

Interaction of Ouabain with the Na⁺ Pump in Intact Epithelial Cells

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ABSTRACT To determine the specificity and efficacy of [³H]ouabain binding as a quantitative measure of the Na⁺ pump (Na⁺,K⁺-ATPase) and as a marker for the localization of pumps involved in transepithelial Na⁺-transport, we analyzed the interaction of [³H]ouabain with its receptor in pig kidney epithelial (LLC-PK₁) cells. When these epithelial cells are depleted of Na⁺ and exposed to 2 μM [³H]ouabain in a Na⁺-free medium, binding is reduced by 90%. When depleted of K⁺ and incubated in a K⁺-free medium, the ouabain binding rate is increased compared with that measured at 5 mM. This increase is only demonstrable when Na⁺ is present. The increased rate could be attributed to the predominance of the Na⁺-stimulated phosphorylated form of the pump, as K⁺ is not readily available to stimulate dephosphorylation. However, some binding in the K⁺-free medium is attributable to pump turnover (and therefore, recycling of K⁺), because analysis of K⁺-washout kinetics demonstrated that addition of 2 μM ouabain to K⁺-depleted cells increased the rate of K⁺ loss. These results indicate that in intact epithelial cells, unlike isolated membrane preparations, the most favorable condition for supporting ouabain binding occurs when the Na⁺,K⁺-ATPase is operating in the Na⁺-pump mode or is phosphorylated in the presence of Na⁺.

When LLC-PK₁ cells were exposed to ouabain at 4°C, binding was reduced by 97%. Upon rewarming, the rate of binding was greater than that obtained on cells kept at a constant 37°C. However, even at this accelerated rate, the time to reach equilibrium was beyond what is required for cells, swollen by exposure to cold, to recover normal volume. Thus, results from studies that have attempted to use ouabain to eliminate the contribution of the conventional Na⁺ pump to volume recovery must be reevaluated if the exposure to ouabain was done in the cold or under conditions in which the Na⁺ pump is not operating.

There is convincing evidence that the activity of the "Na pump" is dependent upon the enzyme Na⁺,K⁺-ATPase (5, 21) and that the cardiac glycoside ouabain inhibits transepithelial Na⁺ transport by binding specifically to and inhibiting this enzyme (21). Indeed, the amount of [³H]ouabain binding has been used to quantify the number of Na⁺-transport sites per cell (2). In addition, a radioautographic technique has recently been developed to localize [³H]ouabain binding sites (23) and, therefore, by inference, the Na⁺ pump. However, the Na,K-ATPase may operate in several different modes, not all of which, though sensitive to ouabain, need involve Na⁺ transport (8). In addition, Boardman et al. (3) reported an increase in specific ouabain binding to HeLa cells that was not correlated with increased ion transport and was concluded to be binding to nonfunctioning pumps. Therefore, it is not clear that Na⁺

transport is required for ouabain to interact with the enzyme, and it remains to be established that ouabain binds only to functional pump sites.

It is important to identify the conditions under which ouabain binds to sites in transporting epithelia, for at least two reasons. Results obtained with [³H]ouabain radioautography identify the cell types involved in Na⁺ transport (7, 20, 22, 23) and the transepithelial route for Na⁺ moving through the active pathway (20). If, however, [³H]ouabain can bind to nonoperating pumps or to pumps operating in other than a Na⁺-transport mode, then it becomes difficult to interpret the radioautograph. Likewise, determination of the number of Na⁺ pumps by [³H]ouabain binding before and after specific manipulations (9) requires verification that the amount of binding is related to functioning pumps and any measured changes can

be attributed to a change in either the total number or to the turnover of Na,K-ATPase units pumping Na⁺.

Studies of the interaction of ouabain with partially purified preparations of the enzyme indicate that ouabain can bind to the enzyme in an incubation medium that contains no Na⁺. Wallich et al. (25) showed that the most rapid rate of binding to the enzyme occurred in the presence of Mg⁺⁺ and inorganic phosphate and that this enzyme-ouabain complex was more stable than the one formed in the presence of Mg⁺⁺, ATP, and Na⁺. The evidence indicates, therefore, that ouabain can bind to different forms of the enzyme (25).

In purified membrane preparations, enzyme sidedness is lost and so ouabain could have access to all forms of the enzyme. This may not be the case in intact cells where access is restricted to the outer surface because ouabain does not readily penetrate the cell membrane (12). However, if in the different conformational states of the enzyme the position of the glycoside binding site in the intact cell membrane is unchanged (i.e., if it remains on the outside irrespective of the ligand-induced conformation), then an inactive pump, or one pumping ions other than Na⁺, could still bind ouabain, possibly in a manner indistinguishable from Na⁺-stimulated binding. Such a mechanism would be consistent with the observation of Cala et al. (4) that the amount of ouabain bound to frog skin, when Na⁺ transport was completely inhibited by amiloride, was similar to that bound when transepithelial sodium transport was normal.

It can be argued, therefore, that some ouabain binding to epithelial cells may be unrelated to Na⁺ transport either because of binding to pumps functioning in other modes or because of binding to nonfunctioning pumps. Such binding can be classified as specific because the ouabain does bind to the Na⁺,K⁺-ATPase, but the localization obtained or the number determined may not relate to the actual role of those pumps in the measured Na⁺ transport. Thus, attempts to quantify the pump (2), determine if external agents, such as hormones, modify the pump (9), or determine the transepithelial route of Na⁺ transport by localization of [³H]ouabain binding sites (20, 22, 23) would be compromised.

It is difficult to investigate this problem in an intact epithelium where underlying submucosal elements such as smooth muscle, nerves, and blood vessels make it difficult to achieve a Na⁺-free environment adjacent to the epithelial cells. In addition, these submucosal cellular elements also have Na⁺ pumps whose activity, though unrelated to that of the epithelial cells involved in transepithelial Na⁺ transport, provide a potential source of error in estimations of ouabain binding by the epithelial cells. To overcome these problems, we have examined the dependency of ouabain-binding on Na⁺ transport, using a cultured epithelial cell line derived from porcine kidney (LLC-PK₁; 13). These cells have ouabain-sensitive Na⁺ pumps and a polarized Na⁺-transport mechanism, as in epithelia in vivo (19). The results show that when Na⁺ transport is prevented either by removing Na⁺ from the incubation medium or by inhibiting cellular metabolism, ouabain binding is virtually eliminated. It can be concluded, therefore, that in intact cells essentially all ouabain binds to pumps that are functioning, at the time of binding, in the Na⁺-transport mode.

MATERIALS AND METHODS

LLC-PK₁ cells were grown in monolayer cultures on 35-mm-diameter dishes (Falcon Plastics Co., Los Angeles, Calif.) for 3 d in Dulbecco's Modified Eagle's Medium (DMEM; Grand Island Biological Co., Grand Island, N. Y.), supplemented with 10% fetal calf serum (Microbiological Associates, Bethesda, Md.),

penicillin (100 U/ml), and streptomycin (100 µg/ml). To begin a binding experiment, the cells were washed three times with bicarbonate-buffered Na⁺-Ringer's solution (24). Each dish was then washed repeatedly over a 120-min period with either the Na⁺-Ringer's solution or with Na⁺-free Ringer's prepared using a choline-bicarbonate-buffered choline Ringer's solution in which choline chloride completely replaced NaCl. In some experiments K⁺-free media were used, the osmolarity being maintained by substituting the appropriate Cl⁻ salt for KCl. After initial experiments to determine optimal conditions for ouabain binding, ouabain was added to each dish to give a final concentration of 2 µM (5µCi/ml [³H]ouabain). The ouabain concentration was checked spectrophotometrically and was found to be 90-97% of that specified by the supplier (New England Nuclear Corp., Boston, Mass.). Incubations at 37°C were carried out in a CO₂ incubator (Forma Scientifica, Marietta, Ohio). Incubations at 4°C were performed by placing dishes on a bed of ice and the containers covered by a plexiglass hood to maintain an atmosphere of 5% CO₂. At the end of the appropriate exposure time, dishes were washed rapidly three times with ice-cold, ouabain-free Ringer's solution of the same composition as that used during the incubation period. This washing was repeated twice more over the next 10 min. Experiments revealed that this treatment was adequate for the removal of unbound ouabain from the samples (Fig. 1). The dishes were drained and gently blotted at the edges, and 1 ml of 1 N NaOH was added to dissolve the cells. 24 h later, aliquots of the cell digest were taken for scintillation counting. Efficiency was determined by the external standard method.

In some experiments, cellular K⁺ was determined. Because various amounts of medium remained on the dishes after medium had been poured off at the end of incubation, dishes from experiments with media containing K⁺ were washed briefly (30-45 s) in ice-cold K⁺-free choline chloride before exposure to 2 ml of 0.1 M HNO₃ for several hours. Dishes from experiments with K⁺-free incubation media were simply drained, surplus medium was removed from the perimeter of the dish with Whatman No. 542 filter paper, and then K⁺ was extracted for several hours into 1 ml of 0.1 M HNO₃. K⁺ concentration in the extracts was measured with an EEL flame photometer (Evans Electro Selenium Ltd., Halstead, Essex, England).

In one experiment, the rate of loss of K⁺ into K⁺-free medium was determined. Dishes were incubated for timed intervals in 3 ml of K⁺-free medium. The medium was then removed and replaced by fresh medium. K⁺ concentration in the medium, which reflects the rate of loss of K⁺ from the cells, was measured on undiluted samples with an EEL flame photometer. Readings obtained were corrected for the small effect on the readings of the very high Na⁺ concentration in the aspirated samples. Because the volume of medium in the dish was known, the amount of K⁺ loss from the cells could be calculated.

RESULTS

To define optimum conditions for ouabain binding to LLC-PK₁ cells we analyzed the time and concentration dependence

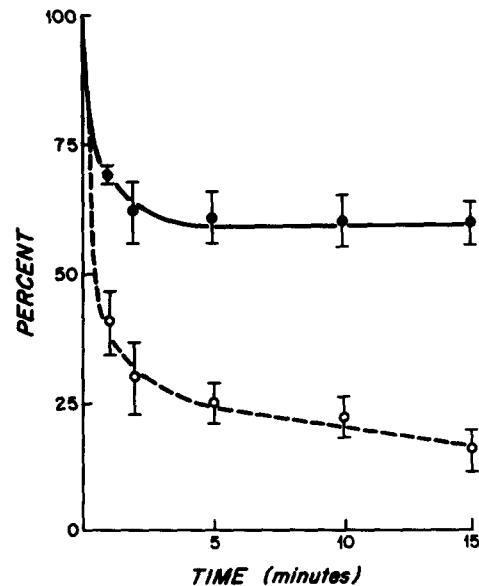
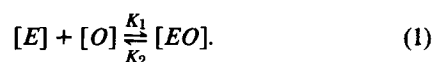


FIGURE 1 Washout curve for [³H]ouabain (●) and [¹⁴C]inulin (○). Confluent monolayers were exposed to label for 1 h and then washed at specified intervals in ice-cold ouabain-free Ringer's. Washes were saved and analyzed. Data expressed as percent remaining in tissue as a function of time. n = 6.

of [³H]ouabain binding. Fig. 2 shows the binding over the course of 90-min exposure to ouabain concentrations ranging from 0.1 to 50 μM. As detailed by Shaver and Stirling (22), a model can be formulated using the bimolecular reaction:



here [E] is the free enzyme concentration; [O], ouabain concentration; [EO], ouabain-enzyme concentration; and K_1 , K_2 , the association and dissociation rate constants. The integrated form of this equation can be expressed as previously shown (22) as:

$$[EO] = \alpha(1 - e^{-\beta t}), \quad (2)$$

where

$$\alpha = E_T[O]/([O] + K_2/K_1), \quad (3)$$

$$\beta = K_1[O] + K_2, \text{ and} \quad (4)$$

$$E_T = [E] + [EO]. \quad (5)$$

The curves in Fig. 2 are plots of Eq. 2. Best fit for α and β was established as described by Shaver and Stirling (22) using a nonlinear method (17). At equilibrium, $\alpha = [EO]$ and the total number of binding sites/cell can be calculated from Eq. 3 as described by Shaver and Stirling (22), plotting ouabain binding against ouabain concentration and obtaining the best-fit curve by the same nonlinear routine as used for Fig. 2. The values obtained are shown in Table I. The value for E_T of 2.92×10^6 molecules/cell is similar to that reported by Baker and Willis (2) for HeLa cells and guinea pig and rabbit kidney cells in culture, and K_m is similar to the value of 3.3×10^{-6} M reported by Almendares and Kleinzeller (1). In all subsequent experiments, incubations were conducted at 2 μM ouabain.

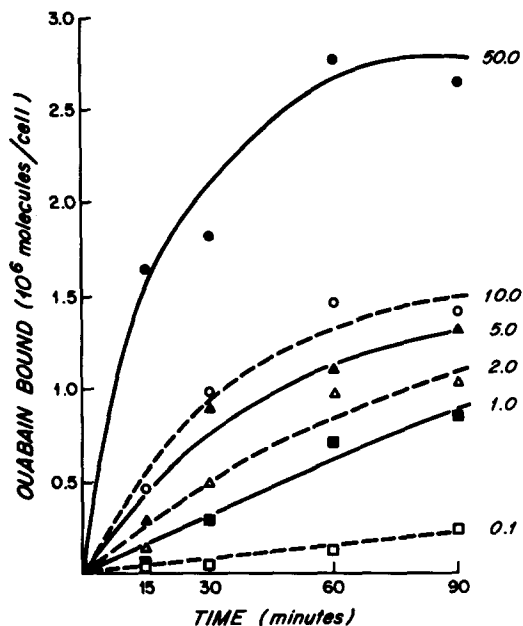


FIGURE 2 Dependence of ouabain binding to LLC-PK₁ cells on time at different ouabain concentrations. Line is best fit to Eq. 2 (see text). Points are means from measurements on confluent monolayers from four separate passages. Numbers with lines indicate ouabain concentration in micromolars. Binding is expressed as the number of molecules $\times 10^6$ /cell.

TABLE I

Kinetic Parameters for Ouabain Binding to LLC-PK₁ Cells *

Constant	Parameter
E_T	2.92×10^6 molecules/cell
K_m	5.94×10^{-6} M
K_1	3.77×10^3 /(M \times min)
K_2	2.24×10^{-2} /min

* Values obtained using data from Fig. 1 and solving for Eq. 2 and 3 (see text).

This concentration was chosen because it gave adequate binding at short time intervals and the rate of uptake was relatively linear over the course of 60 min.

To ensure accurate measurement of bound label, we constructed a detailed washout curve for both [³H]ouabain and [¹⁴C]inulin to determine the optimal time for clearing unbound label from the extracellular space. As shown in Fig. 1 the washout of ouabain, at 4°C, is essentially complete in 5 min. Continued washing in ouabain-free Ringer's for up to 15 min had little effect on the amount of label remaining in the tissue. In contrast, [¹⁴C]inulin was lost throughout the wash period. On the basis of these observations, for each experimental protocol we conducted a 10-min wash before dissolving the cells for scintillation counting.

To determine the amount of ouabain bound in the absence of Na⁺, we exposed cells to 2 μM ouabain in Ringer's solution in which all the Na⁺ was replaced by choline. Before exposure to ouabain, the cells were washed repeatedly over a 2-h period with the choline-Ringer's. The results (Table II) show that after 30 and 60 min of exposure to ouabain the amount bound without Na⁺ is 11% of that bound with 150 mM Na⁺-Ringer's. Substitution of choline for Na⁺ had not destroyed cellular ouabain-binding sites for returning the cells to Na⁺-Ringer's after 3 h in choline-Ringer's resulted in a return to normal binding levels (Table II). That the value at 30 min was similar to the value at 60 min in cells not depleted of Na⁺ indicates that the return of medium Na⁺ appears to stimulate ouabain binding. The value of 110,000 sites per cell in the Na⁺-free medium, after a 60-min exposure, could represent ouabain bound to "specific" sites (e.g., the Na⁺ pump) or could result from nonspecific binding. To determine the magnitude of nonspecific binding, we conducted two standard tests for determining the specificity. The first was to raise the K⁺ concentration in the Ringer's from 5 to 10 mM. This had no effect on binding in the choline-Ringer's. The second was to preincubate the cells in 10⁻³ M unlabeled ouabain for 30 min and then to expose them to [³H]ouabain for 30 and 60 min. Under these conditions, any counts per minute are considered to be nonspecific and this amount of binding can then be subtracted from the total counts per minute bound under normal conditions (18). The final adjusted values are shown in Table III. In both Na⁺ and choline-Ringer's the amount bound was decreased only slightly. Thus, there is little nonspecific binding of ouabain to cells incubated in Na⁺-Ringer's.

The choline-Ringer's contained 5 mM K⁺ and, because K⁺ is known to reduce the rate of ouabain binding (2, 20), the large reduction in ouabain binding in the absence of Na⁺ (Tables II and III) could have resulted from the receptor being very sensitive to K⁺ in the absence of medium Na⁺. However, removal of K⁺ from the choline-media had no effect on binding (Table IV). Therefore, the low binding in Na⁺-free Ringer's cannot be attributed to an inhibitory effect of medium K⁺ on binding.

TABLE II
Ouabain Binding to LLC-PK₁ Cells: Effect of Na⁺-Ringer's vs. Na⁺-free Choline-Ringer's *

Time min	Na ⁺ -Ringer's	Choline-Ringer's	Choline-Ringer's (3 h) → Na ⁺ - Ringer's
30	0.62 ± 0.03 (11)‡	0.07 ± 0.01 (11)	1.03 ± 0.02 (5)
60	0.94 ± 0.05 (5)	0.11 ± 0.01 (5)	

* Binding expressed as 10⁶ molecules of ouabain/cell.
‡ Number in parentheses is number of dishes.

TABLE III
Binding after Correction for "Nonspecific" Binding in 10⁻³ M Ouabain

Time min	Na ⁺ -Ringer's	Choline-Ringer's
30	0.60	0.05
60	0.92	0.09

TABLE IV
Ouabain Binding to LLC-PK₁ Cells: *
Effect of K⁺ in the Presence and Absence of Na⁺

K ⁺ mM	Choline-Ringer's‡	Na ⁺ -Ringer's‡
0	0.15 ± 0.02	1.06 ± 0.08
5	0.14 ± 0.04	1.06 ± 0.04

* Cells exposed to 2 μM [³H]ouabain for 60 min.
‡ 10⁶ molecules of ouabain/cell, mean ± SEM, n = 9.

It was of interest that after a 60-min incubation there was no difference in binding in Na⁺-Ringer's with or without K⁺ (Table IV). To verify that medium K⁺ did indeed have an effect on the rate of the ouabain-receptor interaction in LLC-PK₁ cells, we examined the effect of K⁺ on binding. Cells were washed with a K⁺-free medium for 2 h and then exposed to 2 μM ouabain in a K⁺-free medium. As shown in Fig. 3, binding in a K⁺-free medium reached an equilibrium value sooner than it did in 5 mM K⁺, but the final value in the two conditions did not differ significantly.

If the absence of medium K⁺ inhibited pump turnover as would be expected for a Na⁺:K⁺ exchange pump, the more rapid binding of ouabain by cells initially depleted of potassium by 120-min incubation in a K⁺-free medium (as compared with controls incubated throughout with a 5 mM K⁺ in the medium) would be inconsistent with the hypothesis that pump sites must turn over before ouabain is bound. This issue was investigated by analyzing cellular K⁺ (Table V) and examining the rate of loss of K⁺ from cells to medium (Fig. 4). Though cells lost a substantial fraction of their K⁺, they still retained ~20% of this ion after a 120-min incubation. Of the remaining K⁺, 38% was lost over the next 30 min by control tissue in K⁺-free medium without ouabain, but 59% was lost after exposure to 2 μM ouabain for 30 min. Thus, ouabain caused a highly significant increase (*P* < 0.001) in the rate of loss of K⁺ under these conditions. This finding from cell analysis is confirmed by the results of medium K⁺. As Fig. 4 illustrates, K⁺ was lost at a constant rate from cells during a 150-min incubation in a K⁺-free medium. The addition of ouabain after 120 min increased the amount of K⁺ in the medium from the control value of 0.042 mol/10⁶ cells to 0.058 mol/10⁶ cells, an increase of 0.016 mol/10⁶ cells, in excellent agreement with the difference in

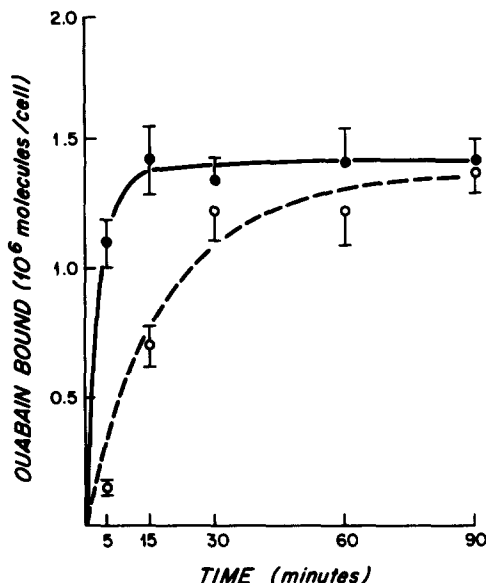


FIGURE 3 [³H]Ouabain binding to LLC-PK₁ cells in 0 or 5 mM K⁺ at 2 μM ouabain. Confluent monolayers were washed in Na⁺-Ringer's containing either 0 or 5 mM K⁺. Then binding assay was begun and dishes were analyzed for binding at times indicated. n = 10. ●, 0 mM K⁺; ○, 5 mM K⁺.

TABLE V
Effects of Ouabain on Loss of K⁺ from LLC-PK₁ Cells
Incubated in a K⁺-free Medium

Protocol	K ⁺ Loss mol K ⁺ /10 ⁶ cells *
Control‡ (5 mM K ⁺)	0.393 ± 0.004
120 min K ⁺ -free	0.092 ± 0.003
150 min K ⁺ -free	0.057 ± 0.001§
150 min K ⁺ -free (2 μM ouabain added after 120 min)	0.038 ± 0.002§

* Values are mean ± SEM, n = 4.

‡ Dishes washed 45 s in ice-cold K⁺-free isosmotic choline chloride solution to remove extracellular K⁺.

§ Δ = 0.019; *P* < 0.001.

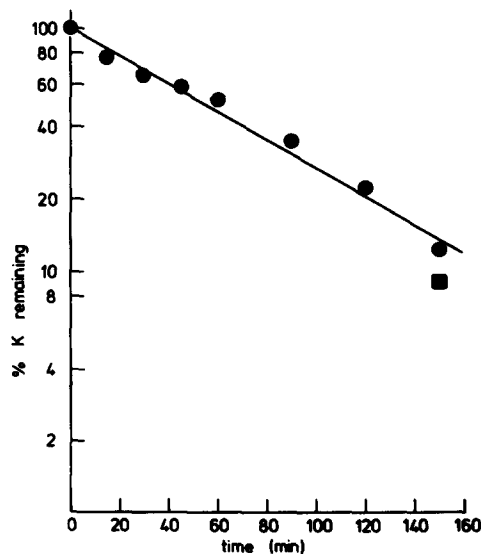


FIGURE 4 The rate of loss of K⁺ from cells to a K⁺-free medium (●). Some dishes were exposed to ouabain (2 μM) at 120 min, and the loss of K from these cells at 150 min is shown by ■.

cellular K^+ under these conditions of $0.019 \text{ mol}/10^6 \text{ cells}$ (Table V). These results clearly demonstrate that ouabain accelerated the loss of K^+ from the tissue and indicate that recycling of K^+ between cells and medium has been inhibited by the glycoside. Thus, even in a K^+ -free medium, sufficient K^+ is available to the transport sites to allow some turnover of the pump to occur. Under these particular conditions, therefore, functioning pump sites would be available for ouabain binding.

A binding experiment was also conducted in media containing various concentrations of Mg^{++} and PO_4^{--} . The osmolarity was maintained by Tris or choline chloride, buffered with choline bicarbonate. Binding in a NaCl, Mg^{++} -free Ringer's was less than in the same Ringer's with $1 \text{ mM } Mg^{++}$ (Table VI). Varying the PO_4^{--} concentration between 2 and 5 mM did not have any effect on the equilibrium value for $2 \mu\text{M}$ ouabain. In Na^+ -, K^+ -free Ringer's containing $1 \text{ mM } Mg^{++}$ and $1 \text{ mM } PO_4^{--}$, the binding was slightly elevated but similar to that obtained in the standard Na^+ -free Ringer's (Tables II-IV).

We also analyzed the effect of temperature on ouabain binding. Lowering the incubation temperature from 37° to 4°C virtually inhibits Na^+ transport and, therefore, pump turnover. As shown in Table VII, exposure of cells to $2 \mu\text{M}$ ouabain for 60 min at 4°C resulted in a dramatic decrease in the amount of ouabain bound as compared with exposure at 37°C . Thus, almost no specific ouabain binding occurs over a 60-min time-course at 4°C . What was surprising was the amount bound after the cooled tissue was rewarmed (Table VII). This value, obtained after only 30 min at 37°C , is equal to the binding value obtained after a 60-min exposure in cells that were not subjected to a cooling period (Table VII). One possible explanation for this discrepancy is that in the cells at 4°C the ouabain had already diffused to the vicinity of the binding site. The higher value at 30 min would then simply reflect an increase in the initial rate of binding through elimination of the lag time for diffusion of ouabain to the basolateral intercellular space. To examine this, we analyzed the time-course of binding in cells first cooled in ouabain-free Na^+ -Ringer's and then returned to a $37^\circ\text{C } Na^+$ -Ringer's containing $2 \mu\text{M } [^3\text{H}]$ -ouabain. This was compared to binding in cells kept at 37°C and then exposed to ouabain in the same manner (Fig. 5). The

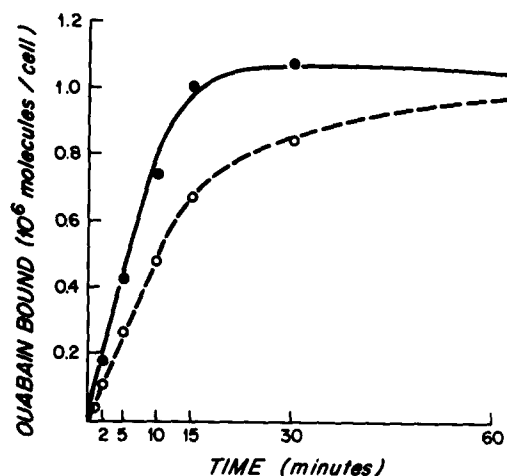


FIGURE 5 $[^3\text{H}]$ Ouabain binding to LLC-PK₁ cells that have been cooled to 4°C and then rewarmed (to 37°C) in the presence of ouabain (●). This is compared with the binding curve obtained in a parallel set of cells that were kept at 37°C throughout the experiment (○). $n = 4$.

binding rate in rewarmed cells was clearly greater. Assuming that ouabain diffused to its site at the same rate in both conditions, these results indicate that cells bind ouabain more rapidly during a recovery from exposure to cold than they do when metabolizing at a constant 37°C .

DISCUSSION

The present results are important for an understanding of results from studies using ouabain as a marker for the Na^+ pump. If significant amounts of ouabain bound to cellular sites nonspecifically or to a cellular membrane pump that was not actively transporting Na^+ , or to a pump operating in other than a Na^+ -transport mode (8), then the interpretation of studies utilizing ouabain to elucidate the number of active pumps, the specific cell localization of the pumps, or the role of this pump in cellular volume regulation would be severely compromised. That in isolated enzyme preparations ouabain can bind to the Na^+ , K^+ -ATPase in the absence of Na^+ has been clearly established (25). In fact, the fastest binding rate is observed in a medium containing only Mg^{++} and inorganic phosphate as the supportive ligands and the maximum binding under these conditions is higher than with ATP and Na^+ . Other ligand conditions, without Na^+ , also support significant levels of binding (25). If ouabain binding to intact cells followed similar patterns, then ouabain binding to cells could be unrelated to its specific inhibitory effects on the Na^+ pump. However, our results clearly show that these other types of ligand-supported bindings do not occur in intact LLC-PK₁ cells. Without Na^+ the amount of ouabain that binds, whether it is considered to be specific for the Na^+ pump or nonspecific, is extremely small.

When Na^+ is in the medium, it can be assumed that the overwhelming majority of pump sites are involved in the transport of Na^+ . Whether or not ouabain binds to a Na^+ pump actually turning over cannot be decided from these studies. Some conclusions can, however, be drawn about the state of the pump (enzyme) in which ouabain binding is optimal. Enzyme preparations do not bind cardiac glycosides when incubated in buffer alone (18). Ligands that favor phosphorylation of the enzyme promote binding ($Na^+ + \text{ATP} + Mg^{++}$; $Mg^{++} + P_i$) and those that reduce the level of phosphoenzyme (K^+) reduce ouabain binding. Thus, it appears that

TABLE VI

Ouabain Binding to LLC-PK₁ Cells Incubated in Various Concentrations of Mg^{++} and PO_4^{--} *

	Na-Ringer's	K^+ -free choline-Ringer's
	mM	
0 Mg	0.62	
1 Mg^{++}	1.27	
1 Mg^{++} , 2 PO_4^{--}	1.30	
1 Mg^{++} , 5 PO_4^{--}	1.22	
1 Mg^{++} , 1 PO_4^{--}		0.18

* Results expressed as 10^6 molecules of ouabain/cell. 60-min exposure.

TABLE VII

Ouabain Binding to LLC-PK₁ Cells at 37°C or 4°C *

Na^+ -Ringer's		
		4°C , 60 min; 37°C , 30 min
37°C , 60 min	4°C , 60 min	
1.09 ± 0.05	0.03 ± 0.01	1.11 ± 0.04

* 10^6 molecules of ouabain/cell, $n = 5$.

phosphorylation is a prerequisite for ouabain binding (10). That medium Na^+ was required for binding and that incubation in Mg^{++} plus inorganic phosphate did not promote significant levels of binding in LLC-PK₁ cells indicates that in such intact cells the predominant interaction between ouabain and the pumps occurs at a site that is only accessible when the pump has been phosphorylated in the presence of Na^+ , and that other phosphorylated states of the pump either do not occur or are not conducive to ouabain-enzyme interaction in intact cells. This would be consistent with the fact that the most rapid rate of binding occurs in a K^+ -free medium. Under these conditions the pump will be phosphorylated because of the presence of Na^+ and intracellular stores of ATP. In fact, binding may be enhanced by a higher cell Na^+ concentration (or greater Na:K ratio), which would tend to stimulate phosphorylation and pump activity (14). Conversion of this phosphorylated form of the enzyme to the dephosphorylated form will be slower because of the very low K^+ . Because ouabain binds to the phosphorylated form of the enzyme, the accumulation of the ouabain-enzyme complex will be faster than when K^+ is present (11).

The results of the washout of K^+ from LLC-PK₁ cells bring up two other important points. First of all, extensive washes over prolonged time periods are necessary to dramatically reduce cellular K^+ . After a 120-min wash in K^+ -free medium, the cells still retain 20% of the original total. The remaining K^+ could be difficult to remove because it is bound or is in an inaccessible cellular compartment. This K^+ would not be expected, necessarily, to be involved in the normal Na:K exchange mechanism of the pump. The results also demonstrate, however, that the measured rate of washout (Fig. 4) must also have as one component the recycling of K^+ that has diffused from the cell and then is pumped back in exchange for Na^+ . This scheme seems necessary because addition of ouabain to the K^+ -free medium accelerates the rate of loss of K^+ from the cells. Thus ouabain blocks the pumps, and the K^+ that diffuses out of the cell is not returned.

Under normal physiological conditions it seems reasonable to conclude, therefore, that ouabain binds to pumps that are involved in Na^+ transport and not to inactive pumps or to nonspecific sites. Thus quantitative measurement of ouabain binding provides a direct measurement of the number of active pumps. Even though the phosphoenzyme may not as yet have "pumped" when binding occurs, the presence of 5 mM K^+ in the extracellular space (or whatever the physiological $[\text{K}^+]$ may be) would guarantee rapid dephosphorylation and thus ion transport, were ouabain not present. These results also strongly suggest that the Na^+ pumps revealed in epithelia by autoradiographic localization of [³H]ouabain binding sites (reviewed in reference 7) are indeed involved in Na^+ transport at the time of labeling and that their position accurately reflects the pathway that Na^+ follows during transepithelial transport.

A possible criticism of this view comes from the data of Cala et al. (4). Using the split-skin preparation, they found that ouabain binding in amiloride-inhibited skins (no net transepithelial transport) occurred at a slower rate but eventually reached the same level as in skins involved in transepithelial transport. However, in this preparation, Na^+ bathes both sides of the skin. Inasmuch as there is some finite permeability of the basolateral membrane to Na^+ , Na^+ recycling between serosa and cell can occur, the pump will turn over (albeit much more slowly than with transepithelial Na^+ transport), and ouabain will bind.

The lack of any ouabain binding to cells at 4°C also bears on the significance of the use of ouabain as a marker for the pumps but, more importantly, relates directly to interpretation of data from studies of cellular volume regulation. Because cooling the tissue alters many cellular processes, the actual mechanism of the inhibition is not precisely identified but the pump is inhibited under these conditions as judged both by inhibition of transepithelial sodium transport and by changes in cellular cations and volume (16), and this inhibition is in agreement with the observed lack of binding. In some studies of cellular volume regulation, cells were swollen by incubation in the cold (15, 26), and it has been argued that the presence of ouabain in the media under these conditions will inhibit the Na^+ - K^+ pump and thereby prevent its activity when cells are rewarmed and metabolism restored. However, Shaver and Stirling (22) clearly established that, in kidney slices, [³H]-ouabain binding was negligible when the incubation temperature was 4°C. In confirmation, our data demonstrate that when the problems of diffusion and accessibility of sites, inherent in tissue slices, are overcome by use of monolayer cell cultures, the pump still does not bind ouabain at 4°C. Binding does not commence until the tissue is rewarmed. Thus, the pumps will initially turn over and thereby extrude Na^+ that has accumulated during the swelling phase at the low temperature. Indeed, the binding rate was faster in rewarmed cultures of pig kidney cells, possibly as a consequence of stimulation of pump turnover resulting from the higher intracellular Na^+ . Even so, it took 7 min for half the pump sites to be bound (Fig. 5). Recovery of volume in kidney slices after swelling in the cold requires <5 min (16). Therefore, at a time when volume recovery is complete in kidney slices, half the pump sites are still functional in the LLC-PK₁ cells exposed to 2 μM ouabain. Even if the ouabain concentration is raised 25-fold, half-maximal binding in metabolizing kidney slices takes 10 min (22). Thus, results from studies that have attempted to use ouabain to eliminate the contribution of the conventional Na^+ pump to volume recovery must be reevaluated. Similarly, interpretation of results from experiments in isolated epithelia exposed to ouabain in the absence of transepithelial sodium transport may be more complicated than has been appreciated (6).

In summary, the results of the present experiments demonstrate that the binding of ouabain to cultured pig kidney epithelial cells requires conditions that favor Na^+ transport, and it is concluded that for ouabain to bind to the cells the Na^+ pump must be functioning in the Na^+ -transport mode.

We gratefully acknowledge the technical assistance of Lorna Brass and Quincy Moy.

This research was supported by National Institutes of Health grants HL-06664, AM-19406, AM-00342, AM-27064, and AM-03564. A. D. C. Macknight was supported in part by the Medical Research Council of New Zealand.

Received for publication 29 May 1980, and in revised form 6 November 1980.

REFERENCES

1. Almendares, J. A., and A. Kleinzeller. 1971. The ouabain inhibition of sugar transport in kidney cortex cells. *Arch. Biochem. Biophys.* 145:511-519.
2. Baker, P. F., and J. S. Willis. 1972. Binding of the cardiac glycoside ouabain to intact cells. *J. Physiol. (Lond.)* 224:441-462.
3. Boardman, L., M. Huett, J. F. Lamb, J. Q. Newton, and J. M. Polson. 1974. Evidence for the genetic control of the sodium pump density in HeLa cells. *J. Physiol. (Lond.)* 241: 771-794.
4. Cala, P. M., N. Cogswell, and L. J. Mandel. 1978. Binding of [³H]ouabain to split frog skin. The role of the Na^+ , K^+ , ATPase in the generation of short circuit current. *J. Gen. Physiol.* 71:347-368.

5. Dunham, P. B., and R. B. Gunn. 1972. Adenosine triphosphatase and active cation transport in red blood cell membranes. *Arch. Intern. Med.* 129:241-247.
6. Eriij, D., and M. W. Smith. 1973. Sodium uptake by frog skin and its modification by inhibitors of transepithelial sodium transport. *J. Physiol. (Lond.)*, 288:221-239.
7. Ernst, S. A., and J. W. Mills. 1980. Autoradiographic localization of tritiated ouabain-sensitive sodium pump sites in ion transporting epithelia. *J. Histochem. Cytochem.* 28:72-77.
8. Glynn, I. M., and S. J. D. Karlish. 1975. The sodium pump. *Annu. Rev. Physiol.* 37:13-55.
9. Grinstead, S., and D. Eriij. 1974. Insulin unmasks latent sodium pump sites in frog muscle. *Nature (Lond.)*, 251:57-58.
10. Hansen, O. 1977. Ouabain used as a tool for trapping and characterizing phosphorylation products of Na-K-ATPase. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 45(A4):191-197.
11. Hobbs, A. S., and P. B. Dunham. 1978. Interaction of external alkali metal ions with the Na-K pump of human erythrocytes. *J. Gen. Physiol.* 72:381-402.
12. Hoffman, J. F. 1966. The red cell membrane and the transport of Na⁺ and K⁺. *Am. J. Med.* 41:666-673.
13. Hull, R. N., W. R. Cherry, and G. W. Weaver. 1976. The origin and characteristics of a pig kidney cell strain; LLC-PK. *In Vitro (Rockville)*, 12:670-677.
14. Joiner, C. H., and P. K. Lauf. 1978. Modulation of ouabain binding and potassium pump fluxes by cellular sodium and potassium in human sheep erythrocytes. *J. Physiol. (Lond.)*, 283:177-196.
15. Macknight, A. D. C. 1968. Water and electrolyte contents of rat renal cortical slices incubated in potassium-free media and media containing ouabain. *Biochim. Biophys. Acta.* 150:263-270.
16. Macknight, A. D. C., and A. Leaf. 1977. Regulation of cellular volume. *Physiol. Rev.* 57: 510-573.
17. Marquardt, D. W. 1963. An algorithm for least-squares estimation of nonlinear parameters. *J. Soc. Indust. Appl. Math.* 11:431-441.
18. Matsui, H. and A. Schwartz. 1968. Mechanism of cardiac glycoside inhibition of the Na⁺,K⁺-dependent ATPase from cardiac tissue. *Biochim. Biophys. Acta.* 151:655-663.
19. Mills, J. W., J. M. Dayer, A. D. C. Macknight, and D. A. Ausiello. 1979. Localization of ³H-ouabain sensitive Na⁺-pump sites in cultured pig kidney epithelial cells (LLC-PK₁). *Am. J. Physiol.* 236:C157-C162.
20. Mills, J. W., S. A. Ernst, and D. R. DiBona. 1977. Localization of Na⁺-pump sites in the frog skin. *J. Cell Biol.* 75:88-110.
21. Schwartz, A., G. E. Lindenmayer, and J. C. Allen. 1975. The sodium-potassium adenosine triphosphatase: pharmacological, physiological and biochemical aspects. *Pharmacol. Rev.* 27:3-124.
22. Shaver, J. L. F., and C. Stirling. 1978. Ouabain binding to renal tubules of the rabbit. *J. Cell Biol.* 76:278-298.
23. Stirling, C. E. 1972. Radioautographic localization of sodium pump sites in rabbit intestine. *J. Cell Biol.* 53:704-714.
24. Stoner, L. C., M. B. Burg, and J. Orloff. 1974. Ion transport in cortical collecting tubules: effect of amiloride. *Am. J. Physiol.* 227:453-459.
25. Wallich, E. T., G. E. Lindenmayer, L. K. Lane, J. C. Allen, B. J. R. Pitts, and A. Schwartz. 1977. Recent advances in cardiac glycoside-Na⁺-K⁺ ATPase interaction. *Fed. Proc.* 36: 2214-2218.
26. Whittembury, G., and F. Proverbio. 1970. Two models of Na extrusion in cells from guinea pig kidney cortex slices. *Pfluegers Arch. Eur. J. Physiol.* 316:1-25.