphatase. Biochim. Biophys. Acta **94:** 81–88, 1965.
140. SCHONER, W., PAULS, H., AND PATZELT-WENCZLER, R.: Biochemical characteristics of the sodium pump: Indications for a half-of-sites reactivity of ($Na^+ + K^+$)-ATPase. *In* Myocardial Failure, ed. by G. Riecker, A. Weber, and J. Goodwin, pp. 104–119, Springer-Verlag, Berlin, 1977.
141. SCHOOT, B. M., SCHOOTS, A. F. M., DE PONT, J. J. H. H. M., SCHUURMANS STEKHOVEN, F. M. A. H., AND BONTING, S. L.: Studies on ($Na^+ + K^+$)activated ATPase. XLI. Effects of N-ethylmaleimide on overall and partial reactions. Biochim. Biophys. Acta **483:** 181–192, 1977.
142. SCHUURMANS STEKHOVEN, F. M. A. H., VAN HEESWIJK, M. P. E., DE PONT, J. J. H. H. M., AND BONTING, S. L.: Studies on ($Na^+ + K^+$)-activated ATPase. XXXVIII. A 100,000 molecular weight protein as the low-energy phosphorylated intermediate of the enzyme. Biochim. Biophys. Acta **422:** 210–224, 1976.
143. SCHUURMANS STEKHOVEN, F., AND BONTING, S. L.: Transport adenosine triphosphatases. Properties and functions. Physiol. Rev. **61:** 1–76, 1981.
144. SCHWARTZ, A., MATSUI, H., AND LAUGHTER, A. H.: Tritiated digoxin binding to ($Na^+ + K^+$)-activated adenosine triphosphatase: Possible allosteric site. Science **160:** 323–325, 1968.
145. SCHWARTZ, A., LINDENMAYER, G. E., AND ALLEN, J. C.: The sodium-potassium adenosine triphosphatase: Pharmacological, physiological and biochemical aspects. Pharmacol. Rev. **27:** 1–134, 1975.
146. SCHÖN, R., SCHÖNFELD, W., AND REPKE, K. R. H.: Zur Characterisierung des Ouabain-bindenden Konformationszustandes der ($Na^+ + K^+$)aktivierten ATPase. Acta biol. med. germ. **24:** K61–65, 1970.
147. SCHÖN, R., SCHÖNFELD, W., MENKE, K. -H., AND REPKE, K. R. H.: Mechanism and role of Na^+/Ca^{2+} competition in (Na,K)-ATPase. Acta biol. med. germ. **29:** 643–659, 1972.
148. SCHÖNFELD, W., SCHÖN, R., MENKE, K. -H., AND REPKE, K. R. H.: Identification of conformational states of transport ATPase by kinetic analysis of ouabain binding. Acta biol. med. germ. **28:** 935–956, 1972.
149. SEN, A. K., TOBIN, T., AND POST, R. L.: A cycle for ouabain inhibition of sodium- and potassium-dependent adenosine triphosphatase. J. Biol. Chem. **244:** 6596–6604, 1969.
150. SIEGEL, G. J., KOVAL, G. J., AND ALBERS, R. W.: Sodium-potassium-activated adenosine triphosphatase. VI. Characterization of the phosphoprotein formed from orthophosphate in the presence of ouabain. J. Biol. Chem. **244:** 3264–3269, 1969.
151. SIEGEL, G. J., AND JOSEPHSON, L.: Ouabain reaction with microsomal (sodium-plus-potassium)-activated adenosine triphosphatase. Characteristics of substrate and ion dependencies. Eur. J. Biochem. **25:** 323–335, 1972.
152. SKOU, J. C.: The influence of some cations on an adenosine triphosphatase from peripheral nerves. Biochim. Biophys. Acta **23:** 394–401, 1957.
153. SKOU, J. C.: Further investigations on a $Mg^{++} + Na^+$-activated adenosintriphosphatase, possibly related to the active, linked transport of Na^+ and K^+ across the nerve membrane. Biochim. Biophys. Acta **42:** 6–23, 1960.
154. SKOU, J. C.: Enzymatic basis for active transport of Na^+ and K^+ across cell membrane. Physiol. Rev. **45:** 596–617, 1965.
155. SKOU, J. C., BUTLER, K. W., AND HANSEN, O.: The effect of magnesium, ATP, P_i and sodium on the inhibition of the (Na^++K^+)-activated enzyme system by g-strophanthin. Biochim. Biophys. Acta **241:** 443–461, 1971.
156. SKOU, J. C.: The ($Na^+ + K^+$)-activated enzyme system and its relationship to transport of sodium and potassium. Q. Rev. Biophys. **7:** 401–434, 1975.
157. SKOU, J. C., AND ESMANN, M.: Eosin, a fluorescent probe of ATP-binding to the ($Na^+ + K^+$)-ATPase. Biochim. Biophys. Acta **647:** 232–240, 1981.
158. SKOU, J. C., AND ESMANN, M.: Effect of magnesium ions on the high-affinity binding of eosin to the ($Na^+ + K^+$)-ATPase. Biochim. Biophys. Acta **727:** 101–107, 1983.
159. SMITH, R. L., ZINN, K., AND CANTLEY, L. C.: A study of the vanadate-trapped state of the (Na,K)-ATPase. Evidence against interacting nucleotide site models. J. Biol. Chem. **255:** 9852–9859, 1980.
160. SMITH, T. W., AND BARRY, W. H.: Monovalent cation transport and mechanisms of digitalis-induced inotropy. *In* Current Topics in Membranes and Transport, ed. by J. F. Hoffman, and B. Forbush, III, vol. 19, pp. 843–884, Academic Press, New York, 1983.
161. SWEADNER, K. J.: Two molecular forms of ($Na^+ + K^+$)-stimulated ATPase in brain. Separation, and difference in affinity for strophanthidin. J. Biol. Chem. **254:** 6060–6087, 1979,
162. TANIGUCHI, K., AND IIDA, S.: Two apparently different ouabain binding sites of (Na^+-K^+)ATPase. Biochim. Biophys. Acta **288:** 98–102, 1972.
163. TANIGUCHI, K., AND IIDA, S.: The role of phospho-lipids in the binding of ouabain to sodium- and potassium-dependent adenosine triphosphatase. Mol. Pharmacol. **9:** 350–359, 1973.
164. TOBIN, T., AND SEN, A. K.: Stability and ligand sensitivity of [^{3}H]ouabain binding to ($Na^+ + K^+$)-ATPase. Biochim. Biophys. Acta **198:** 120–131, 1970.
165. TOBIN. T., AKERA, T., HOGG, R. E., AND BRODY, T. M.: Ouabain binding to sodium- and potassium-dependent adenosine triphosphatase: Inhibition by the β,γ-methylene analogue of adenosine triphosphatase. Mol. Pharmacol. **6:** 278–281, 1973.
166. TOBIN, T., AKERA, T., LEE, C. Y., AND BRODY, T. M.: Ouabain binding to ($Na^+ + K^+$)-ATPase. Effects of nucleotide analogues and ethacrynic acid. Biochim. Biophys. Acta **345:** 102–117, 1974.
167. TOBIN, T., AKERA, T., AND BRODY, T. M.; Studies on the two phosphoenzyme conformations of Na^++K^+-ATPase. Ann. N. Y. Acad. Sci. **242:** 120–131, 1974b.
168. WALLICK, E. T., ANNER, B. M., RAY, M. V., AND SCHWARTZ, A.: Effect of temperature on phosphorylation and ouabain binding to N-ethylmaleimide-treated ($Na^+ + K^+$)-ATPase. J. Biol. Chem. **253:** 8778–8786, 1978.
169. WALLICK, E. T., LANE, L. K., AND SCHWARTZ, A.: Regulation by vanadate of ouabain binding to ($Na^+ + K^+$)ATPase. J. Biol. Chem. **254:** 8107–8109, 1979.
170. WALTER, H.: Permeability of plasma membrane vesicles to ouabain and Mg^{2+} as a factor determining rate of binding of ouabain to Na^+ and K^+ dependent ATPase. Z. Naturforsch. **34C:** 1224–1231, 1979.
171. WELLSMITH, N. C., AND LINDENMAYER, G. E.: Two receptor forms for ouabain in sarcolemma-enriched preparations from canine ventricle. Circ. Res. **47:** 710–720, 1980.
172. WHITTMAN, R., AND CHIPPERFIELD, A. R.: Ouabain binding to the sodium pump in plasma membranes isolated from ox brain. Biochim. Biophys. Acta **307:** 563–577, 1973.
173. WILSON, W. E., SIVITZ, W. I., AND HANNA, L. T.: Inhibition of calf brain membranal sodium- and potassium-dependent adenosine triphosphatase by cardioactive sterols. A binding site model. Mol. Pharmacol. **6:** 449–459, 1970.
174. WITHERING, W.: An account of the Foxglove, and some of its medical uses: With practical remarks on dropsy, and other diseases. *In* Cardiac Classics, C. C. Mosby Co., St. Louis, 1941 (original publication, 1785).
175. YODA, A., AND HOKIN, L. E.: On the reversibility of binding of cardiotonic steroids to a partially purified (Na + K)-activated adenosinetriphosphatase from beef brain. Biochem. Biophys. Res. Commun. **40:** 880–886, 1970.
176. YODA, A.: Structure-activity relationships of cardiotonic steroids for the inhibition of sodium- and potassium-dependent adenosine triphosphatase. I. Dissociation rate constants of various enzyme-cardiac glycoside complexes formed in the presence of magnesium and phosphate. Mol. Pharmacol. **9:** 51–60, 1973.
177. YODA, A., YODA, S., AND SARRIF, A. M.: Structure-activity relationships of cardiotonic steroids for the inhibition of sodium- and potassium-dependent adenosine triphosphatase. II. Association rate constants of various enzyme-cardiac glycoside complexes. Mol. Pharmacol. **9:** 766–773, 1973.
178. YODA, A., AND YODA, S.: Structure-activity relationships of cardiotonic steroids for the inhibition of sodium- and potassim-dependent adenosine triphosphatase. III. Dissociation rate constants of various enzyme-cardiac

glycoside complexes formed in the presence of sodium, magnesium, and adenosine triphosphatase. Mol. Pharmacol. **10:** 494–500, 1974.

179. YODA, A., AND YODA, S.: Influence of certain ligands on the dissociation rate constant of cardiac glycoside complexes with sodium- and potassium-dependent adenosine triphosphatase. Mol. Pharmacol. **10:** 810–819, 1974b.
180. YODA, A.: Association and dissociation rate constants of the complexes between various cardiac monoglycosides and Na,K-ATPase. Ann. N. Y. Acad. Sci. **242:** 598–616, 1974c.
181. YODA, A.: Binding of digoxigenin to sodium- and potassium-dependent adenosine triphosphatase. Mol. Pharmacol. **12:** 399–408, 1976.
182. YODA, A., AND YODA, S.: Interaction between ouabain and the phosphorylated intermediate of Na,K-ATPase. Mol. Pharmacol. **22:** 700–705, 1982.