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STABILITY AND LIGAND SENSITIVITY OF [^{3}H]OUABAIN BINDING TO ($Na^+ + K^+$)-ATPase

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SUMMARY

Specific [^{3}H]ouabain binding to guinea pig kidney ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) at 37° was determined. At less than $1 \cdot 10^{-6}$ M ouabain specific binding to the native enzyme was negligible. Specific binding occurred in two ways. Binding was stimulated by Mg^{2+} or Mg^{2+} and P_i which greatly increased the affinity of the enzyme for ouabain. This binding was saturated at 10 μM ouabain and reduced by Na^+ or EDTA. The Na^+ inhibition showed cooperative effects, apparent K_i values between 2.5 and 70 mM and appeared to be indirect. K^+ at low concentrations stimulated, then at higher concentrations inhibited this binding. The inhibition appeared to be competitive. Binding in the presence of 200 mM Na^+ required ATP and Mg^{2+}. In the presence of ATP and Mg^{2+}, binding was stimulated by 0–16 mM Na^+ and further increased by 200 mM Na^+. $SrCl_2$ or ADP did not support binding in the presence of Na^+. The half-life of the enzyme–ouabain complex was 9 h at 0°. On warming, the rate of dissociation increased exponentially with temperature to $t_{\frac{1}{2}} = 3$ min at 37°. The experiments suggest that there is one type of specific binding site and that the binding pathways are reciprocally related with respect to Na^+. They suggest different binding sites and conformations of maximum affinity for Na^+ and ouabain. The Na^+-stimulated pathway requires phosphorylation. Phosphorylation and/or ouabain binding appear to alter the affinity of the Na^+ sites. Any single non-covalent bond type appears insufficient to explain the enzyme–ouabain interaction.

INTRODUCTION

Cardiac glycosides are specific inhibitors of the ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) which is believed to be associated with cation transport in most mammalian tissues[1,2]. This inhibition is stoichiometric and relatively stable[3,4]. It is asymmetric in that it occurs only from the extracellular medium[5] and appears under some conditions to be at least partially competitive with K^+ (refs. 1 and 6). In keeping with these observations, early results suggested that cardiac glycosides inhibited this enzyme by binding to the phosphorylated form[4,7,8]. Recently[9,10], we

have outlined the conditions under which ouabain alters the reactivity of this enzyme at 0°. Binding was not measured directly but inferred from the altered reactivity. Changes in affinity for ouabain were inferred from differences in the rate of action of ouabain as binding at 0° was essentially irreversible[10]. From these observations, a scheme for ion transport and ouabain inhibition of this enzyme was proposed. Two major forms, the native and phospho forms of the enzyme were postulated with linked changes in carrier presentation and ligand affinity occurring during the transport cycle[10].

For this communication we used [^{3}H]ouabain to measure ouabain binding. Binding at 37° was readily reversible and we present data obtained under equilibrium and steady-state conditions suggesting the existence of two major conformations of the enzyme with linked changes in affinity for ouabain and Na^+. Evidence is presented to support the coupling of changes in affinity and orientation of binding sites to phosphorylation of the enzyme under certain conditions. Also, the stability of the enzyme–ouabain complex is at present controversial. Published reports suggest reversibility[1,6,11], difficulty in reversal[10,12] or an interaction which is "essentially irreversible" at physiological temperature and pH (ref. 13). A dependence of the dissociation rate constant of the enzyme–ouabain complex on temperature is demonstrated, which may partially explain the discrepancies in the literature. A preliminary report has been communicated[14].

MATERIALS AND METHODS

Enzyme preparation

Guinea pig kidney ($Na^+ + K^+$)-ATPase was prepared by the method of POST AND SEN[15]. It was washed 3 times with a solution containing 25.0 mM imidazole, 12.5 mM histidine, 0.1 mM EDTA (pH 7.5 ± 0.1) to reduce endogenous Mg^{2+} and Na^+. Specific activity of the enzyme was 100–250 μmoles P_i per mg protein per h at 37°, and more than 90% of the activity was ($Na^+ + K^+$)-dependent.

Binding of [^{3}H]ouabain

Binding was determined by the method of MATSUI AND SCHWARTZ[4]. All reactions were carried out in 15-ml corex centrifuge tubes containing 0.2–0.4 mg protein and 10 μmoles each of imidazole and glycylglycine (pH 7.4 ± 0.1). Subsequent additions of reagents were made in 0.1-ml volumes to give the stated concentrations in the final volume of 1.0 ml. Unless otherwise stated, incubations were at 37° for 3 min in the presence of ATP or for at least 20 min in its absence. The tubes were then centrifuged at 48 000 × *g* for 5 min at 0°. The supernatant was discarded, the remaining droplets removed with a Kimwipe[4] and the complete pellet solubilized in 0.2 ml of Nuclear Chicago Solubilizer. Counting was in 10 ml of scintillation medium (100 mg of 1,4-bis-(5-phenyloxazolyl-2)benzene, 4 g of 2,5-diphenyloxazole in a total volume of 1 l of toluene) in a Nuclear Chicago or Packard Tri-Carb liquid scintillation spectrometer. Protein was determined by the method of LOWRY *et al.*[16]. Binding of [^{3}H]ouabain is expressed as pmoles [^{3}H]ouabain per mg protein. Specific activity of the [^{3}H]ouabain was 100 C/mole.

Chemicals and reagents

[^{3}H]ouabain was obtained from New England Nuclear, Boston, Mass Carrier ouabain was from Sigma Chemical Company, St Louis, Mo The sodium salts of ATP and ADP (Sigma) were converted to their respective Tris salts by passing over an AG 50W-X8 (Bio-rad Laboratories, Richmond, Calif) column in the Tris form Other chemicals and reagents were obtained or prepared as described previously[10]

TABLE I

NONSPECIFIC BINDING OF [^{3}H]OUABAIN TO TWO PREPARATIONS OF ($Na^+ + K^+$)-ATPase

Enzyme, 10 mM imidazole–glycylglycine and 0 25 μM [^{3}H]ouabain were incubated for 20 min at 37° in the presence of various ligands or after treatment as indicated Binding was measured as indicated in MATERIALS AND METHODS

Preparation	*Additions*	*Concn (mM)*	*pmoles [^{3}H]ouabain per mg protein*
A	NaCl	16 0	8 01
	NaCl	100 0	6 31
	KCl	16 0	8 90
	Ouabain (unlabeled)	2 5	8 10
	Tris ATP	2 0	9 70
	Heat-denatured enzyme	—	10 30
	$MgCl_2$ *plus*	0 4	
	H_3PO_4	1 0	265 00
B	Tris EDTA	10 0	5 70
	NaCl	200 0	4 75
	KCl	16 0	4 75
	—	—	6 40
	$MgCl_2$ *plus*	4 0	
	H_3PO_4	1 0	153 70

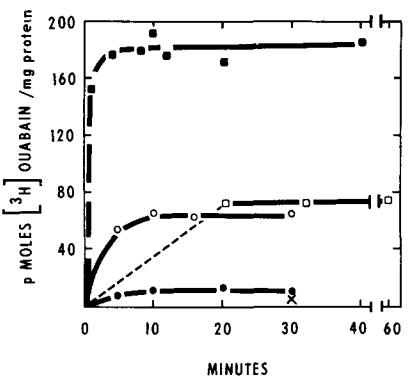
RESULTS

Nonspecific binding

Previous experiments[9,10] showed a slow binding of ouabain to the native enzyme and inhibition of this binding by Na^+ or ATP Table I confirmed and extended these findings The various ligands and treatments of Table I produced comparable levels of binding which are a small fraction (2–4%) of the possible binding The amount of this binding varied directly with the concentration of [^{3}H]ouabain added, consistent with the counts being in pellet water[4] In the experiments reported here, nonspecific binding was usually that occurring in the presence of 200 mM Na^+, although in some experiments 16 mM K^+ or 5 0 mM EDTA were used Specific binding was any increment above the background estimated in this way Native enzyme exposed to 2 5 10^{-7} M [^{3}H]ouabain appeared to bind a small amount (1%) of ouabain specifically (see also Fig 3) In another experiment at 22° this binding did not increase with time (but see ref 3)

Time-course, type and extent of Na^+-inhibited binding

Specific binding occurs in two ways[10] One is nucleotide-dependent[4,10] and stim-



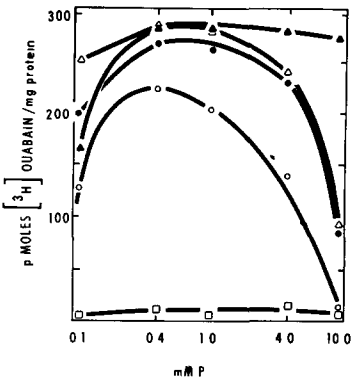


Fig 1 Time-course of [^{3}H]ouabain binding by the Na^+-inhibited pathway The reaction tubes contained enzyme, 10 mM imidazole–glycylglycine and 2 5 10^{-7} M [^{3}H]ouabain After the indicated times at 37° binding was stopped by centrifugation as described under MATERIALS AND METHODS Further additions were as below ■—■, binding in the presence of 1 mM Mg^{2+} and 1 mM P_i, □—□, binding in this medium with 60 mM Na^+ added, ○—○, binding in the presence of 1 mM Mg^{2+}, ●—●, binding in the presence of 1 mM Mg^{2+} *plus* 1 mM K^+, ×, binding in the presence of 1 mM Mg^{2+} *plus* 16 mM K^+

Fig 2 Optimal concentrations of Mg^{2+} and P_i for [^{3}H]ouabain binding Enzyme, 10 mM imidazole–glycylglycine and 2 5 10^{-7} M [^{3}H]ouabain were incubated for 20 min at 37° and the reaction stopped by centrifugation P_i concentration was varied as indicated on the horizontal axis at different Mg^{2+} concentrations The Mg^{2+} concentrations were □—□, no added Mg^{2+}, ○—○, 0 1 mM Mg^{2+}, ●—●, 0 4 mM Mg^{2+}, △—△, 1 mM Mg^{2+}, and ▲—▲, 4 mM Mg^{2+}

ulated by Na^+ The other[10] is stimulated by Mg^{2+} *plus* P_i and inhibited by Na^+ The Na^+-inhibited pathway was first investigated The time-course of ouabain binding by this pathway to $(Na^+ + K^+)$-ATPase was slow at 0° and 24° (refs 10 and 13) Fig 1 shows the time-course of [^{3}H]ouabain binding to $(Na^+ + K^+)$-ATPase at 37° under various conditions Binding was relatively slow and appeared to reach an equilibrium in 20 min in all cases Once reached, equilibrium was maintained for at least 1 h The addition of Na^+ or K^+ reduced the binding In another experiment Mg^{2+}-dependent binding was reduced following addition of EDTA Mn^{2+} also stimulated a binding which was reduced by Na^+ In all subsequent experiments in the absence of nucleotides, at least 20 min were allowed for binding to equilibrate

TABLE II

MAXIMAL [^{3}H]OUABAIN BINDING IN PRESENCE OF Mg^{2+} AND P_i

Enzyme, 10 mM imidazole–glycylglycine and 2 5 μM [^{3}H]ouabain were incubated with additions as indicated Tubes containing ATP were incubated for 3, and those containing P_i for 20 min ($P < 0.01$)

Additions	*Concn (mM)*	*pmoles [^{3}H]ouabain per mg protein ± S E*
$MgCl_2$	4 0	409 61 ± 12 6 ($n = 6$)
H_3PO_4	1 0	
NaCl	200	
$MgCl_2$	2	340 25 ± 15 4 ($n = 6$)
ATP	2	

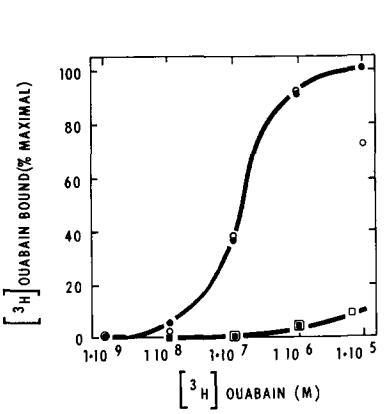
Specific binding in the presence of Mg^{2+} and P_i was substantially higher than the maximal binding by the Na^+-stimulated pathway (Table II) Maximal binding appeared to occur in the presence of 4 mM Mg^{2+} (Fig 2), being 15% higher than a curve at 10 mM Mg^{2+} in another experiment P_i alone did not support any substantial binding Thus 4 mM Mg^{2+} and 1 mM P_i were routinely used where maximal binding was required Later it was found that this binding could be further stimulated by K^+ (Fig 6)

Apparent affinities of the enzyme for ouabain

The lower curve of Fig 3 shows that specific binding of ouabain to the native enzyme occurred in the absence of added ligands and increased with increasing ouabain concentration However extrapolation of this curve suggests that the concentration for half saturation is high Addition of optimal concentrations of Mg^{2+} and P_i greatly increased the affinity of the enzyme for ouabain to give an apparent K_m of 1.8×10^{-7} M The data suggest a greater than 200-fold change in the apparent K_m of the enzyme for ouabain Using the relationship $E = -2.303\ RT \log K$ the standard free-energy change for the interaction at highest apparent affinity was about 9.5 kcal/mole From a linear transformation of the same data, binding at 1×10^{-5} M ouabain was estimated as 94% of binding at infinite concentration

The Na^+ inhibition of ouabain binding

Though Na^+ has been shown to inhibit ouabain binding[3,9,10,13] occurring by the Mg^{2+} *plus* P_i pathway, there are no reports that ouabain binding is reversed by Na^+ The time-course of the inhibition by Na^+ in Fig 1 suggested that the effect of Na^+ was to shift an equilibrium Fig 4 was an experiment designed to test this possibility



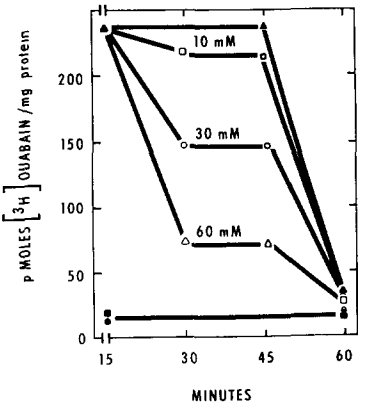


Fig 3 Effect of Mg^{2+} *plus* P_i on affinity of the enzyme for [^{3}H]ouabain Two enzyme preparations were incubated at 37° with the indicated concentrations of [^{3}H]ouabain in the presence and absence of 4 mM Mg^{2+} and 1 mM P_i ○ ●, Mg^{2+} and P_i □ ■, no added ligands Binding is expressed as per cent maximal to allow comparison of two different experiments

Fig 4 Na^+ inhibition of ouabain binding Enzyme was incubated at 37° for the indicated times in the presence of 2.5×10^{-7} M [^{3}H]ouabain and various ligands ●–●, no added ligands, ■–■, 200 mM Na^+ ▲, in the upper left quandrant shows binding in the presence of 4 mM Mg^{2+} + 1 mM P_i after 15 min incubation At 15 min 0, 10, 30, or 60 mM Na^+ were added and binding estimated at 30 and 45 min At 45 min 200 mM Na^+ were added to all tubes and the binding estimated at 60 min

[^{3}H]ouabain was bound in the presence of Mg^{2+} and P_i, then followed by different concentrations of Na^+ Na^+ reduced the binding to a new equilibrium level which was dependent on the concentration of Na^+ Subsequent addition of 200 mM Na^+ lowered the binding to a level close to that for nonspecific binding The discrepancies may be accounted for by the 3-min half-life of the enzyme–ouabain complex (Fig 9) The control points show that no binding of ouabain to the native enzyme occurred over the time period of this experiment

Fig 5 shows the effect of varying the concentrations of Mg^{2+}, P_i and ouabain on the shape of the Na^+-inhibition curve At low concentrations of ligands antagonistic to Na^+ (Mg^{2+}, P_i, ouabain), the inhibition was weakly cooperative ($n = 1$ 11) Also, the concentration dependence of the inhibition approximates the concentration at which Na^+ stimulates phosphorylation Increasing the concentration of antagonistic ligands (particularly ouabain) exposed a strongly cooperative[17] interaction ($n = 2$ 5) (ref 18) At the same time, the apparent affinity of the enzyme for Na^+ dropped from 2 5 to 70 mM

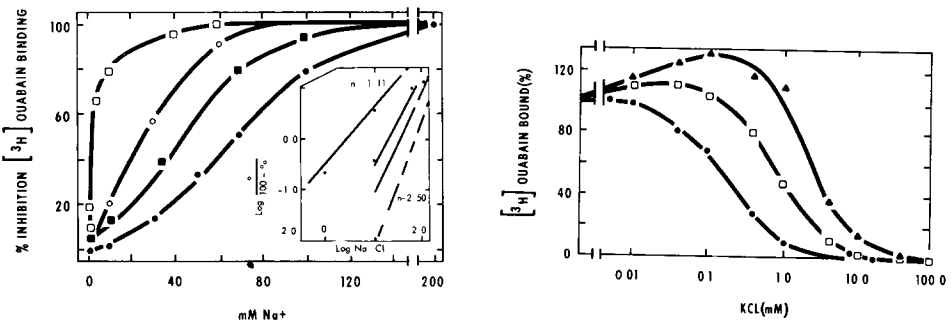


Fig 5 Na^+ inhibition of ouabain binding effect of varying the antagonistic ligands [^{3}H]ouabain was allowed to bind to the enzyme under different conditions for 20 min □—□, indicates ouabain binding in the presence of 0 1 mM Mg^{2+} and 2 5 10^{-7} M ouabain 4 mM Mg^{2+}, 1 mM P_i and 2 5 10^{-7} M (○—○), 2 5 10^{-6} M (■—■), or 2 5 10^{-5} M (●—●) ouabain with Na^+ varied as indicated Binding is plotted as per cent of the value at 0 0 mM NaCl The inset shows a Hill plot of the same data

Fig 6 K^+ and ouabain binding effect of different ligand concentrations [^{3}H]ouabain was allowed to bind under different conditions for 20 min ●—●, ouabain binding in the presence of 0 4 mM Mg^{2+} and 2 5 10^{-6} M ouabain, □—□, 4 mM Mg^{2+}, 1 mM P_i and 2 5 10^{-7} M ouabain, ▲—▲, 4 mM Mg^{2+}, 1 mM P_i and 2 5 10^{-6} M ouabain when K^+ was varied as indicated Binding is plotted as per cent of the value at 0 0 mM KCl

Effect of K^+ on ouabain binding

The interaction between K^+ and ouabain has been suggested to be competitive[6], partially competitive[1] and noncompetitive[19] Also, K^+ has been shown to inhibit [^{3}H]ouabain binding occurring by both pathways[13] Since both K^+ and ouabain have their highest affinities extracellularly for the phospho form of the enzyme, they will tend to favor this conformation of the enzyme[17] Thus separate K^+ and ouabain sites would allow K^+ to stimulate ouabain binding Fig 6 shows that K^+ at low concentrations in the presence of Mg^{2+} and P_i stimulated ouabain binding At higher concentrations it inhibited Plotting the inhibitory portions of these curves as per cent maximal binding showed them to be parallel

The Na^+-stimulated pathway requirement for ATP

The time-course of the Na^+-stimulated binding is shown in Fig 7 Binding was supported for 8 min by 1 mM ATP, whereas at 0 1 mM ATP it dropped rapidly from an initial high value The Mg^{2+}-dependent ATPase of the preparation was sufficient to account for hydrolysis of the lower levels of ATP in 3 min Thus, low levels of ATP only supported a transient pulse of [^{3}H]ouabain binding In other experiments, 1 0 mM ADP supported 50–70% of the binding observed with 1 0 mM ATP as reported by others[3,13] These observations suggest ouabain binding in the absence of phosphorylation However, the lower curves in Fig 7 show that, while 0 05 mM ATP supported a substantial, rapidly peaking pulse of ouabain binding, a pulse with 0 05 mM ADP peaked later and was only one eighth of that with ATP Since these enzyme preparations are contaminated with adenylate kinase activity, ATP from this source would account for the later peaking and small rise of the peak The experiment also suggests that ouabain–enzyme formed by this pathway has the same half-life as that formed by the equilibrium pathway (Fig 9) Binding by this pathway thus appeared to depend on the availability of ATP

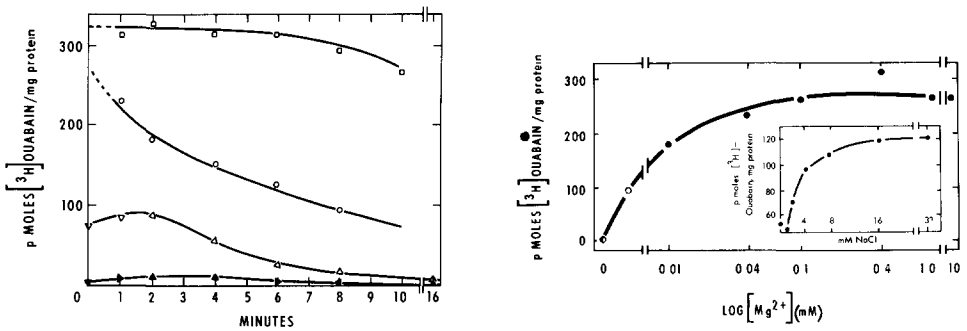


Fig 7 Time-course and substrate requirement of Na^+- stimulated ouabain binding Enzyme, 10 mM imidazole–glycylglycine, 60 mM NaCl, and 2 5 10^{-7} M [^{3}H]ouabain were incubated at 37 □—□, binding after addition of 1 mM ATP and 1 mM Mg^{2+} ○—○, 0 1 mM ATP and 0 1 mM Mg^{2+}, △—△, 0 05 mM ATP and 0 1 mM Mg^{2+}, ▲—▲, 0 05 mM ADP and 0 1 mM Mg^{2+}

Fig 8 Effect of Mg^{2+} on ouabain binding in the presence of Na^+ and ATP Enzyme, 200 mM NaCl, 4 mM ATP and 2 5 10^{-6} M [^{3}H]ouabain were incubated with the indicated concentrations of Mg^{2+} for 3 min at 37 ●—●, added Mg^{2+}, ○—○, no added Mg^{2+}, ◐, 5 mM Tris EDTA In the inset enzyme, 2 mM ATP and 2 mM Mg^{2+} and 2 5 10^{-7} M [^{3}H]ouabain were incubated with the indicated concentrations of Na^+ for 3 min at 37

Requirement for Mg^{2+}

Individually ATP or Na^+ did not support binding[3,10] If the Na^+-stimulated binding required phosphorylation of the enzyme, then Na^+–enzyme–ATP should show a requirement for Mg^{2+} This dependence on Mg^{2+} is shown in Fig 8 In the presence of EDTA no binding occurred, but residual Mg^{2+} supported considerable binding and low levels of Mg^{2+} were adequate to support maximal binding Table III shows that binding was reduced by addition of EDTA and recovered on addition of excess Mg^{2+} Mg^{2+} presumably acted by allowing ATP to phosphorylate the enzyme

Effects of Na^+

The effects of Na^+ further support this hypothesis Na^+ stimulates phosphory-

TABLE III

Mg^{2+} REQUIREMENT OF Na^+-STIMULATED BINDING

Enzyme, 10 mM imidazole–glycylglycine and 0.25 μM [^{3}H]ouabain were incubated with sequential addition of the indicated ligands

Additions	*Concn (mM)*	*Time added (min)*	*Time centrifuged (min)*	*pmoles [^{3}H]ouabain per mg protein*
$MgCl_2$	0.1			
H_3PO_4	4.0	0	15	181.7
+ NaCl	200.0	15	30	33.7
+ ATP	4.0	30	33	161.9
+ EDTA	10.0	33	36	62.5
+ $MgCl_2$	10.0	36	39	227.2

lation at 0–16 mM (ref. 20), and at higher concentrations (200 mM) it accelerates ouabain binding to the phospho-enzyme[10]. Similarly with squid axons[12], high Na^+ extracellularly accelerated the ouabain inhibition of the pump. If ouabain were binding to the phospho-enzyme, a similar sensitivity to Na^+ might be expected. The experiments show that Na^+ stimulated ouabain binding at concentrations which stimulate phosphorylation of the enzyme (Fig. 8 inset) and again at concentrations

TABLE IV

[^{3}H]OUABAIN BINDING WITH 20 AND 200 mM NaCl

Enzyme, 10 mM imidazole–glycylglycine, 1 mM $MgCl_2$, 0.25 μM [^{3}H]ouabain and 1.3 mM ATP were incubated with NaCl as indicated for 3 min at 37° ($P < 0.05$)

NaCl concn (mM)	*pmoles [^{3}H]ouabain per mg protein ± S.E.*
20	130.5 ± 11.4 ($n = 7$)
200	159.9 ± 10.6 ($n = 7$)

TABLE V

[^{3}H]OUABAIN BINDING IN PRESENCE OF Sr^{2+} OR Sr^{2+} *plus* NaCl

Enzyme, 10 mM imidazole–glycylglycine and $2.5 \cdot 10^{-7}$ M [^{3}H]ouabain were incubated in the presence and absence of 100 mM NaCl for 20 min at 37°. Nonspecific binding was 3.80 and maximal binding 80.86 pmoles [^{3}H]ouabain per mg protein

Additions	*Concn (mM)*	*pmoles [^{3}H]ouabain per mg protein*
$SrCl_2$	1.0	0.63
	5.0	0.18
	10.0	0.13
NaCl *plus* $SrCl_2$		
	1.0	2.28
	5.0	1.17
	10.0	0.76

which increased binding to the phospho-enzyme (Table IV) $SrCl_2$ has been reported to support ouabain binding in the presence of Na^+ (ref 3) This was not observed in our system (Table V)

Temperature-dependent stability of ouabain–enzyme complex

In a previous report[10], we indicated the difficulty of reversing the ouabain inhibition at 0° whereas the experiments reported here suggest rapid dissociation of the enzyme–ouabain complex at 37° We therefore investigated the effect of temperature on the stability of the enzyme–ouabain complex Fig 9 shows the relative stability of the enzyme-ouabain complex at 0° and its decreasing stability as the temperature was raised The dissociation rate constant increased exponentially with increasing temperature The data fit an Arrhenius plot with an activation energy of about 28 30 kcal/mole

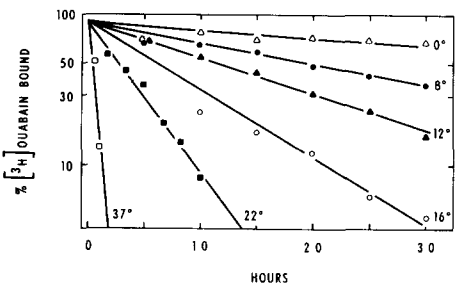
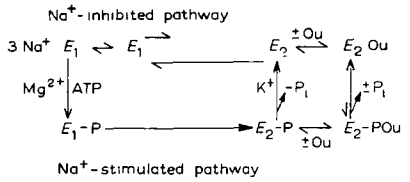


Fig 9 Stability of enzyme-ouabain complex at different temperatures Enzyme, 4 mM $MgCl_2$, 1 mM H_3PO_4 and 2 5 10^{-7} M [^{3}H]ouabain were incubated for 20 min at 37 The tubes were then cooled to the indicated temperature and 10 mM EDTA and 5 10^{-4} M unlabeled ouabain added The reaction was stopped by centrifugation at the indicated time points At 22 and 37° only unlabeled ouabain was added At 37° the reaction was stopped by rapidly freezing (20 sec) the tubes in an acetone–dry ice mixture After thawing at 0 the tubes were centrifuged as before The pH of the system did not change (7 4 ± 0 1 pH units) with temperature

DISCUSSION

These experiments have shown that ouabain binding at 37° is reversible (Fig 9), whereas binding at 0° was apparently irreversible[10] The increased dissociation rate constant at higher temperatures explains this discrepancy (Fig 9) At 37° binding will depend on the rates of association and dissociation Little specific binding of ouabain to the native enzyme was observed at the lower ouabain concentrations (Fig 3) The experiments have shown two pathways by which the rate of association was increased to give substantial binding By one pathway Mg^{2+} and P_i stimulated a binding which was reduced by Na^+ and high K^+ The binding energy of the ligand molecules determined the equilibrium at which the system remained The other pathway was stimulated by Na^+ It required ATP and Mg^{2+} and binding was maximal at concentrations of Na^+ which completely inhibited the other pathway Maintenance of binding by this pathway required ATP The enzyme–ouabain complexes formed by both pathways appear to be the same Both result in inhibition of the enzyme, are reduced by K^+ (refs 4 and 13), allow incorporation of P_i into the enzyme[9,10], have similar half-

lifes (Figs 7 and 9) and apparent affinities (Fig 3 and ref 3) These pathways and products are related in the following scheme



The Na^+-stimulated pathway is from the literature[9,10,13] A transient intermediate E_1–P formed immediately after the Na^+-stimulated phosphorylation has been demonstrated[9], and a dephosphoform of this (E_1) has been postulated[9] This form should be Na^+ sensitive (to allow Na^+ stimulation) and have a low affinity for ouabain Native enzyme had little affinity for ouabain (Fig 3), and at low concentrations of Mg^{2+} the enzyme had its highest apparent affinity for Na^+ as measured by displacement of ouabain (Fig 5) Thus native enzyme was designated E_1 E_2–P in the scheme is the conformationally changed phospho-enzyme with which ouabain has been shown to react rapidly[4,9,10] As suggested by SCHWARTZ *et al* [3], it appears that "the conformational state of the enzyme is probably of primary significance in glycoside binding" Further, when bound ouabain appears to maintain "a structure of the enzyme which is normally a consequence of phosphorylation"[13] Because of these considerations, ouabain (Ou) in the Na^+-inhibited pathway is shown binding to the postulated[9,13] conformationally equivalent dephospho-enzyme, E_2 E_2 is unstable[10,13] unless maintained by ouabain or phosphorylation and tends to revert to the E_1 form as indicated in the scheme

The apparent affinity of native enzyme (E_1) for ouabain and Na^+ may be altered by the two pathways outlined In experiments at 0°, Mg^{2+} and P_i increased the rate of association of the enzyme with ouabain[10] They appear to do this by affecting the allosteric equilibrium between E_1 and E_2 Due to its relative stability, this form (E_2Ou) accumulates and at equilibrium there was a greater than 200-fold increase in the apparent affinity of the enzyme for ouabain (Fig 3) At the same time there was a drop in the apparent affinity of the enzyme for Na^+ This change in affinity for Na^+ also suggests the equivalence of E_2Ou and E_2–P since the latter also appears to have a low affinity for Na^+ (see below)

Na^+ and K^+ apparently inhibited this pathway by different mechanisms The inhibition by Na^+ was strongly cooperative in the presence of high concentrations of antagonistic ligands suggesting an allosteric interaction The interaction was less cooperative at lower concentrations of antagonistic ligands This is a standard heterotropic effect of an allosteric activator[17] It supports the suggestion that Na^+ and ouabain have their highest affinities for different conformations of the enzyme which are in allosteric equilibrium Further, the concentration dependence of the Na^+ inhibition at highest apparent affinity approximates the Na^+ stimulation of phosphorylation which is also cooperative[21] This suggests that Na^+ inhibits the Na^+-inhibited pathway by binding to the sites at which it stimulates phosphorylation Thus the pathways are reciprocally related with respect to Na^+

The stimulation by 200 mM Na^+ of ouabain binding occurring by the Na^+-

stimulated pathway (Table IV and *cf* ref 10) also indicates different binding sites for Na^+ and ouabain The K^+ stimulation of ouabain binding is consistent with both having their highest affinity for the E_2 form of the enzyme Whether this stimulation at low concentrations indicates distinct binding sites is difficult to say The inhibition curves (Fig 6) when normalized are parallel, suggesting a direct competitive inhibition Thus the experiments show different binding sites for Na^+ and ouabain, the latter possibly shared by K

Na^+ inhibited the Na^+-inhibited pathway by stabilizing E_1 It has been suggested that, in the presence of saturating Na^+, phosphorylation is required to produce ouabain binding[22] The present findings support this hypothesis In the presence of Na^+, binding required free Mg^{2+} and ATP and was not supported by ADP (Figs 7, 8 and Table III) Binding was stimulated by Na^+ consistent with its occurring to E_2–P (Fig 8, Table IV) This biphasic effect of Na^+ is of considerable interest Firstly, Na^+ stimulated binding at concentrations which stimulate the phosphorylation step This stimulation is presumably intracellular[23] Secondly, at a much higher concentration (200 mM) Na^+ again stimulated ouabain binding (Table IV) This action is extracellular[12] and occurs to the phospho-enzyme[10] The data suggest that following phosphorylation the Na^+ binding sites have changed their orientation from intracellular to extracellular Since the second phase of Na stimulation is not seen at 100 mM Na^+ the reorientated sites appear to have lost a large part of their Na^+ affinity This observation corresponds with the drop in apparent affinity for Na^+ produced by ouabain in the Na -inhibited pathway These changes in affinity and orientation of the Na^+ sites are consistent with the transport role of this enzyme Hydrolysis of ATP maintains E_2–P The steady-state nature of E_2–P (ref 20) gives binding by this pathway its requirement for substrate and the pathway is that of active Na^+ transport

The conclusion that ouabain binding in the presence of Na^+ depends on phosphorylation contrasts with those of other investigators It has been reported that Na^+ and ATP or ADP or Na^+ and Si^{2+} will support ouabain binding[3,13] The high sensitivity of Na^+–*E*–ATP to Mg^{2+}, the presence of adenylate kinase activity in these membrane preparations[24], temperature[13] and tissue[3] differences may explain the discrepancies

When the system is Na^+ stimulated, free enzyme formed after the dissociation of ouabain recycles through the Na^+-inhibited pathway Under physiological conditions this pathway has been suggested to transport K^+ (refs 10 and 13) The mechanism of its partial reversal to produce the Na^+-inhibited pathway is not clear Though P_i accelerates bindings by this pathway, it has not been shown to phosphorylate the native enzyme[10,20] The K^+ stimulation of ouabain binding (Fig 6) would appear to rule out formation of the K^+-sensitive E_2–P from P_i as a mechanism of this pathway This pathway may be related to the Na^+–Na^+ exchange of red cells[25] Both are energy independent, inhibited by ATP and high intracellular Na^+ (ref 26) The Na^+ inhibition of both is relieved by P_i No K^+-sensitive intermediates appear to be involved in the mechanism of either The ATP requirement of the Na^+–Na^+ exchange is the only apparent discrepancy

The exponential decay of the enzyme–ouabain complex over the temperature range studied suggests one type of specific binding site Using the approximate free energy of interaction of the enzyme–ouabain complex (Fig 3) and the enthalpy value obtained from an Arrhenius plot of the data in Fig 9, the entropy change for the

interaction was estimated at 123 entropy units This high value is consistent with a large conformational change Any single-bond type cannot explain the characteristics of the interaction Simple hydrophobic bonding is not compatible with the positive ΔH which does not diminish with temperature and the large positive entropy change[27] Hydrogen bonding is also unlikely Presumably, a number of noncovalent-bond types are involved in the interaction

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