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OUABAIN BINDING TO THE SODIUM PUMP IN PLASMA MEMBRANES ISOLATED FROM OX BRAIN

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SUMMARY

1. A quantitative study has been made of the binding of ouabain to two $(Na^+ + K^+)$-ATPase preparations from ox brain.

2. The binding with $Na^+ + ATP + Mg^{2+}$ was similar to that with Mg^{2+} but no ATP regarding the affinity and the number of receptor sites.

3. The binding with $Na^+ + ATP + Mg^{2+}$ was dissimilar to that with Mg^{2+} but no ATP regarding the effects of cations, P_i and pH. With Mg^{2+} but no ATP, cations inhibited in the order $Tl^+ > K^+ > Na^+ > choline^+$, but with $Na^+ + ATP + Mg^{2+}$, only, Tl^+ and K^+ inhibited. P_i stimulated ouabain binding with Mg^{2+} but not with $Na^+ + ATP + Mg^{2+}$. Ouabain binding with Mg^{2+} varied with pH like ATP hydrolysis but with $Na^+ + ATP + Mg^{2+}$, the binding was constant from pH 5.0–8.3.

4. The inhibitory effects of Tl^+, K^+ and of Na^+ with Mg^{2+} were quantitatively similar to their activation of the sodium pump ATPase.

5. A two-site mechanism which passes through a transition state complex is deduced from the results. Intramolecular movements of Na^+ and K^+ are associated with the formation of the complex which decomposes spontaneously thus liberating Na^+ externally and K^+ internally.

INTRODUCTION

There is much speculation regarding the mechanism by which the sodium pump ATPase translocates Na^+ and K^+ across cell membranes (for reviews see refs 1–3). Some suggestions depend on the way cardiac glycosides, usually ouabain, are bound to fragmented membranes. The binding shows many features which are clearly analogous to the pump action[4–6], for example, the requirement for ATP and Mg^{2+}, stimulation by Na^+ and inhibition by K^+. In other respects, it is difficult to make this correlation, for example, the nucleotide specificity is low[7–9] whereas only ATP will support active Na^+ transport and Na^+–Na^+ exchange diffusion[10].

Cardiac glycosides are nevertheless valuable as probes which reveal the different conformations of the transport system. We have therefore studied ouabain binding to two $(Na^+ + K^+)$-ATPase preparations from ox brain, placing particular emphasis

Abbreviation: EGTA, 1,2-di(2-aminoethoxy)ethane-*N,N,N',N'*-tetraacetic acid.

on the affinity and number of receptor sites for the binding and on correlating the observed binding with the pump action. The results lead us to propose a mechanism for the sodium pump which is considerably simpler, yet more chemically orientated, than previous models.

METHODS

Preparation of $(Na^+ + K^+)$-ATPases from ox brain

Native membranes. The membrane fraction was prepared from the grey matter of ox brain to Step 1 of the method described by Tanaka and Strickland[11]. The membranes were resuspended in water to a final concentration of 20–30 mg/ml and stored at −20 °C.

Urea-treated membranes. In order to increase the proportion of $(Na^+ + K^+)$-ATPase, native ox brain membranes were treated with urea[12]. In the original procedure[13], urea treatment was carried out a low temperature for several days, but here a shorter exposure at a higher temperature was employed[14]. Native membranes were suspended (5–7 mg/ml) in a medium containing 2.5 M urea and 100 mM ATP, pH 7.6, and after incubating at 37 °C for 15 min[14], they were collected by centrifuging at $23000 \times g$ for 1 h. They were resuspended in a similar volume of ATP (100 mM, pH 7.6) and centrifuged as before. The membranes were washed once more in the same way, resuspended in water to a final concentration of 6–8 mg/ml and stored in the frozen state.

Measurement of ouabain binding

The binding of [^{3}H]ouabain (N.E.N., Boston, Mass., U.S.A.) was measured essentially as described by Akera and Brody[15]. The membrane suspensions (1 mg/ml) were shaken at 37 °C in solutions containing 2 mM 1,2-di(2-aminoethoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and 20 mM imidazole–HCl (pH 7.6) *plus* the substances shown in the legends to the figures and tables. By cooling to 0 °C, binding was stopped in 2-ml aliquots, which were then centrifuged at $100000 \times g$ for 30 min. The pellets were resuspended in 1.5 ml 5% aqueous Triton X-100, and 1-ml aliquots of the resuspended pellets, supernatants and the original suspensions were assayed for radioactivity using an emulsion scintillant (1% PPO in toluene–Triton X-100; 70:30, v/v). In all experiments, the overall recovery of ouabain was checked: bound ouabain was estimated from the counts in the pellet. In order to avoid any difficulty arising from non-specific binding[15,16], we have employed ouabain concentrations below 10^{-6} M.

Standard assay procedures

The measurement of ATPase activity and protein were carried out as described previously[17]. The standard assay medium contained 100 mM NaCl, 10 mM KCl, 3 mM ATP, 3 mM $MgCl_2$, 20 mM imidazole–HCl (pH 7.6) and, as required, 0.1 mM ouabain and 2 mM EGTA.

Reagents

The sources of the reagents were the same as previously described[17].

RESULTS

Ouabain binding to native membranes

The first experiments were with native membranes isolated from ox brain cortex. In all experiments, the Ca^{2+}-chelating agent, EGTA (2 mM), was added in order to relieve the inhibition caused by traces of Ca^{2+} (ref. 18). The ouabain-sensitive ATPase was raised by EGTA from 5.3 to 15.7 μmoles P_i released/mg protein per h (see Table IV).

The time course of ouabain binding. To see whether equilibrium could be reached between ouabain and its receptor sites, the time-course was measured. Measurements were made under conditions which support generation of the phosphorylated intermediate of the sodium pump, namely in the presence of 115 mM Na^+ + 3 mM ATP + 5 mM Mg^{2+} and as a control with 5 mM Mg^{2+} and minimal (4 mM) Na^+. For both conditions, there was a gradual increase in binding with a half time of about 8 min, and a steady level was reached after 45–60 min (Fig. 1). The plateau shows that there was a finite number of receptor sites, and suggests that the binding was of a specific kind like that seen at low ouabain concentrations with intact cells[16]. The striking feature of Fig. 1 is that the binding capacity was the same whether or not Na^+ + ATP was added. In view of this similarity, measurements were made of the dependence of binding on ouabain concentration.

The constants for binding under equilibrium conditions. Binding was measured under equilibrium conditions (after 60 min) with different ouabain concentrations,

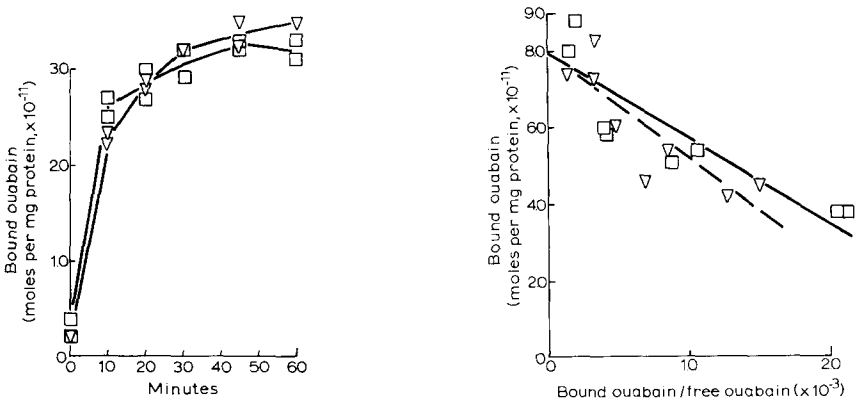


Fig. 1. Time course of ouabain binding to ox brain membranes. The binding of [^{3}H]ouabain ($5 \cdot 10^{-8}$ M) was measured at 37 °C in the presence of 2 mM EGTA and 20 mM imidazole–HCl buffer (pH 7.6) *plus* 100 mM NaCl, 3 mM ATP and 5 mM $MgCl_2$ (□), or 5 mM $MgCl_2$ (▽) (see Methods).

Fig. 2. Ouabain binding to ox brain membranes. The binding of [^{3}H]ouabain at concentrations of $5 \cdot 10^{-8}$ M to $5 \cdot 10^{-6}$ M was measured after 1 h at 37 °C in the presence of 2 mM EGTA and 20 mM imidazole–HCl (pH 7.6) *plus* 100 mM NaCl, 3 mM ATP and 5 mM $MgCl_2$ (□) or 5 mM $MgCl_2$ (▽) (see Methods). The dissociation constant (K) and the number of binding sites (n) were calculated from the bound ouabain (B) and the free ouabain (F) from the linear relationship[19] $B = n - K(B/F)$. The straight lines were calculated by the method of least squares. Collected data are shown in Table I.

so that the dissociation constant and the number of receptor sites could be evaluated[19] for the reaction

(receptor–ouabain) complex $\rightleftharpoons$ receptor + ouabain

Straight lines were obtained by plotting bound ouabain against bound ouabain/free ouabain (Fig. 2) indicating that there is reversible binding to a single class of receptor sites. The maximum amount of ouabain (in moles per mg protein) which would be bound at infinite ouabain concentration with $Na^+ + ATP + Mg^{2+}$ was $6.58 \cdot 10^{-11} \pm 0.59 \cdot 10^{-11}$, and the value of $6.93 \cdot 10^{-11} \pm 0.47 \cdot 10^{-11}$ with Mg^{2+} but no ATP was similar. Taking an average value of $6.8 \cdot 10^{-11}$ mole per mg, this result shows that the number of receptor sites, assuming one molecule of ouabain per site, was $(6.8 \cdot 10^{-11} \times 6.23 \cdot 10^{23}) = 4 \cdot 10^{13}$ per mg protein.

The dissociation constants for binding with $Na^+ + ATP + Mg^{2+}$ and with Mg^{2+} but no ATP were also comparable (Table I). When Mg^{2+} was omitted but still with ATP and Na^+ (15 and 115 mM, respectively), binding was characterised by lower affinities and small changes in the number of sites (Table I).

TABLE I

OUABAIN BINDING TO OX BRAIN MEMBRANES

The binding of [^{3}H]ouabain ($0.5 \cdot 10^{-7}$–$5 \cdot 10^{-7}$ M) was measured after incubation at 37 °C for 1 h in a medium containing 2 mM EGTA and 20 mM imidazole–HCl (pH 7.6) *plus* additions as shown above (see Methods). The dissociation constants and the numbers of receptor sites were calculated from the observed binding at different ouabain concentrations (see legend to Fig. 2). The results are expressed as the mean ± S.D.

Concentrations of additions (mM)			*Number of experiments*	*Dissociation constant* ($M \times 10^8$)	*Maximum amount of ouabain bound* (*moles per mg protein* $\times$ *10*$^{-11}$)
ATP	Na^+	Mg^{2+}			
3	115	5	6	2.35 ± 1.38	6.58 ± 0.59
—	4	5	5	3.02 ± 0.51	6.93 ± 0.47
3	115	—	5	3.68 ± 1.44	4.18 ± 0.31
3	15	—	3	7.48 ± 0.98	3.30 ± 1.30

The main finding seems clear that there were two conditions for ouabain binding —with $Na^+ + ATP + Mg^{2+}$ and with Mg^{2+}, which were indistinguishable as regards the number of sites or dissociation constant. More important is the point that comparable binding was observed under conditions such that the phosphorylated intermediate could not be formed.

Inhibition and stimulation ot the binding to native membranes

In an attempt to distinguish binding by the two pathways, conditions were varied in three ways to test the effects of cations (Na^+, K^+, Tl^+ and choline), of P_i and of pH.

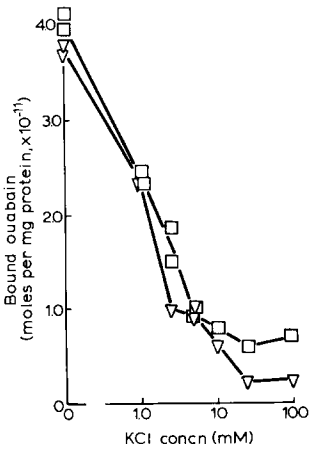
Effects of Na^+, K^+ and choline. The effects of cations on the binding of cardiac glycosides are important because they provide a means of correlating binding with function of the sodium pump[4–6]. The first tests were made with K^+ which was found to inhibit binding equally with 115 mM Na + 3 mM ATP + 5 mM Mg^{2+} and

with 5 mM Mg^{2+} + 4 mM Na^+ (Fig. 3), half-maximal inhibition being given by 2 mM and complete inhibition by 10 mM. The concentration dependence on K^+ resembles[20] that for activation of the sodium pump by external K^+. It is striking that K^+ should act in this manner vis-à-vis ouabain binding with Mg^{2+} but no ATP. The result with $Na^+ + ATP + Mg^{2+}$ is in accord with the well-known antagonism[4–6] between cardiac glycoside binding and K^+.

In contrast to the similar effect of K^+ under the two conditions there was a clear difference in the response to Na^+. In the first place, there was complete inhibition of Mg^{2+}-dependent binding with 100 mM Na^+ (Fig. 4), half-maximal inhibition being obtained with 20 mM. This value is markedly close to the Na^+ concentration which gives half-maximal activation of the sodium pump[21]. Choline also caused some inhibition with Mg^{2+} but no ATP, but even at 100 mM this was less than 50% (Fig. 4). Thus, the effectiveness in inhibiting binding was in the order $K^+ > Na^+ > choline^+$.

To check the possibility that inhibition with Na^+ really arose from Cl^-, the experiment was repeated with sodium gluconate. The inhibitory effects, however, were the same (Fig. 5). To check further that osmotic effects, such as those observed with *p*-nitrophenyl phosphatase[22], might give rise to inhibition, non-electrolytes were examined. Glucose (or sucrose) did not inhibit binding with Mg^{2+} but no ATP (Fig. 5). All these results show that the effects of Na^+ and K^+ on ouabain binding are specific actions for these ions, and do not arise from changes in ionic strength, osmolarity or nature of the anion.

There was little effect when sodium gluconate and choline chloride were tested with $ATP + Mg^{2+}$ (Table II). We never completely omitted Na^+, and binding was not significantly altered when the Na^+ concentration was lowered from 115 mM to 15 mM or when 100 mM NaCl was replaced by sodium gluconate or choline chloride.



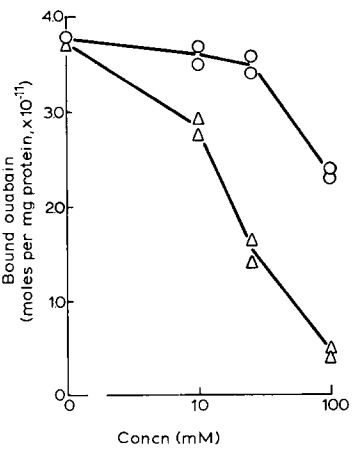
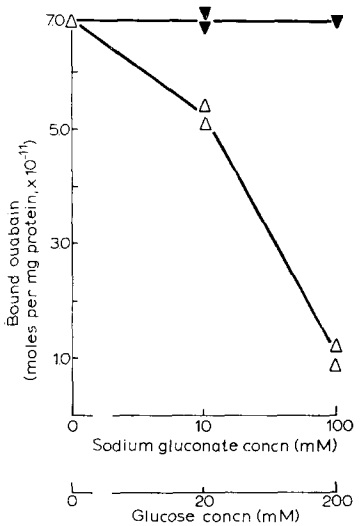


Fig. 3. Effect of K^+ on ouabain binding to ox brain membranes. The binding of ouabain ($5 \cdot 10^{-8}$ M) was measured after 1 h at 37 °C in the presence of $Na^+ + ATP + Mg^{2+}$ (□) and Mg^{2+} (▽) *plus* K^+ as shown, as described in the legend to Fig. 1.

Fig. 4. Effects of Na^+ and choline on ouabain binding to ox brain membranes. The binding of ouabain in the presence of Mg^{2+} *plus* Na^+ (△) and choline (○) chlorides, as shown, was measured as described in the legend to Fig. 3.

Na^+ is held to be necessary to support maximum binding with $ATP + Mg^{2+}$, and a low Na^+ concentration (15 mM) appeared to be sufficient[4]. The Na^+-dependent ATPase activity was unaltered when NaCl was replaced by sodium gluconate, again showing that the nature of the anion is not generally important[23].



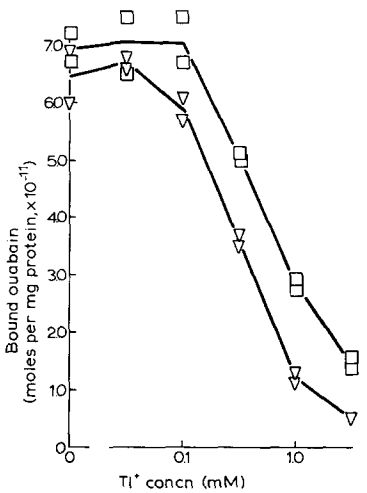


Fig. 5. Effects of sodium gluconate and glucose on ouabain binding to ox brain membranes. The binding of ouabain ($1.5 \cdot 10^{-7}$ M) in the presence of Mg^{2+} *plus* gluconate (△) and glucose (▼), as shown, was measured as described in the legend to Fig. 3.

Fig. 6. Effect of Tl^+ on ouabain binding to ox brain membranes. The binding of ouabain in the presence of $Na^+ + ATP + Mg^{2+}$ (□) and Mg^{2+} (▽) *plus* thallous acetate, as shown, was measured as described in the legend to Fig. 5.

TABLE II

EFFECTS OF CATIONS AND ANIONS ON OUABAIN BINDING TO OX BRAIN MEMBRANES

The binding of ouabain ($5 \cdot 10^{-8}$ M) was measured as described in the legend to Table I with additions of ATP and other substances as shown. The results are the mean ±S.D. of four experiments (except control, mean of two experiments).

Concentrations of additions (mM)					*Bound ouabain (moles per mg protein × 10⁻¹¹)*
$MgCl_2$	*ATP*	*NaCl*	*Sodium gluconate*	*Choline chloride*	
5	—	4	—	—	5.1 ± 0.9
5	3	15	—	—	5.8 ± 0.9
5	3	115	—	—	4.5 ± 0.7
5	3	15	100	—	4.6 ± 0.5
5	3	15	—	100	5.2 ± 0.5
—	3	15	—	—	3.1 ± 0.1
—	3	115	—	—	3.1 ± 0.2
—	—	4	—	—	0.8

Inhibition by Tl^+. K^+ strongly inhibited binding, and it seemed worthwhile to examine the effect of Tl^+, which resembles K^+ in activating the ATPase, in being actively transported in erythrocytes and in causing breakdown of the phosphorylated intermediate[24–26]. The only difference compared with K^+ seems to be[26,27] the generally greater affinity of membranes for Tl^+. We confirmed that Tl^+ activates the Na^+-dependent ATPase; a K_m of 0.1–0.5 mM was found for Tl^+ and 1.0–3.0 mM for K^+. Addition of EGTA did not alter these K_m values which are consistent with previous results[20,21,26,27]. Tl^+ at a concentration of 3 mM completely inhibited ouabain binding and in this respect resembles K^+. However, on varying the Tl^+ concentration there was less sensitivity with $Na^+ + ATP + Mg^{2+}$ than with Mg^{2+} but no ATP (Fig. 6); the concentrations required to reduce the binding by 50% were 1.0 mM and 0.3 mM, respectively. These different values are in contrast to the same value for the two conditions found with K^+.

Effect of P_i. There seems good evidence that in the presence of Mg^{2+} and ouabain, P_i becomes bound to the sodium pump[28–30] and the product appears to be identical with that formed from ATP in the presence of Na^+ and Mg^{2+}. There is, moreover, some stimulation of ouabain binding by P_i which has been attributed to formation of the phosphate intermediate[5,6]. If binding depended on a reaction of P_i with protein, then the amount of binding should be much greater with $Mg^{2+} + P_i$ than with Mg^{2+} alone. It was therefore necessary to reinvestigate the effect of P_i on binding.

At concentrations of up to 10 mM, P_i had no effect on binding with $Na^+ + ATP + Mg^{2+}$. In contrast, with Mg^{2+} but no ATP the binding was stimulated (Fig. 7) to a small, but consistently observed (Fig. 8) extent (25%), and there appeared

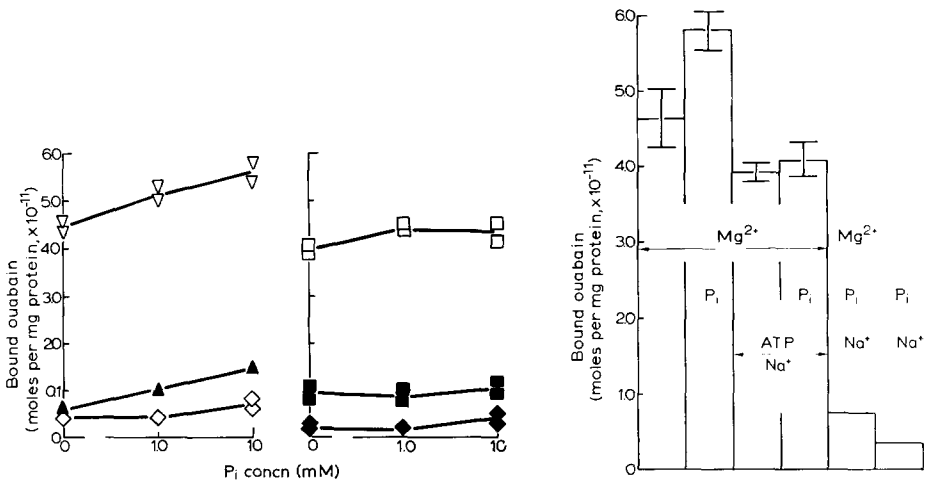


Fig. 7. Effect of P_i on the binding of ouabain to ox brain membranes. The binding of ouabain in the presence of P_i as shown was measured as described in the legend to Fig. 3. Left hand figure: Mg^{2+} (▽), Mg^{2+} + 10 mM KCl (▲), 10 mM KCl (◇). Right hand figure: $Na^+ + ATP + Mg^{2+}$ (□), $Na^+ + ATP + Mg^{2+}$ *plus* 10 mM KCl (■), control, no additions (◆).

Fig. 8. Effect of P_i on the binding of ouabain to ox brain membranes. The binding of ouabain was measured with the additions shown as described in the legend to Fig. 7, with and without 10 mM P_i.

still to be some P_i-dependent binding even when Na^+ or K^+ was added, although the level was very small (Figs 7 and 8). We have not made a detailed analysis of these results because of the small magnitude of the effect of P_i.

Effect of pH. The third attempt to characterise the binding was by varying pH. In these experiments, the usual buffer (20 mM imidazole–HCl) was replaced by 20 mM Tris–maleate buffer at the required pH for the measurement of both binding and ATPase activity.

With Mg^{2+} but no ATP, ouabain binding was sensitive to pH over the range pH 5.0–8.3 (Fig. 9). The broad maximum resembled, but was not identical to, the maximum observed in $(Na^+ + K^+)$-ATPase activity with and without EGTA (Fig. 10). Paradoxically, ouabain binding in the presence of $Na^+ + ATP + Mg^{2+}$ was largely insensitive to pH between pH 5.0 and 8.3 (Fig. 9). The effect of pH on binding with Mg^{2+} resembles its effect on the stability of bound ouabain in *Electrophorus* microsomes[5]. Thus there seems to be a difference in response to pH with the binding by the two pathways.

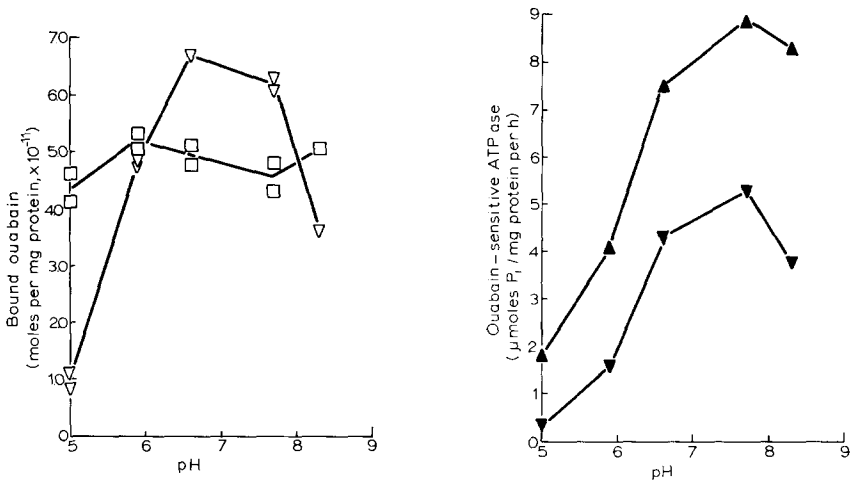


Fig. 9. Effect of pH on ouabain binding to ox brain membranes. The binding of ouabain was measured as described in the legend to Fig. 5, using 20 mM Tris–maleate buffers at the pH shown, in the presence of $Na^+ + ATP + Mg^{2+}$ (□) and Mg^{2+} (▽).

Fig. 10. Effect of pH on the sodium pump ATPase. The ouabain-sensitive ATPase was measured as described above (see Methods), using 20 mM Tris–maleate buffers at the pH shown: ▼, without EGTA; ▲, with 2 mM EGTA.

Effects of Mg^{2+} and EGTA on ouabain binding

Effect of Mg^{2+}. Binding with Mg^{2+} is shown as a function of total and free Mg^{2+} in Fig. 11. It increased as the total Mg^{2+} concentration was raised to 1 mM, and higher concentrations (up to 12 mM) were without further effect. The free Mg^{2+} concentration was calculated from an apparent stability constant of $2.5 \cdot 10^4$ for MgEGTA[31], assuming that EGTA is the only chelating species present. The strong ouabain binding at low Mg^{2+} concentrations suggests that the sodium pump lipoprotein has a high affinity for Mg^{2+} as previously concluded by Siegel and Josephson[32].

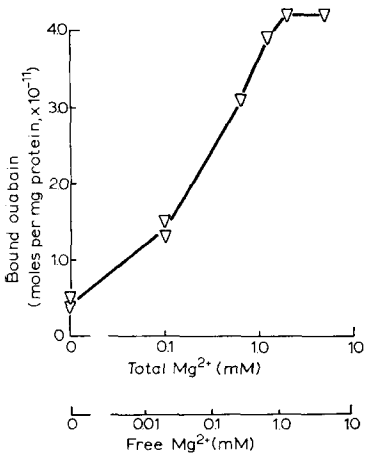


Fig. 11. Effect of Mg^{2+} concentration on ouabain binding to ox brain membranes. Ouabain binding in the presence of Mg^{2+} at the concentrations shown was measured as described in the legend to Fig. 3. The results are shown as ouabain binding as a function of free Mg^{2+} (lower scale) and total Mg^{2+} (upper scale).

Effect of EGTA. Since EGTA was routinely added in the present study, it was necessary to examine its effect on ouabain binding. When EGTA was omitted, we found no change in the extent of ouabain binding in each one of the six conditions tested despite the fact that the absolute magnitude varied twofold under the influence of other conditions of the assay (Table III). Specht and Robinson[33] have also shown that ouabain binding is not affected by certain other chelating agents. As chelating agents stimulate the $(Na^+ + K^+)$-ATPase, it follows that there is no obligatory correlation between ATPase activity and the capacity to bind ouabain under comparable conditions. Since Ca^{2+} inhibits from inside cells and ouabain from

TABLE III

EFFECT OF EGTA ON OUABAIN BINDING TO OX BRAIN MEMBRANES

The binding of ouabain ($1.5 \cdot 10^{-7}$ M) was measured as described in the legend to Table I, with additions of ATP and other substances as shown. The results are the mean ±S.D. of four experiments.

Concentrations of additions (mM)				*Bound ouabain (moles per mg protein × 10⁻¹¹)*	
$MgCl_2$	*ATP*	*NaCl**	*P_i*	*No EGTA*	*2 mM EGTA*
5	3	111	—	5.5±0.6	5.8±0.7
5	3	11	—	7.8±0.5	7.5±0.3
5	—	—	—	7.5±0.5	7.9±0.3
5	—	—	10	9.7±0.4	9.3±0.7
5	3	111	10	5.5±0.7	5.5±0.7
—	3	11	—	2.9±0.6	3.9±0.5

* The presence of mM EGTA increases the Na^+ concentration by approximately 4 mM.

TABLE IV

ATPase ACTIVITY OF NATIVE AND UREA-TREATED OX BRAIN MEMBRANES

ATPase activities were measured as described in Methods. The results shown are the mean of duplicate assays which differed by less than 5% from the mean.

Membranes	*Assay medium*	*ATP hydrolysis (μmoles P_i released/mg protein per h)*					
		Control			*Plus EGTA*		
		Total	*Ouabain-insensitive*	*Ouabain-sensitive*	*Total*	*Ouabain-insensitive*	*Ouabai sensitiv*
Native	Complete	13.9	8.6	5.3	20.3	5.5	15.7
Native	No K^+	9.6	9.1	0.5	6.9	6.2	0.7
Urea-treated	Complete	10.3	3.4	6.9	27.8	4.8	22.2
Urea-treated	No K^+	2.4	2.1	0.3	5.0	5.2	0

outside, it also follows that there is spatial separation of their sites of action. Thus ouabain binds to an allosteric site which is unaffected when inhibition at the active centre is relieved by EGTA.

Besides stimulating the sodium pump, EGTA inhibited the ouabain-insensitive component, suggesting that sequestration of Ca^{2+} leads to inhibition of a Ca^{2+}-stimulated, ouabain-insensitive ATPase (Table IV).

Enrichment of the sodium pump in membranes

The proportion of ouabain-sensitive ATPase in the membranes can be raised by exposure to urea (see Methods) to 65% or, with EGTA, to 80% (Table IV). When K^+ was omitted no ouabain-sensitive ATPase was observed (Table IV). Ouabain binding was measured with this enriched preparation to check the main results described above.

Ouabain binding at equilibrium. Again, the rate of reaction of ouabain was

TABLE V

OUABAIN BINDING TO UREA-TREATED OX BRAIN MEMBRANES

The binding of ouabain was measured as described in the legend to Table I, and the dissociation constant and number of binding sites were calculated as described in the legend to Fig. 2. Where possible, the results are shown as the mean ±S.D.

Concentrations of additions (mM)				*Number of experiments*	*Dissociation constant ($M \times 10^8$)*	*Maximum amount of ouabain bound (moles per mg protein $\times 10^{-11}$)*
ATP	*Na^+*	*Mg^{2+}*	*P_i*			
3	115	5	—	4	1.38±0.38	15.8±3.0
—	4	3	1	4	1.70±0.35	12.2±3.1
3	—	—	—	2	7.95	18.6
—	4	3	—	1	2.22	16.0
3	115	—	—	1	2.93	13.0

investigated to ensure that equilibrium was achieved. The time course of ouabain binding to pump-enriched membranes resembled that for native membranes, and a steady level of ouabain binding was observed after one hour. The dissociation constants and numbers of receptor sites were also measured as described above. The dissociation constant (Table V) found with $Na^+ + ATP + Mg^{2+}$ agrees satisfactorily with those obtained with native ox brain membranes (Table I), and with deoxycholate-treated ox brain microsomes[34]. With Mg^{2+} but no ATP the value was not significantly different from that found with $Na^+ + ATP + Mg^{2+}$. In contrast, there was a lower affinity in the presence of $Na^+ + ATP$ or of ATP, both without Mg^{2+} (Table V). The number of receptor sites found in the pump-enriched membranes was greater than the number in the native enzymes, but unlike the native membranes this number was similar for all of the five conditions tested (Tables I and V).

Inhibition and stimulation of ouabain binding. In order to check the effects of cations and anions, ouabain binding to pump-enriched membranes was measured as before, and it is not necessary to describe the results at length.

K^+ caused a similar inhibition of ouabain binding in the presence of $Na^+ + ATP + Mg^{2+}$ and of Mg^{2+} without ATP, and again, with Mg^{2+}, Na^+ inhibited less than K^+, but more than choline. Sodium gluconate reduced ouabain binding in the presence of Mg^{2+} exactly like NaCl, and in the same conditions sucrose and glucose did not cause significant changes in ouabain binding.

All these results, with a preparation enriched with the sodium pump, confirm those obtained with the native microsomes, and it therefore follows that the binding is to the sodium pump and not an artifact related to the presence of unwanted protein.

DISCUSSION

Specificity of ouabain binding to the sodium pump

The first point to consider is whether ouabain is uniquely bound to the sodium pump. There is good evidence that this is so, since the number of receptor sites with pump-enriched (urea-treated) membranes was greater than with native membranes, approximately in proportion to the higher $(Na^+ + K^+)$-ATPase activity. The linearity of Scatchard plots further suggests that the sites were of a single class, and whether binding was supported by Mg^{2+} or by $Na^+ + ATP + Mg^{2+}$, the dissociation constant was comparable to the inhibitor constant for the effect of ouabain on ATPase activity. Moreover, whatever conditions were employed to stimulate binding, the number of receptor sites was constant. None of these observations is consistent with ouabain binding to anything other than the sodium pump.

The two pathways for ouabain binding

It is thought that a conformational change must occur before ouabain can become bound and this is the point of studying ouabain as a ligand[4–7]. We have shown that it is protein to which ouabain binds[17], suggesting that only the protein moiety of the phospholipoprotein which makes up the $(Na^+ + K^+)$-ATPase is likely to undergo conformational changes. Our main new finding is that binding supported by $Na^+ + ATP + Mg^{2+}$ and by Mg^{2+} without ATP was similar with respect to ouabain affinity and the number of receptor sites. However, there were two important differences, *viz.* the response to cations and pH.

The effects of cations were consistent with the existence of two pathways for ouabain binding: sodium-stimulated and sodium-inhibited[6]. With Mg^{2+} but no ATP, cations inhibited binding and their effectiveness fell in the order: $Tl^+ > K^+ > Na^+ > choline^+$. Iso-osmotic replacements of cations with non-electrolytes, and of Cl^- with gluconate, all showed that these were specific effects of cations.

The effect of pH on binding was also different according to the conditions of formation of the complex. There was a clear paradox in that binding with $Na^+ + ATP + Mg^{2+}$, which might more reasonably be correlated with the normal function of the pump[4], was unaffected by pH, whereas the binding with Mg^{2+} but no ATP responded to pH changes in a way which resembled the overall hydrolysis of ATP.

Mechanism of ouabain binding

There is no doubt that very complicated schemes are required to account for all aspects of ouabain binding, which occurs under a wide variety of conditions[7]. We do not propose to add to the schemes which have been devised[4–6,15,35–37], simply because they seem unnecessarily complicated, when it is recognised that some of the conformations which bind ouabain are not part of the natural reaction path. Thus it seems unlikely that the conformation revealed by the effects of P_i represents a step in the normal mechanism in view of the similar effects[7] of phosphate and arsenate. It is more likely that this represents a side reaction[32]. Again, since the true substrate is an $ATP\text{–}Mg^{2+}$ complex[12], the conformation seen with ATP or $Na^+ + ATP$ may not be a natural intermediate.

The results show that ouabain binds reversibly to the sodium pump and that equilibria such as

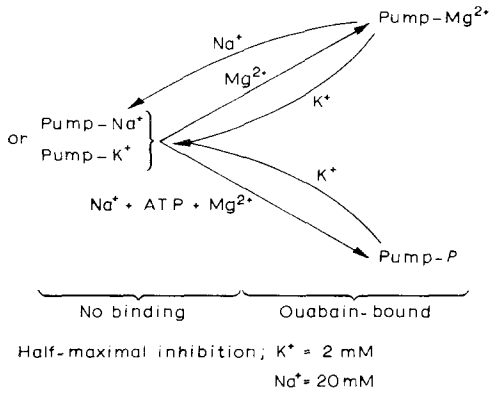
$$Pump\text{–}Mg^{2+}\text{-ouabain} \rightleftharpoons Pump\text{–}Mg^{2+} + ouabain \quad (1)$$

are displaced to the right in the presence of cations which inhibit the binding by the reactions

$$Pump\text{–}Mg^{2+} + Na^+ \rightleftharpoons Pump\text{–}Na^+ + Mg^{2+} \quad (2)$$

$$Pump\text{–}Mg^{2+} + K^+ \rightleftharpoons Pump\text{–}K^+ + Mg^{2+} \quad (3)$$

The most important results of the present study can thus be represented by the following sequence of reactions:



(4)

The facts on ouabain binding relevant to the mechanism of the pump are summarised in this simple scheme.

The mechanism of the sodium pump

These facts (Eqn 4) provide a basis for a pump mechanism if one assumption is made, *viz.* that Na^+ and K^+ can be bound simultaneously to the pump under physiological conditions in the intact cell with concentrations for half maximum saturation of 2 mM for external K^+ and 20 mM for internal Na^+. This is a reasonable assumption in light of the quantitative agreement vis-à-vis the interdependence of Na^+ and K^+ transport and the influence of the ions on ouabain binding.

The salient points of the mechanism are:

(1) The binding site for Na^+ faces the inside and for K^+ outside, and can be occupied simultaneously: Na^+ and K^+ bind without ATP being required.

(2) When these sites are occupied there is then a phosphorylation with ATP+ Mg^{2+} to form a transient, unstable complex in which there is an intramolecular shift in position of the cation binding sites such that they become orientated with K^+ facing the inside and Na^+ the outside.

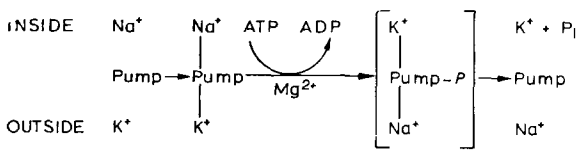
(3) The complex has an affinity for K^+ shown by a half-maximum value of 2 mM.

(4) The complex contains K^+, and therefore dephosphorylation takes place, thus liberating K^+ internally and Na^+ externally.

(5) The dephosphorylation is a consequence of the formation of the complex. Therefore, the complex is not an intermediate in the biochemical sense but in the chemical sense of a transient complex commonly postulated in the field of reaction mechanisms[38]. It is regarded as a high energy transition state and not an isolable intermediate.

(6) The mechanism is formally a two-site system, which is consistent with the kinetics of ATP hydrolysis[39].

The proposed mechanism can be represented in the following way:



(5)

Four features of the mechanism are particularly interesting. First, the conformational change leading to an intramolecular shift in position of Na^+ and K^+ is generated by the transient incorporation of P_i into an acyl phosphate (see ref. 3). It is clear that replacement of a hydroxyl group by phosphate could readily modify the tertiary structure of protein, as found, for example, in the phosphorylation of nucleoproteins[40]. Second, the degree of charge separation as the pump operates will be small. Third, if external K^+ is not present the transition state cannot occur: instead the phosphorylated intermediate will be formed as already amply characterised with Na^+ alone (see refs 1–3). Fourth, half-saturation values for Na^+ and K^+ transport can be predicted from the results on ouabain binding. Quantitatively, the inhibitory effects of Na^+ and K^+ on ouabain binding should represent their affinity

for the enzyme (see Eqns 1–3 and Figs 3–5). Whilst competition between ouabain and cations[6] and between Na^+ and K^+ (ref. 41) have not been taken into account, the results do show agreement between the predicted and observed K_m values of 2 mM for K^+ and 20 mM for Na^+ (refs 20, 21).

It is clear that the proposed transition state complex possesses many of the properties attributed to the putative E_2P conformation[5,6]. Although it is necessary to emphasise that there is a fundamental difference in the concept of the transition state complex and the E_2P intermediate, their superficial similarity may explain the division of opinion on whether the phosphorylated intermediate is part of the normal reaction path[1–3,42,43]. We believe that the isolable phosphorylated intermediate is not part of the natural mechanism, which instead involves the transition state complex.

The question arises whether this mechanism can be distinguished from previous suggestions. It differs from Skou's two-site model[1] in positing the formation of a transition state complex as distinct from a biochemical intermediate and in not requiring a pump–ATP–Mg^{2+} complex as a prerequisite for attachment of Na^+ and K^+ to the membrane. The most discussed models[2,3,5,6] suggest a sequence of reactions by which Na^+ and K^+ are successively transported by a one-site system. However, some of the most recent observations[37,44,45] are consistent with our results and conclusions. On the other hand, it might be argued that a one-site model best explains our result which showed that K^+ inhibited ouabain binding equally with $Na^+ + ATP + Mg^{2+}$ and with Mg^{2+} alone. This difficulty was overcome by examining the effect of Tl^+ on ouabain binding.

Tl^+ can replace K^+ in the sodium pump, but the affinity[24–27] for Tl^+ is much greater than for K^+. We found that Tl^+ inhibited binding but, in contrast to K^+, not equally by the two pathways. The K_m for Tl^+ activation is 0.2 mM[26,27] which is close to the K_m value of 0.3 mM predicted for the proposed two-site mechanism (see Eqns 1–3 and Fig. 6). A higher value of about 1.0 mM would be expected for the one-site model from the half-maximum inhibition of the binding with $Na^+ + ATP + Mg^{2+}$.

In conclusion, by making only one assumption a simple mechanism for the sodium pump has been deduced. The three most important features of this mechanism are that it is based on conventional views of chemical reaction mechanisms, it quantitatively predicts activation of ATP hydrolysis by cations and it accounts for the close and obligatory coupling between simultaneous, active Na^+ and K^+ movements.

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