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MODULATION OF OUABAIN BINDING AND POTASSIUM PUMP FLUXES BY CELLULAR SODIUM AND POTASSIUM IN HUMAN AND SHEEP ERYTHROCYTES

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SUMMARY

1. Erythrocytes were treated with nystatin to alter internal Na (Na_i) and K (K_i) composition. Although the rates of K pumping and [^{3}H]ouabain binding were altered dramatically, the relationship between glycoside binding and K pump inhibition was unaffected.

2. Human cells with high Na_i and low K_i exhibited an increased rate of ouabain binding as compared to high K_i, low Na_i cells; this paralleled the stimulated K pump activity of high Na_i cells.

3. At constant K_i, increasing internal Na stimulated K pump and ouabain binding rates concomitantly.

4. At low Na_i, increasing K_i inhibited both K pumping and ouabain binding. However, at high Na_i, increasing K_i from 4 to 44 mM stimulated the rate of glycoside binding, parallel to its effect of increasing the rate of active K influx.

5. Anti-L, an isoantibody to low K (LK) sheep red cells, increased the rate of ouabain binding via its stimulation of K pump turnover. Since the latter effect is the result of affinity changes at the internal cation activation site(s) of the pump (Lauf, Rasmusen, Hoffman, Dunham, Cook, Parmelee & Tosteson, 1970), the antibody's effect on ouabain binding reflected the positive correlation between the rates of K pump turnover and glycoside binding.

6. These data provide the first evidence in intact cells for the occurrence of a Na_i-induced conformational change in the Na/K pump during its normal operational cycle.

INTRODUCTION

Early investigations of the interaction of red cells with cardiac glycosides revealed a connexion between the development of transport inhibition and the ionic constituents of the bathing medium (Glynn, 1957; 1964; Schatzmann, 1965; Hoffman, 1966). The effects of external Na and K on the rate of [^{3}H]ouabain binding have been shown to be related to the ability of these ions to inhibit or stimulate active K influx. External K depresses the rate of binding (Hoffman, 1966; Baker & Willis, 1972; Sachs, 1974) with a concentration dependence similar to its activation of the K pump (Sachs, 1967; Garrahan & Glynn, 1967*a*, *b*). External Na, in the presence of K, stimulates glycoside binding (Hoffman, 1966; Beauge & Adragna, 1971; Baker & Willis, 1972). This effect of sodium is believed to be due to its competition with K

for external activation site for the pump (Baker & Willis, 1972; Beauge & Adragna, 1971; Sachs, 1974), although a separate site for sodium activation of glycoside binding may exist (Gardner & Frantz, 1974; Hobbs & Dunham, 1975).

These effects of external cations on [^{3}H]ouabain binding to intact cells are basically consistent with a large body of data on the cation modulation of glycoside binding to microsomal (Na+K) ATPase preparations. In the presence of Mg and ATP, Na stimulates the rate and increases the level of glycoside binding; the addition of K to the system retards binding (Matsui & Schwartz, 1968; Albers, Koval & Siegel, 1968; Sen, Tobin & Post, 1969; Skou, Butler & Hansen, 1971; Schönfeld, Schon, Menke & Repke, 1972). Under these circumstances, ouabain binding appears to be associated with the phosphoprotein which is formed in the presence of Mg^{2+}, ATP, and Na and dephosphorylated by K^+ (Post, Sen & Rosenthal, 1965; Albers *et al.* 1968). Corroborating this concept are reports that ATP analogues not phosphorylating the enzyme do not support glycoside binding (Erdmann & Schoner, 1973; Tobin, Akera & Brody, 1974; Schönfeld *et al.* 1972), although there are contradictory findings (Hoffman, 1969; Schönfeld *et al.* 1972). There is general consensus that the dependence of glycoside binding on ionic conditions is a reflexion of the fact that the (Na+K) ATPase undergoes ion dependent changes in conformation during its catalytic cycle which are related in some way to the translocation of ions, and that the cardiac glycoside receptor is associated to one (or more) of these conformational states (Sen *et al.* 1969; Allen, Lindenmayer & Schwartz, 1970; Schönfeld *et al.* 1972; Schwartz, Lindenmayer & Allen, 1975).

Despite the wealth of data concerning [^{3}H]ouabain binding to microsomal and 'purified' (Na+K) ATPase preparations, such investigations lack the asymmetric ionic environment basic to the operation of the Na/K transport system. Accordingly, the membrane orientation (sidedness) of the conformation changes reflected in the ouabain binding reaction cannot be explored in such preparations, except by indirect methods. The only study to date of the effects of internal Na and K on ouabain binding to erythrocytes is that of Bodemann & Hoffman (1976*a*, *b*, *c*). However, that investigation employed resealed ghost membranes and very low concentrations of internal Na and K, so that the extrapolation to physiologic conditions is somewhat complicated.

Here we report studies of [^{3}H]ouabain to intact human and sheep erythrocytes in which cation contents were altered by treatment with the polyene antibiotic nystatin. Changes in the rate of ouabain binding brought about by internal cation modulation were related to K pump turnover by parallel measurement of active K influx in these cells, which were normal with respect to water, haemoglobin, and ATP contents. The increased rate of ouabain binding to LK sheep red cells produced by anti-L was shown to be secondary to the increase in K pump turnover mediated by the antibody induced affinity change of the internal cation activation sites of the transport system (Lauf *et al.* 1970).

A preliminary report of these findings has been published (Joiner & Lauf, 1977).

METHODS

The general aspects of media preparation, handling of cells, as well as the measurement of [^{3}H]ouabain binding and unidirectional K influx have been described in the preceeding paper (Joiner & Lauf, 1978). When tetra methyl ammonium chloride (TMA Cl) (Eastman Kodak Co., Rochester, N.Y.), was used to replace Na or K in media, the hygroscopic salt was dried under vacuum and stored in a dessicator to allow accurate weighing; a 166 mM solution of TMA Cl was determined to be isosmotic.

Cellular water contents

The volume fraction of water (F_{W}^{V}) of cells was calculated from wet and dry weights of cell suspensions of known haematocrit and specific gravity according to the relation published by Cook (1967). It was found that water contents of nystatin treated cells could be accurately predicted from their mean corpuscular haemoglobin concentration (MCHC), using an equation described by Garay & Garrahan (1973). The later method was employed in most of the experiments presented here, since it avoided the cumbersome and time consuming wet and dry weight determinations.

Alteration of cellular cations by nystatin treatment

Intracellular cation contents were altered by treatment of cells with the polyene antibiotic nystatin (Mycostatin: E. R. Squibb and Sons, New York), using a modification of the method described by Cass & Dalmark (1973). The procedures for nystatin treatment of human and sheep cells differed with respect to ionic conditions, but were similar in their general aspects. Nystatin was added to the various treatment media as a 20 mg/ml. solution in dimethyl sulphoxide (DMSO) to yield a final nystatin concentration of 30–80 μg/ml. The flasks were mixed well and cooled to 0 °C in a rotatory shaker bath. Cells washed thrice in 166 mM-TMA Cl were adjusted to 50 % suspension and added with swirling to the nystatin containing media to give a final haemotocrit of 2 %. The incubation proceeded with shaking for 30–40 min; cell volumes, as monitored by MCHC determinations, were stabilized after 10 min. There was no detectable haemolysis. A flask of control cells containing DMSO without nystatin was similarly prepared.

For washing, cells were spun and washed seven times at room temperature with treatment media containing BSA, 50 mg/100 ml., which had been added to the media as a 12 g/100 ml. solution dialysed against deionized water. After each centrifugation, medium was added to the tubes up to the brim to assure removal of any traces of drug from the sides of the tube; only then was the wash supernatant aspirated, taking care to remove all droplets adhering to the sides of the tube. Although the first two washes produced some haemolysis (1–2 %), the cells were subsequently stable and exhibited virtually no haemolysis. After washing in treatment medium, the cells were washed three times in the incubation medium to be used in the subsequent experiment. The water contents of the treated and washed cells were estimated by measurement of MCHC, as described above. Several samples taken during the incubation with ^{42}K or [^{3}H]ouabain demonstrated the stability of cell volumes and allowed the calculation of cation concentrations in m-mole/l. cell water.

In experiments with human cells, when Na and K were not altered reciprocally, TMA Cl was used to maintain intracellular ionic strength. TMA was chosen since it has been shown not to compete with Na or K at the outer aspect of the cation pump (Sachs, 1967) and not to be different from choline at the inner aspect (Knight & Welt, 1974). Since nystatin-treated cells were considerably less permeable to TMA than to Na or K (though more so than to choline), hypertonic solutions were used to increase the driving force for TMA entry. This resulted in cells with water contents closer to normal (after washing and resuspension in isotonic incubation medium) than those treated in isotonic TMA solutions. Under these circumstances, TMA also functioned as an 'impermeant' species to prevent colloid osmotic swelling of the cells, so that the 27 mM-sucrose used by Cass & Dalmark (1973) for this purpose could be omitted from the media. For these experiments the media for nystatin treatment consisted of (mM):

NaCl: X
KCl: Y
TMA Cl: 190–X–Y
Glucose: 10.

Nystatin was present at 80 μg/ml.; lower concentrations of the drug did not allow the entry of TMA and higher concentrations produced unimpeded cell swelling and lysis.

Table 1 lists water contents, cations, and K influx measurements for human cells treated with nystatin at both 30 and 80 μg/ml. concentrations. After washing, the nystatin treated cells were close to normal volume, judged by water contents. In general, passive K permeabilities, reflected in the rate coefficients for ouabain-insensitive K uptake (${}^{i}k_{K}^{L}$), were slightly higher than normal, though not to the extent that cells lost or gained significant amounts of cation over the incubation period or that the estimation of active K influx was impaired.

In several experiments, human cells were treated with nystatin in K-free media, washed and then suspended in media containing 5 mM-K plus 10^{-4} M-ouabain. After 1 hr at 37 °C, these cells had $[K]_i$ of 1·5–2·0 m-mole/l. cell H_2O, indicating that nystatin affected all of the cells in the population, leaving so significant portion with high intracellular K.

TABLE 1. Water and cation contents and K influx in human red cells after nystatin treatment. Control values at the top of the table are the means of eight separate experiments; numbers in parentheses represent the range of the determinations. Water contents were estimated from MCHC, except for control cells, where 0·70 was used since this represented the average from other determinations. The first row of figures listed for experiment D108 represents control cells which were handled the same as nystatin treated cells, except that DMSO without nystatin was added to a 2 % suspension, followed by all of the washing steps. Cation concentrations (mM) throughout this report are given as m-mole/l. cell water

	F_W^V l. water / l. cells	$[K]_i$ (mM)	$[Na]_i$ (mM)	${}^{i}M_K^P$ m-mole / l. orig. cells	${}^{i}k_K^L$ (hr^{-1})
Control cells	(0·70)	(130–145)	(6·0–17·2)	1·48 (1·23–1·85)	0·079 (0·038–0·101)
D125	0·707	4	156	2·88	0·077
nystatin 30 μg/ml.	0·717	153	4	0·572	0·080
D108	0·702	143 (control)	6	0·900	0·055
nystatin	0·653	90	4	0·969	0·070
80 μg/ml.	0·658	90	6	1·53	0·069
	0·674	86	13	2·97	0·082
	0·688	86	26	3·77	0·072

The experiments with sheep cells were designed to avoid replacement of Na and/or K with TMA, since the greater osmotic fragility of these cells (Lauf & Dessent, 1973) as compared to human erythrocytes made the problems of the control of cell volume all the more troublesome. Thus Na and K were varied reciprocally, with their sum equalling 150 mM. Nystatin was present in final concentrations of 30 μg/ml., and 50 mM-$MgSO_4$ (60 m-osmole) was included as an impermeant species, since it was discovered that this compound was more effective in preventing the osmotic swelling of sheep cells during nystatin treatment than sucrose. Cells treated under these conditions exhibited little haemolysis during treatment, had virtually normal volumes after washing, and could be restored to nearly normal cation permeabilities as shown in Table 2.

ATP was measured in nystatin treated LK sheep red cells according to the method of Lowry, Passonneau, Hasselberger & Schulz (1964). Samples were taken after a nystatin treatment and incubation which simulated the conditions of the K influx and [^{3}H]ouabain binding experiments. Table 3 shows that control cells had only slightly lower ATP concentrations than cells stored in the cold for a similar period. Cells made high in K_i had similar levels of nucleotide. Cells with high Na_i showed a drop in ATP content, presumably due to their high rate of K pumping. Nevertheless, this small decrease would not be expected to impede the operation of the Na/K pump.

TABLE 2. Water and cation contents and active and passive K influx in LK sheep red cells after nystatin treatment. Control cells were taken from five separate experiments, involving only brief washing in incubation media before ^{42}K exposure. Water content F_W^V was estimated from MCHC. Anti-L sera were S-39 for control cells and S-44 for nystatin treated cells

	F_W^V (l. water / l. cell)	$[K]_i$ (mM)	$[Na]_i$ (mM)	$^iM_K^P$ (m-mole / l.orig. cells)		$^ik_K^L$ (hr^{-1})	
				Control	Anti-L	Control	Anti-L
Control cells	(0·70)	(17–25)	(125–140)	0·125	0·203	0·033	0·030
				0·133	0·241	0·039	0·033
				0·176	0·345	0·022	0·018
				0·149	0·686	0·047	0·015
				0·223	0·678	0·029	0·019
D 124	0·666	2	155	0·540	1·12	0·02	0·058
nystatin	0·671	6	150	0·439	1·44	0·091	0·048
30 μg/ml.	0·663	32	137	0·168	0·886	0·062	0·039
	0·662	150	10	0·005	−0·002	0·043	0·038
D 122	0·660	1·5	165	0·438	1·09	0·071	0·030
nystatin	0·660	25	125	0·110	0·938	0·041	0·025
30 μg/ml.	0·647	165	10	0·007	0·081	0·054	0·024

TABLE 3. ATP contents of nystatin-treated LK sheep erythrocytes. Cells were treated with nystatin (30 μg/ml.) as described in the text, washed and suspended in a medium containing (mM): 100 NaCl, 5 KCl, 50 TMA Cl, 10 glucose, and 10 imidazole HCl, pH 7·4 at 37 °C. Cation samples were taken after warming to 37 °C ($t = 0$) and after 60 min incubation. Control cells were incubated and washed as nystatin-treated cells, except that the drug was absent. Cold stored cells were kept at 4 °C in their serum during the treatment period and were assayed for ATP along with the other samples. ATP measurements were made at the end of 60 min. Water contents (F_W^V) were estimated from MCHC, measured after 30 min incubation; control cells had MCHC = 0·335 kgHb/l. cells and were assumed to have F_W^V of 0·70

Sample	$[K_i]$ (mM)		$[Na]_i$ (mM)		F_W^V	ATP (mM)
	$t = 0$	$t = 60$	$t = 0$	$t = 60$		
Cold stored cells	—	—	—	—	0·683	1·22
High Na_i	1·13	2·02	138	138	0·653	0·61
High K_i	124	123	3·9	5·2	0·660	1·03
Control	15·3	15·0	125	128	(0·70)	0·93

General experimental design

The experiments described in this paper were designed to relate the alterations in Na/K pump activity mediated by variations in internal cation composition to the concurrent changes produced in the rate of [^{3}H]ouabain binding to the cells. To this end, cells were treated with nystatin as described above, washed and divided into three aliquots. To one suspension, [^{3}H]-ouabain was added and samples taken at several intervals to yield the time course of glycoside binding. To the other two aliquots, ^{42}K was added with and without 10^{-4} M unlabelled ouabain. Samples were taken at two timed intervals over 45 min and counted for radioactivity. From the uptake of isotope in these two suspensions, the rate of active K influx could be calculated, as described in the preceding paper. No back flux correction was necessary for these short incubation periods (Joiner & Lauf, 1978).

Attempts to define rigorous second order or pseudo-first order association rate coefficients for both human and sheep red cells met with failure. Kinetic plots of binding data yielded such

curved lines that it was unreasonable to draw straight lines to define rate constants. Thus in order to evaluate the effects of alterations in K pump rate on the ouabain/receptor interaction, direct observations of the rates of [^{3}H]ouabain binding were relied upon throughout this work. Changes in the rate of binding were accordingly assumed to reflect changes in the receptor 'affinity' for glycoside.

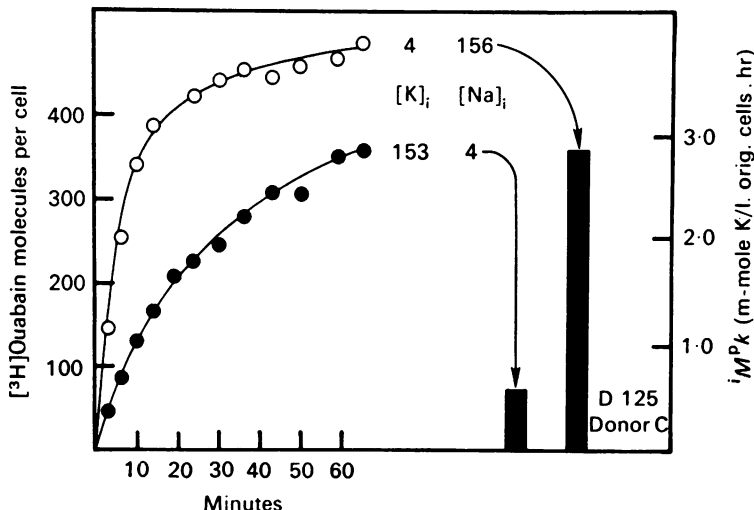

Fig. 1. Rates of [^{3}H]ouabain binding and active K influx in human erythrocytes with altered internal cation composition. Cells were treated with nystatin (30 μg/ml.) in a medium containing (mM): (Na or K) Cl, 155; $MgSO_4$, 25; glucose, 10. After washing six times in the same medium, without nystatin but with BSA, 50 mg/100 ml. cells were washed three times and suspended in a solution containing (mM): NaCl, 100; TMA Cl, 50; glucose, 10; imidazole HCl, 10, pH 7·4 at 37 °C. After warming to 37 °C, both batches of cells were divided into three lots. To the first lots, ^{42}K and [^{3}H]ouabain (4×10^{-7} M, final) were added; the second lots received ^{42}K alone and the third ^{42}K and unlabelled ouabain to give 10^{-4} M. The final K_o was 2 mM, which is at least twice the K_m for external K activation in the presence of $[Na]_o$ of 100 mM. The graph depicts [^{3}H]ouabain binding from single samples taken at the indicated time points. The histograms represent active K influx calculated from duplicate samples at three time points from the second and third lots of cells (see Methods for more details). Values for K_i and Na_i are averages of determinations from each of the three lots and are expressed as m-mol/l. cell water. Water contents were estimated for each batch of cells by measurement of MCHC on a fourth lot of cells at the end of the incubation; F^V_W was 0·71 for high Na cells and 0·72 for high K cells.

RESULTS

Internal cation alterations in human red cells

In order that the practical limits of maximal and minimal K pump rates be achieved, human red cells were treated with nystatin to obtain very high Na_i and low K_i or low Na_i and high K_i. [^{3}H]Ouabain binding and active K influx were measured in parallel under identical external conditions and are shown in Fig. 1. The histograms demonstrate that the high Na_i, low K_i cells exhibited much greater K pump activity than low Na_i, high K_i cells, as expected from the known effects of internal cations (Sachs, 1967, 1977; Garay & Garrahan, 1973). Associated with the high K pump rate in the high Na_i cells was a dramatically stimulated rate of [^{3}H]-

ouabain binding relative to that of high K_i cells. Thus, *stimulating* K transport from the *inside* of the membrane resulted in an *increased* rate of ouabain binding, unlike the effects of external cations. As mentioned earlier, external cations which activate K pumping depress glycoside binding (Hoffman, 1969; Beauge & Adragna, 1971; Sachs, 1974).

Since the alteration of internal cations represented a significant perturbation of red cells, it was important to ascertain that the correlation between [^{3}H]ouabain binding K pump inhibition was unaffected by these experimental circumstances. Accordingly, ^{42}K influx was measured simultaneously with the [^{3}H]ouabain binding depicted in Fig. 1, to estimate the degrees of K pump inhibition produced by the various amounts of ouabain bound. The results are depicted in Fig. 2. There was virtually no difference

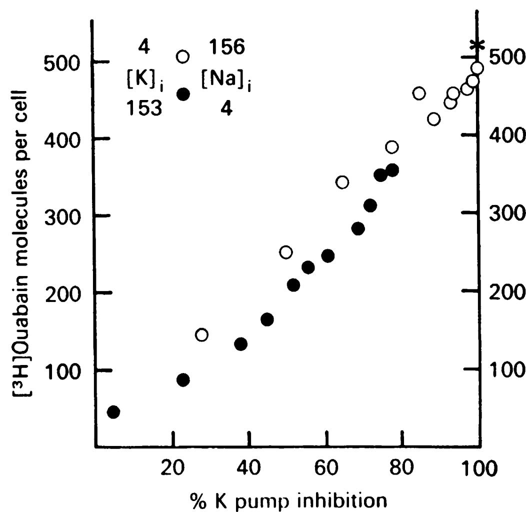

Fig. 2. Correlation of [^{3}H]ouabain binding with K pump inhibition in human erythrocytes with altered internal cation composition. The data are from the experiment described in Fig. 9. The asterisk (*) marks the maximal number of [^{3}H]ouabain molecules binding to untreated cells. The concentrations of internal cations in the treated cells are given in the Figure.

in the number of [^{3}H]ouabain molecules required to produce a given level of K pump inhibition in high Na cells, as compared to high K_i cells, even though the magnitude of the active K influxes of the two cell samples differed by a factor of nine. This means that the differences in K pump activity between high Na_i and high K_i human red cells must be attributed to differences in the turnover rates of the K pumps of the cells, and not to an alteration in the number of functional pump sites. This is an important point if changes in [^{3}H]ouabain binding rate are to be correlated with changes in K pump turnover.

The design of the experiment depicted in Fig. 1 did not permit distinction between individual effects of Na_i and K_i on the rate of [^{3}H]ouabain binding, since the ions were varied reciprocally. Accordingly, a series of experiments was undertaken in which Na_i or K_i was varied while the other remained constant, with TMA Cl maintaining intracellular osmolarity and ionic strength constant. This allowed further dissection of the

relationship between the rates of glycoside binding and K pumping as affected from the inside of the membrane.

The effects of internal Na on K pumping and [^{3}H]ouabain binding were investigated in cells with constant K_i (slightly lower than normal), using TMA to maintain intracellular isotonicity. The histograms of Fig. 3 demonstrate the stimulation of active K influx brought about by increasing internal Na, in agreement with the finding of Sachs (1970), Garay and Garrahan (1973) and Knight & Welt (1974). Concomitant with the changes in K pump rate was a clear *increase* in the rate of [^{3}H]ouabain binding as internal Na was raised.

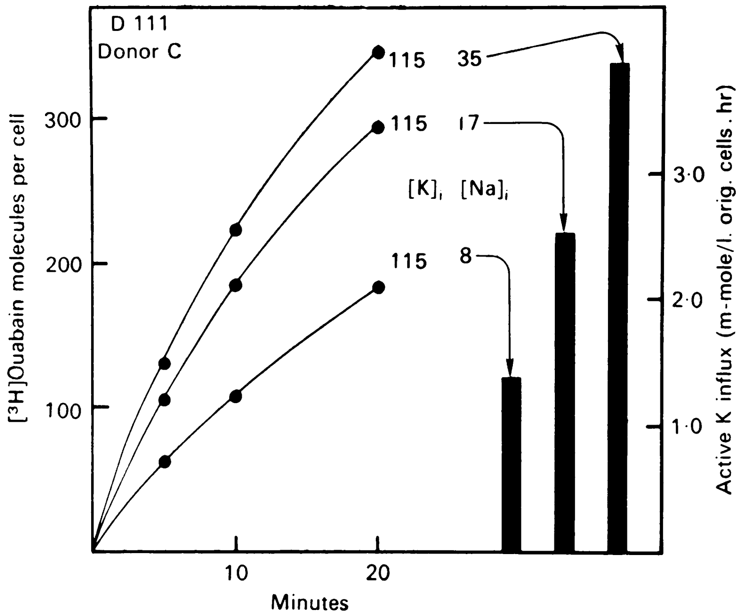

Fig. 3. Effect of internal Na on the rate of [^{3}H]ouabain binding and active K influx in human erythrocytes at constant internal K. Cells were treated with nystatin (80 μg/ml.) in media containing Na, K, and TMA in concentrations which summed to 190 mM (see Methods). After washing, cells were suspended in an isotonic medium containing (mM): NaCl, 40; TMA Cl, 110; glucose, 10; imidazole HCl, 10, pH 7·4 at 37 °C. Each batch of cells was divided into three lots for measurement of [^{3}H]ouabain binding, total K influx, and passive K influx (10^{-4} M unlabelled ouabain). [^{3}H]Ouabain was 5×10^{-7} M, and K_o = 6·5 mM in the tube containing [^{3}H]ouabain; points for [^{3}H]-ouabain binding are the means of duplicate samples. ^{42}K influx was measured in the other two tubes at 8 mM K_o representing saturating concentrations for the external K pump activation site. Internal cation concentrations are given in m-mole/l. cell water and determined as described previously for each batch of cells. F^V_W was 0·65–0·67 v/v. This experiment was representative of four others of similar design.

It will be noted that the effect of Na_i on the glycoside binding rate was not strictly proportional to that on K pumping; that is, increasing Na_i from 17 to 35 mM seemed to stimulate active K influx to a greater extent than it increased the rate of [^{3}H]-ouabain binding. However, the importance of such a discrepancy was minimized by the fact that other parameters (internal and external K, external Na, as well as the particular [^{3}H]ouabain concentration used in a given experiment) imposed limits of

the ability of Na_i to modulate glycoside binding and K pumping. Nevertheless, the effect of Na at the inner face of the membrane of human red cells is clearly shown in Fig. 3. *Increasing* internal Na *increased* the rate of [^{3}H]ouabain binding.

The effects of internal K on Na/K transport in red cells have proved more complex than the 'simple' stimulation by Na_i of the pump mechanism. At low Na_i, K_i appeared to compete for Na at the internal activation site (Garay & Garrahan, 1973), thereby inhibiting Na efflux, apparently by reducing the turnover rate of the pump mechanism, rather than by being ejected from the cell instead of Na_i (Knight & Welt, 1974). However, when the internal activation sites were saturated by high Na_i, internal K stimulated the rate of Na efflux (Garay & Garrahan, 1973; Knight & Welt, 1974). This phenomenon has also been reported for HK goat red cells (Sachs *et al.* 1974*b*), but it is not yet understood at the molecular level. Nevertheless the dual

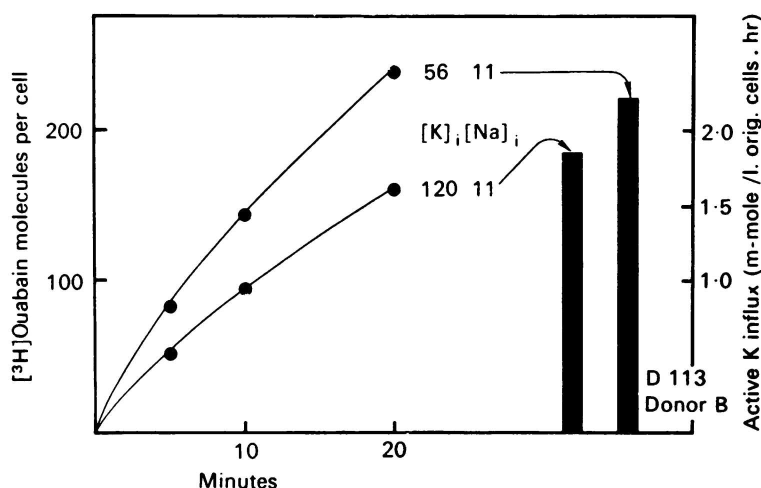

Fig. 4. Effect of high K on the rates of [^{3}H]ouabain binding and active K influx at constant low internal Na. The experimental design was similar to that described for Fig. 3 except that different Na and K concentrations were used for nystatin treatment. [^{3}H]Ouabain was $4{\cdot}2 \times 10^{-7}$ M, with K_o = 8 mM. ^{42}K influx was measured with K_o = 7 mM. [^{3}H]Ouabain binding points are the means of duplicate determinations. F_W^V for these cells was estimated to be 0·66–0·67 v/v.

effect of K_i on Na/K pump activity allowed a rather rigorous test of the direct relationship between K pump turnover and glycoside binding rate which was proposed on the basis of the effect of Na_i on [^{3}H]ouabain binding. This hypothesis predicted dual effects of internal K on the glycoside binding rate to correlate with its effects on Na/K pumping.

The well known inhibitory effect of internal K on K pump activity in human red cells was demonstrated in cells with relatively low Na_i (11 mM) in Fig. 4. The K_i concentrations were relatively high, so that the magnitude of the effect was not maximal. Nevertheless, the decrease in K pump rate observed by raising internal K was clearly associated with a decrease in [^{3}H]ouabain binding rate. This was consistent with the previously described results showing binding rates and K pump turnover rates to be *positively* correlated.

The stimulatory effect of K_i on Na efflux at high internal Na (Knight & Welt,

1974) has been attributed to an increase in the maximal velocity of Na pumping (Garay & Garrahan, 1973). These authors showed that $V_{max.}$ was increased by internal K over the range of 0–40 mM, with no further effects at higher K_i. The cells depicted in Fig. 5 had internal Na greater than 50 mM, above which Na efflux is not augmented by further increase in Na_i even at K_i of 100 mM (Sachs, 1970; Garay & Garrahan, 1973). Thus any effects of K_i on active K influx of these cells must have been on the maximal velocity of Na/K exchange. (There is no reason to expect any significant component of K/K exchange in cells with such high Na_i (Simons, 1974).)

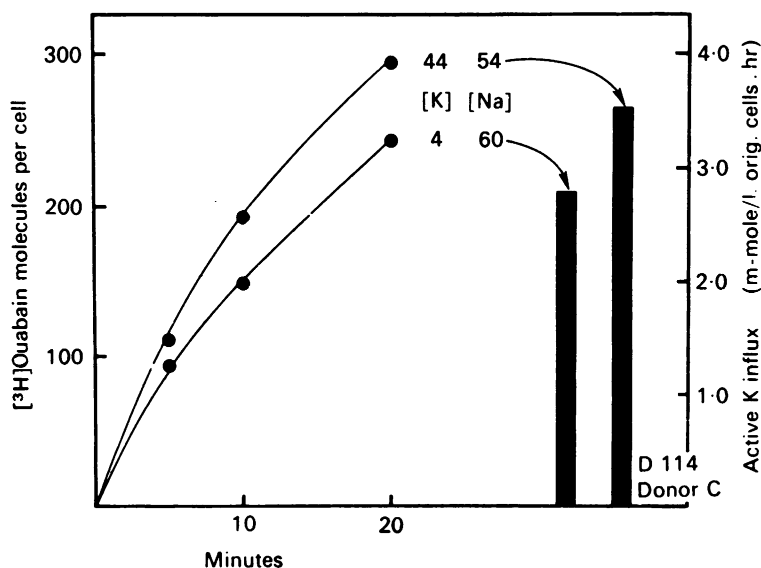

Fig. 5. Effect of low internal K on the rates of [^{3}H]ouabain binding and active K influx at constant high internal Na. The experimental design was similar to those of Figs. 3 and 4. The incubation medium for [^{3}H]ouabain binding and ^{42}K influx contained (mM): NaCl, 50; TMA Cl, 100; glucose, 10; imidazole HCl, 5. K_o was 2 mM for the K influx measurements, 5 mM for [^{3}H]ouabain ($3 \cdot 9 \times 10^{-7}$ M) binding. F^V_W was 0·67. A similar experiment verified these results.

The histograms in Fig. 5 demonstrate a 26 % increase in K pump flux as K_i was raised from 4 to 44 mM at saturating internal Na. Correlated with this stimulation of K pump turnover was a clear increase in the rate at which [^{3}H]ouabain bound to the high K_i cells. Thus, K_i exhibited dual effects on the glycoside binding rate, reflecting its effects on active K influx. This finding strongly suggested that the modulation by K_i of [^{3}H]ouabain binding was, in fact, mediated through the alteration of K pump turnover, rather than through another internal modulation site.

In the present study it is not possible to determine precisely to what extent K/K exchange as opposed to Na/K exchange may have participated in the active K influx measurements in the cells. There are reasons to believe that it was quite low. First, under optimal conditions (red cell ghosts with K_i = 10 mM, Na_i < 1 mM) the maximal rate of K/K exchange was reported to be 0·8 m-mole/l. ghost.hr; internal Na as low as 4 mM virtually abolished this mode of operation of the pump (Simons, 1974). Bodemann & Hoffman (1976*a*) reported that in intact cells, the ouabain-

sensitive component of K efflux (which defines K/K exchange) was less than 8 % of the total K efflux. Knight & Welt (1974) found that even at very low Na_i (about 4·5 mM, at K_i of 120 m-mole/l.cell) K/K exchange was less than 0·5 m-mole/l. cell.hr; the lowest Na_i in the present study was 8 mM, so that K/K exchange would be expected to be even lower.

Regardless of the degree to which K/K exchange contributed to the K influx measurements in this study, the conclusions of the positive relationship between [^{3}H]ouabain binding rate and K pump turnover remain valid. Because K *influx* was measured, changes in the rate of this parameter brought about by *internal* cation alterations reflected changes in the rate at which the K pump apparatus turned over, whether it was extruding Na_i or K_i in exchange for K_o. And at the risk of repetition, if the K pump turnover *increased* by stimulation from the inside of the membrane, the rate of glycoside binding *increased* concomitantly.

Stimulation of [^{3}H]*ouabain binding rate to LK sheep red cells by anti-L*

Early investigations of the effect of anti-L on LK sheep red cells showed an apparent increase in the number of [^{3}H]ouabain binding sites (Lauf *et al.* 1970; Ellory & Tucker, 1970). We have subsequently shown that this is not, in fact, the case; rather, anti-L stimulated the rate at which the existing pumps of the cell turn over (Joiner & Lauf, 1975, 1978). The situation was similar in LK goat red cells, in which anti-L stimulated K pumping two fold, but only increased the number of functional glycoside binding sites from 55 to 70 per cell (Sachs *et al.* 1974*a*). For both sheep and goat red cells, it has been shown that anti-L increased the rate of ouabain binding (Sachs *et al.* 1974*a*; Joiner & Lauf, 1975, 1978). In goat cells this effect was dependent on the presence of K_i (Sachs *et al.* 1974*a*), and thus seemed related to the antibody's effect on internal cation affinities: anti-L increased the affinity of internal activation sites (of both sheep and goat cells) for Na_i relative to K_i, partially relieving the pump's inhibition by K_i and thereby stimulating turnover (Lauf *et al.* 1970; Glynn & Ellory, 1972; Sachs *et al.* 1974*b*).

In light of these findings, we undertook studies to determine if the increased rate of [^{3}H]ouabain binding to anti-L treated cells might be viewed as a manifestation of the control of glycoside binding rate by the turnover of the K pump, as demonstrated for human cells. To test this hypothesis, several experiments were designed to examine the effect of internal cations on K pump activity and [^{3}H]ouabain binding to LK cells in the presence and absence of anti-L.

A typical experiment is depicted in Fig. 6. The procedure consisted of treatment of cells with nystatin to alter internal cations as desired, and then measuring [^{3}H]-ouabain and ^{42}K influx in parallel samples. Thus the rate of [^{3}H]ouabain binding could be correlated with the rate of active K influx obtained for similar cells. As long as the number of pump sites remained unchanged, alterations in K influx, brought about by changing internal cation concentration or by anti-L, reflected changes in the turnover of the K pump sites, and changes in turnover could be examined for their effects on [^{3}H]ouabain binding rate, provided external conditions were constant.

The centre panel of Fig. 6 shows LK cells treated with nystatin, but with nearly normal internal cation concentrations. The increased rate of [^{3}H]ouabain binding to

the antibody treated cells is evident and was associated with the fivefold stimulation of active K influx by anti-L. The lower panel represents cells made very high in K_i and low in Na_i, with the result that ${}^{i}M_{K}^{P}$ both without and with anti-L was almost abolished. In both cases [³H]ouabain binding was concomitantly reduced, indicating that the antibody could not change the apparent affinity of the glycoside receptor for [³H]ouabain independent of K pump stimulation. In addition, the fact that high K_i

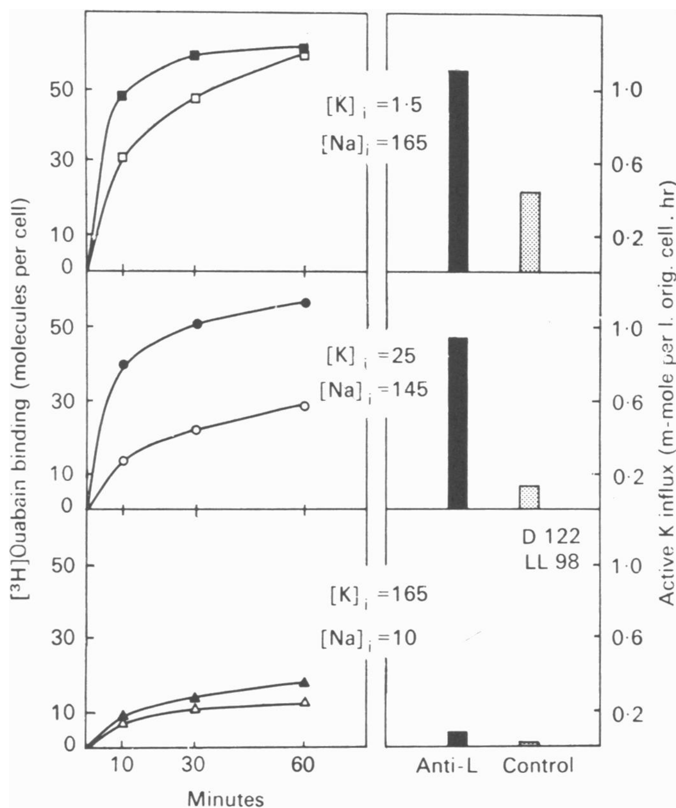

Fig. 6. Effect of alteration of internal cation composition on [³H]ouabain binding and active K influx in LL sheep erythrocytes with and without anti-L. Cells were treated with nystatin (30 μg/ml.) as described in Methods and washed free of the drug. Each batch of cells was divided into two lots and incubated 30 min at 37 °C with or without anti-L (S-44) in a medium containing (mM): NaCl, 100; TMA Cl, 50; glucose, 10; imidazole HCl, 10. After this treatment, each lot was further divided for parallel measurement of [³H]ouabain binding and ⁴²K uptake with and without 10^{-4} M unlabelled ouabain as described for human cells in Fig. 1. [³H]Ouabain was 1×10^{-7} M; KCl was added at the beginning of the binding and flux measurements to give a final concentration of 5 mM in all of the tubes. K_i and Na_i are given as m-mole/l. cell water and are the means of determinations on all of the binding and influx samples, measured as described for Fig. 1. F_{W}^{V} was estimated as 0·65–0·66 v/v.

cells had virtually no active K influx confirmed the finding of Tosteson & Hoffman (1960) that the Na/K transport system of LK (and HK) sheep red cells cannot carry out K/K exchange. This means that the changes in the rate of [³H]ouabain binding

to these cells were related to alterations in K influx via changes in the turnover rate of the pump mechanism and not by a shift in the mode of operation of the pump from Na/K exchange to K/K exchange.

In the upper panel of Fig. 6 cells were made high in Na_i and low in K_i. As expected, active K influx of control cells was increased, along with the rate of [^{3}H]ouabain binding. Additional stimulation of both parameters occurred with anti-L, though not with the relative magnitude of that in cells with normal cation content. This may indicate that the antibody increased the maximal velocity of K pumping. On the other hand, 1·5 mM-K_i may have been sufficient to cause some inhibition of K influx in control cells, so that the affinity change at the internal activation site brought about by anti-L still caused an increase in K influx. Nevertheless, as K_i was increased, the relative effect of anti-L was consistently reduced on both active K influx and [^{3}H]ouabain binding rate. Under no circumstances was there a dissociation between the effects of antibody on the rates of K pumping and [^{3}H]ouabain binding.

The effect of anti-L on [^{3}H]ouabain binding to LK sheep red cells can be summarized as follows. (*a*) Anti-L stimulated the rate of [^{3}H]ouabain binding, (*b*) the number of glycoside receptor sites was unchanged, (*c*) the effect of anti-L could be mimicked (though not completely supplanted) by the stimulation of active K influx by replacement of K_i with Na_i, (*d*) the effect could be eliminated by inhibition of active K influx by high K_i. These facts support the hypothesis that anti-L stimulates active K influx in sheep red cells by reducing the inhibition of the transport system by internal K. This leads to an increased turnover rate of the existing pump sites, which is responsible for the increased rate of [^{3}H]ouabain binding. Thus the effect of anti-L on [^{3}H]ouabain binding to LK sheep red cells supports the general principle proposed for the relationship between glycoside binding rate and K pump turnover in human red cells: when elicited from the inside of the membrane (internal affinity change in the case of the anti-L effect), an *increase* in the K pump turnover results in a *stimulation* of the rate of [^{3}H]ouabain binding.

DISCUSSION

This investigation represents the first attempt to explore the modulation by internal cations of glycoside binding rate in intact erythrocytes. Since the effects of internal alterations on transport were monitored by the *trans* event of K influx, changes in this parameter reflected changes in the turnover rate of the K pump mechanism (see Results). Active K influx was stimulated by several diverse procedures: increasing Na_i at the expense of K_i; increasing Na_i at constant K_i; decreasing K_i at low Na_i; and increasing K_i at high Na_i. In addition, the interaction of anti-L with LK sheep red cells provided another stimulus to K pump turnover (Joiner & Lauf, 1975, 1978) by means of an affinity change of the internal cation activation site(s) (Lauf *et al.* 1970; Glynn & Ellory, 1972). In each case, the stimulation of K pump turnover by internal means led to an increase in the rate of [^{3}H]ouabain binding. Thus the relationship between glycoside binding rate and K pump rate when effected from the inside was opposite to the modulation by external cations, which decreased the binding rate as K pumping was increased (Glynn, 1964; Gardner & Conlon, 1972; Hoffman, 1973; Sachs, 1974).

Our results contrast with those of Bodemann & Hoffman (1976*a*). They proposed that the glycoside binding rate was *inversely* related to K pump turnover, whether modulated by internal or external cations, since they found that increasing internal Na (at very low K_i and in the presence of K_o) reduced the rate of ouabain binding to resealed human red cell ghosts. This important qualitative discrepancy in results may be grounded in the fact that our studies employed intact cells with relatively high K_i as compared to their use of resealed ghosts with low K_i. Unfortunately, there were no common experimental conditions (in terms of internal cation composition) in the two studies from which comparisons may be drawn. It is noteworthy that the two reports did agree on the effect of K_i (at low Na_i) to reduce the rate of ouabain binding to red cells. However, Bodemann & Hoffman (1976*a*) interpreted this action to be the result of the activation of the K/K exchange mode of the pump system. This was consistent with their observed Na_i effect and their proposal that the effects of both internal and external cations on ouabain binding rate were inverse to the cation modulation of K pump rates. Our data with regard to external cations were consistent with the extensive literature on the subject and were therefore omitted from this report. As discussed earlier, these data support the notion of an inverse relationship between ouabain binding and K pump turnover rates, when affected from the *outside* of the membrane. However, the data presented here show clearly that the converse obtained when considering the effects of *internal* cations. We interpreted the effect of K_i to inhibit ouabain binding as arising from its inhibition of active Na/K transport, rather than its activation of K/K exchange, since other workers have shown that this mode of operation of the pump would be expected to contribute insignificantly to active K influx under the experimental conditions employed (Simons, 1974; Knight & Welt, 1974).

Further evidence for the concept of a positive correlation between the rates of glycoside binding and K pumping when modulated internally comes from the experiments with LK sheep red cells treated with anti-L. The effect of the antibody to stimulate active K transport was clearly the result of increased turnover of the existing pump sites of the cells, since there is no increase in the number of functional K pumps (Joiner & Lauf, 1975, 1978). At least part of this stimulation was brought about by a reduction in the sensitivity of the internal cation activation sites of LK cells to inhibition by K_i (Lauf *et al.* 1970; Glynn & Ellory, 1972; Sachs *et al.* 1974*b*), although there may also have been an increase in the maximal velocity of transport in these cells (Joiner & Lauf, 1978). In the present study (Fig. 6) we demonstrated that the antibody stimulation of [^{3}H]ouabain binding rate to LK sheep red cells was strictly related to the antibody's effect on K pump turnover and therefore mediated by internal cation affinity changes. In no instance was there a dissociation between the effects of anti-L on [^{3}H]ouabain binding rate and K pump rate. This eliminated the possibility that anti-L changed the affinity of a static glycoside receptor independently of its stimulation of K transport. Support for this interpretation comes from recently reported experiments on trypsin-treated LK sheep red cells: such cells had normal K pump rates and ouabain binding kinetics, but the stimulatory effects of anti-L on *both* active K influx and [^{3}H]ouabain binding rate were completely abolished (Lauf, Stiehl & Joiner, 1977). Thus the addition of anti-L to LK sheep cells was analogous to increasing internal sodium in human cells. In both cases, the

interaction of the pump with Na_i was augmented, either by an affinity change (LK cells) or higher Na concentration. The net result was the same: K pumping and glycoside binding were increased concomitantly.

The results of this study are consistent with the known characteristics of the Na/K transport system in intact cells as well as the large body of data concerning the effects of cations on glycoside binding to microsomal (Na+K) ATPase preparations. Na has long been known to stimulate Mg-ATP = dependent glycoside binding to microsomal (Na+K) ATPase (Matsui & Schwartz, 1968; Tobin & Sen, 1970; Skou *et al.* 1971; Schönfeld *et al.* 1972). Indirect evidence that Na acting at an internal site (sidedness was inferred from Na affinity) augmented Mg-ATP = supported ouabain binding to purified (Na+K) ATPase comes from the kinetic experiments of Inagaki, Lindenmayer & Schwartz (1974). Recently Clausen & Hansen (1977) have demonstrated in skeletal muscle and adipocytes a positive correlation between [^{3}H]ouabain binding rate and Na/K pump turnover as affected by various pharmacologic agents. The experiments depicted in Fig. 3 of this report provide direct evidence that the rate of ouabain binding to human red cells was stimulated by increasing internal Na, parallel to the well known activation by Na_i of K pumping (Post, Merritt, Kinsolving & Albright, 1960; Sachs, 1970; Hoffman & Tosteson, 1971; Garay & Garrahan, 1973; Knight & Welt, 1974). There are several lines of evidence to suggest that the stimulation by Na_i of both transport and ouabain binding is associated with its role in phosphorylation of the membrane system: internal Na activates the (Na+K) ATPase of erythrocytes (Glynn, 1962); Na is known to augment the phosphorylation of membrane (Na+K) ATPase preparations (Fahn, Koval & Albers, 1968; Post *et al.* 1965; Tobin *et al.* 1974); finally, the ionic conditions which support ouabain binding to microsomal (Na+K) ATPase are associated with the phosphorylation of the enzyme (Sen *et al.* 1969; Lindenmeyer & Schwartz, 1970; Akera & Brody, 1971; Hegyvary, 1976; see also Schwartz *et al.* 1975). The present results provide additional evidence that the interaction of the pump with ATP (whether or not the phospho-enzyme is a 'true intermediate' in the reaction) is a crucial factor in the availability of the glycoside receptor of intact cells.

Within this framework, the effects of K_i on the rate of ouabain binding are easily interpreted in terms of its modulation of the interaction of Na_i with the activation sites of the pump. At low Na_i, K_i competes with Na_i for occupation of the sites, thereby inhibiting activation of the pump mechanism (Garay & Garrahan, 1973; Knight & Welt, 1974; Sachs, 1977). The anti-L effect on ouabain binding to LK sheep cells is also explicable by this mechanism, as mentioned earlier, in that the antibody increases the relative affinity of the activation sites for Na_i. Although the effect of high K_i to increase the $V_{max.}$ of transport in human cells is not well understood (Garay & Garrahan, 1973; Knight & Welt, 1974), the stimulation of glycoside binding by high K_i at saturating Na_i is understandable in the simple terms of increasing the rate of Na_i interaction with the system, whatever the molecular nature of the phenomenon.

The diversity of the ionic conditions under which the tight correlation between the rates of K pump turnover and glycoside binding persisted suggests that a direct association of these two parameters exists. It seems unlikely, for example, that there would be two independent sets of cation sites for the control of pump activation and

glycoside binding, since there is no reason to expect that an independent site modulating glycoside binding would respond in exactly the same way as K pump sites to the $V_{max.}$ effect of high K_i and to alteration of cation affinity by anti-L in LK sheep cells. Rather it is more reasonable to postulate that the glycoside receptor exists or is exposed during a portion of the pump cycle. The concept that the cation modulation of ouabain binding to (Na + K) ATPase preparations reflects various conformational states of the enzyme which occur during its reaction cycle has wide-spread support in the biochemical literature (Albers *et al.* 1968; Sen *et al.* 1969; Allen *et al.* 1970; Schönfeld *et al.* 1972; Repke *et al.* 1974). The general idea has also been invoked to explain the effects of cations on glycoside binding to asymmetric membrane systems (Hoffman, 1973; Bodemann & Hoffman, 1976*a*). The present data are consistent with the known kinetic characteristics of the transport system and the biochemical data on (Na + K) ATPase preparations, and strongly suggest that conformational changes must occur in the normal operation of the Na/K pump.

Such conformational changes were not disclosed by conventional kinetic analysis of intact transporting systems. In the presence of Na and K on both sides of the membrane, the cation affinities of both internal and external activation sites were unaffected by the ionic conditions of the other side of the membrane (Hoffman & Tosteson, 1971; Garay & Garrahan, 1973). This indicated that activation sites were present throughout the pump cycle and did not spend significant time in an occluded conformation (Garrahan & Garay, 1974). The data were consistent only with transport models which postulated that the simultaneous occupation of both internal and external activation sites by Na and K, respectively, was required for pumping to occur (Garrahan & Garay, 1974). Although subsequent studies found that interaction between internal and external sites could be demonstrated in the absence of Na_o and K_i (Whittam & Chipperfield, 1975; Sachs, 1977), they nevertheless confirmed the validity of the simultaneous model of transport and eliminated those models involving a ping-pong mechanism (Sachs, 1977; as pointed out in this reference, the term 'sequential', often used in transport nomenclature to refer to a shuttling carrier mechanism, should be avoided, since it applies, in kinetic usage, to mechanisms identical to the simultaneous model of transport). On the other hand, biochemical studies of microsomal (Na + K) ATPase have suggested a number of discrete conformational changes related to ionic interactions with the enzyme system, such as Na-dependent phosphorylation and K-dependent dephosphorylation. The interpretation of these data has been in terms of reaction sequences involving first Na and then K interactions with the system (Post *et al.* 1965; Fahn *et al.* 1968; Sen *et al.* 1969; Lindenmeyer & Schwartz, 1970), making these models apparently incompatible with the simultaneous model developed from studies with intact cells.

Reconciliation of these apparent discrepancies has begun to emerge from several recent studies. Lindenmeyer *et al.* (1974) have published kinetic experiments with purified (Na + K) ATPase which support the simultaneous existence of Na and K activation sites on the membrane. Sachs (1977) presented kinetic data from transport experiments with human red cells which suggest that Na_i interacts with the pump prior to the interaction with K_o. Finally, the development of flip-flop models for the reaction mechanism of the pump (Repke *et al.* 1974) has allowed the incorporation of the co-existence of reactive sites for Na and K, the formation of a phosphoprotein

intermediate, and a sequence of conformational changes induced by ligands and substrates.

The present report contributes to the integration of the biochemical and physiologic behaviour of the Na/K transport system by demonstrating the existence of a Na_i-induced conformational change in the normal operation of the pump. From this behaviour of the system conclusions about the reaction mechanism of the system may be drawn. The rigid interpretation of the simultaneous model of transport has asserted that transport occurred by a concerted, one-step exchange in the instant that all activation sites were occupied since, as mentioned earlier, kinetic studies demonstrated no affinity changes of activation sites or occluded pump forms (Garrahan & Garay, 1974). However, within the framework of such a pump mechanism it would not be possible to reconcile three fundamental characteristics of ouabain binding: the rate dependence of binding on pump turnover, the stimulation of binding by Na_i and its inhibition by K_o. For binding to be dependent on Na/K pump turnover, it would have to occur *during* the concerted exchange step. This would explain the stimulation by Na_i, but K_o would then be expected to stimulate binding also, since it too would initiate translocation. Thus the inhibitory effect of K_o on ouabain binding could not be explained by such a model. The data therefore require that the conformational change signalled by glycoside binding to the pump complex be initiated by internal Na, and presumably also ATP, *before* the interaction of the system with K_o. This confirms the inference of Sachs (1977) made from kinetic data that Na_i interacts with the pump before K_o. Furthermore, this interpretation is easily incorporated into flip-flop models of Na/K transport (Repke *et al.* 1974), which at present are the most successful at integrating the physiological, biochemical, and structural characteristics of this important biologic system.

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