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Some Characteristics of Na/K-ATPase from Rat Intestinal Basal Lateral Membranes*

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Summary. Basal lateral membrane vesicles were isolated from rat intestinal epithelial cells. The sodium potassium triphosphatase (Na/K-ATPase) of these plasma membranes has been characterized by (1) the molecular weight of the phosphorylated intermediate, (2) the sensitivity of the phosphorylated intermediate to hydroxylamine, (3) its ouabain binding constants, and (4) its susceptibility to digestion by pronase. The phosphorylated intermediate was shown by SDS polyacrylamide gel electrophoresis to be a protein of 100,000 Daltons apparent mol wt. Its extensive hydrolysis in hydroxylamine demonstrated that it was an acyl phosphate. The isolated basal lateral membranes bound ouabain with a dissociation constant, K_m (1.5×10^{-5} M), similar to the inhibitory constant K_I (3×10^{-5} M), measured for ouabain inhibition of the Na/K-ATPase activity. The association rate constant measured for ouabain binding at 22 °C was 1.3×10^3 M^{-1} sec^{-1} and is similar to the association rate constants reported for other tissues and species. The high dissociation rate constant, 3.6×10^{-2} sec^{-1}, is consistent with the insensitivity of the rat to ouabain. Digestion of the intact cells by pronase yielded basal lateral membranes in which the Na/K-ATPase had been unaffected. The phosphorylated intermediate ran as a sharp band at 100,000 Daltons on electrophoresis, and the ouabain dissociation constant appeared to be unchanged. In these membranes, protein stains of polyacrylamide gels revealed digestion of the major high mol wt proteins including the major protein at 100,000 Daltons. This suggests that the Na/K-ATPase represents a minor component, less than 1%, of the basal lateral membrane protein. From these characteristics of the phosphorylated intermediate and the ouabain binding constants, we conclude that the Na/K-ATPase of the basal lateral membranes of rat intestinal epithelial cells is similar to that found in other tissues and species. Estimates of the number of pump sites and the turnover number predict rates of Na transport that are consistent with observed values.

Current models of the active transport of solutes across epithelial cells are based on the premise that there is a polarization of the two faces of the epithelium. In the case of sodium absorption by the intestine, sodium first enters the epithelium across the brush border membrane, down its electrochemical potential gradient from lumen to cell interior by a passive process, and then is pumped out of the cell against an electrochemical gradient across the basal lateral membrane by a sodium/potassium exchange pump. This model of sodium absorption is supported by (i) the distribution of ouabain binding sites and (ii) the distribution of Na/K-ATPase between the isolated basal lateral and brush border membranes. Stirling (1972) found that ^{3}H-ouabain was bound exclusively to the basal lateral membrane of the rabbit intestinal epithelium, and Mircheff and Wright (1976) found that 95% of the total cellular Na/K-ATPase of rat intestinal epithelium was on the basal lateral membrane.

Despite the importance of the sodium/potassium pump (Na/K-ATPase) in the transepithelial transport of sodium and water, and its indirect role in the absorption of sugars and amino acids (Schultz & Curran, 1970), little is known about the properties of pump because of the inaccessibility of the basal lateral membranes to direct study. With the advent of procedures for the isolation of pure intestinal basal lateral membrane vesicles in high yield (Mircheff & Wright, 1976; Mircheff et al., 1979*a*; Mircheff, van Os & Wright, 1980; and Mircheff et al., 1979*b*), we have

* This paper is dedicated to the memory of Professor David H. Smyth, FRS, who died on September 10, 1979.

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undertaken a study to characterize the Na/K-ATPase of the rat intestinal basal lateral membranes. First, we have used gel electrophoresis of ^{32}P labeled basal lateral membranes to obtain the mol wt of the phosphorylated Na/K-ATPase intermediate. Second, we have used ^{3}H-ouabain binding to characterize the ouabain binding site on the basal lateral membrane Na/K-ATPase. Third, we have studied the ouabain sensitivity of the basal lateral Na/K-ATPase. Finally, we have used a pronase digestion procedure to determine the contribution of the Na/K-ATPase to the prominant protein band which comigrates with the phosphorylated intermediate on polyacrylamide gel electrophoresis. We conclude that the properties of the intestinal basal lateral membrane Na/K-ATPase (Na/K-pump) are virtually identical to those in other cells and tissues.

Materials and Methods

Membrane Isolation

Basal lateral membranes were isolated from the jejunum of Wistar rats by the method of Mircheff, van Os, and Wright (1980). Briefly, cells were homogenized by nitrogen cavitation at 300 psi and centrifuged at $450 \times g$ for 10 min. The supernatant was pelleted by high speed centrifugation at $95{,}000 \times g$ for 30 min and resuspended in 40% sorbitol. The 40% sorbitol was overlaid with 25% sorbitol and centrifuged at $95{,}000 \times g$ for 60 min. The band at the 25–40% interface was collected and pelleted. In three experiments the Na/K-ATPase activity increased from 0.48 μM/mg/hr in the homogenate to 6.02 μM/mg/hr in the purified basal lateral membranes, with an average purification of 13.2 ± 3.4 (SE) fold with respect to protein. For some experiments these membranes were purified by digitonin density perturbation, as described by Mircheff et al. (1979b), to obtain a purification of Na/K-ATPase greater than 25-fold with respect to protein. The membranes used in all experiments were suspended in 200 mM sorbitol, 5 mM Tris-Hepes pH 7.5.

Materials and Enzyme Assays

All chemicals were reagent grade. Isotopes, ouabain ^{3}H (10–20 Ci/mM), sucrose ^{14}C (400–700 mCi/mM), and ATP γ ^{32}P (10–40 Ci/mM), were obtained from New England Nuclear, Cambridge, Mass. Na/K-ATPase was assayed as described by Mircheff and Wright (1976) and is reported in units of μmol of PO_4 liberated per hour.

Phosphorylation

Membranes were phosphorylated by the method of Knauf et al. (1974). Membranes were phosphorylated on ice for 15 sec in a medium containing 5 mM Tris-Hepes pH 7.5, $MgCl_2$ 12 μM, ATP γ ^{32}P 0.4–10 μM, and NaCl 50 mM. For controls, either 10 mM KCl was added or the NaCl was replaced with 10 mM KCl and 40 mM choline chloride. After incubation the membranes were precipitated with cold 5% trichloroacetic acid (TCA) containing 5 mM phosphate and 1 mM ATP. The precipitated membranes were washed twice with 5% TCA containing 5 mM phosphate and assayed by liquid scintillation counting.

Treatment with hydroxylamine was performed according to Blostein (1968). Phosphorylated membranes which were precipitated with TCA were washed in 0.1 M HCl and divided into three parts. One was left on ice; one was suspended in 0.6 M NaCl buffered with 50 mM sodium acetate pH 5.0 and kept at 37 °C for 15 min; and the third was placed in 0.6 M hydroxylamine brought to pH 5.0 with NaOH and kept at 37 °C for 15 min. All samples were then precipitated with 5% TCA, containing 5 mM phosphate, and counted.

Acrylamide Gels

The membranes were phosphorylated as described above except that the final wash was in 1% TCA and 1 mM phosphate. The precipitate was solubilized in 8% SDS 1% mercaptoethanol, and 100 μg samples of this protein were electrophoresed on slab gels of $4^{1}/_{2}$% acrylamide by the method of Fairbanks, Steck & Wallach (1971). Electrophoresis was performed at 11 °C to decrease hydrolysis of the phosphate bond. The current was maintained at 15 mA for one hour and then increased to 30 mA. Following electrophoresis the gels were cut into 3 mm slices, placed into scintillation cocktail, and assayed 24 hr later. Recovery of ^{32}P from the gels was 80–90%. Molecular weight standards (Bio Rad, Richmond, Calif.) were run in parallel and these were stained with Coomassie Blue. Membrane samples were also electrophoresed on both Fairbanks, Steck & Wallach gels (1971) and on Laemmli (1970) $7^{1}/_{2}$% gels and stained with Coomassie Blue. Although better resolution of membrane peptides could be obtained with Laemmli gels, the acyl phosphate was unstable at alkaline pH, and therefore this gel system could not be used for phosphorylation studies.

Ouabain Binding

Ouabain binding was determined by a Millipore filtration procedure. Fifty micrograms of membranes were incubated in 100 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 3 mM ATP, 5 mM Tris-Hepes pH 7.5 with (^{3}H)-ouabain and (^{14}C)-sucrose. After the incubation period, a sample of the medium was diluted into buffer (5 mM Tris-Hepes, 200 mM sorbitol, 1 mM ouabain) at 0 °C and left on ice for 1 min. An aliquot of this solution was filtered on Sartorius 0.45 μm filters and the filter was washed with 4 ml of cold ouabain-free buffer. In preliminary experiments, the 1-min wash at 0 °C reduced both the amount and variability of the nonspecific binding. Specific binding was determined by subtraction of the ouabain bound at 10^{-3} M, and no change in this value was detected when the wash time was varied from 10 sec to 5 min. Unless indicated, the values reported for ouabain binding have been corrected for nonspecific binding. The rate and concentration dependence of ouabain binding were measured at 22 and 37 °C. However, at 37 °C the rate of ouabain binding was too rapid to measure accurately with the Millipore filtration technique.

To measure the rate at which bound ouabain is released from the membranes, an aliquot of membranes at equilibrium with ^{3}H ouabain was diluted into 5 mM Tris-Hepes, 200 mM sorbitol, 1 mM ouabain at 22 °C. At specific times, samples of this solution were filtered and the filter was washed once with 4 ml of cold buffer.

The radioactive filters were dissolved in scintillation cocktail and assayed by liquid scintillation counting. Counts were corrected for background and spillover of ^{14}C into the ^{3}H channel.

Protease Treatment

Cells were isolated from the jejunum described by Stern (1966). The isolated cells were suspended in 150 mM NaCl, 0.5 mM azide,

5 mM Hepes pH 7.4 and were incubated at 37 °C for 10 min with or without pronase (Streptomyces griseus, Sigma, St. Louis, Mo.). The cells were then washed twice with 20 volumes of cold isotonic NaCl and basal lateral membranes were isolated as described above[2].

Results

Phosphorylation

Basal lateral membranes were phosphorylated in the presence of 0.4–10 μM ATP labeled with ^{32}P in the gamma position. At ATP concentrations between 0.4 and 1 μM, the incorporation of phosphate into the membranes in the presence of 50 mM NaCl averaged 12 ± 1.2 pmol/mg protein (n=9) and decreased to 1.1 ± 0.1 pmol/mg protein in the presence of 10 mM KCl, i.e., the maximum phosphorylation in the presence of sodium was 11 times greater than in the presence of potassium. As ATP concentration was increased, the absolute amount of phosphate incorporated into the membranes continued to increase, but the increment in phosphorylation due to sodium, i.e., sodium phosphorylation minus potassium, remained constant. For example, sodium-stimulated phosphorylation was only twice potassium-stimulated phosphorylation at 10 μM ATP. The increment in phosphorylation due to sodium was constant at 10.1 ± 0.96 (SE n=4) pmol/mg protein (as ATP increased from 0.6 to 10 μM). For these membranes this corresponds to 2 pmol phosphate/unit of Na/K ATPase activity.

Basal lateral membranes phosphorylated in the presence of ^{32}P ATP were analyzed by SDS polyacrylamide gel electrophoresis. Phosphorylation in the presence of 50 mM NaCl led to incorporation of phosphate into a protein whose apparent mol wt is 100,000 (Fig. 1). The amount of phosphate in this peak was reduced 10-fold either by the addition of 10 mM KCl or by the substitution of 10 mM KCl and 40 mM choline chloride for the NaCl in the incubation medium. The average mol wt for this peak in 6 gels was $105{,}000 \pm 3{,}000$ (SE).

To test that the phosphoproteins were acyl phosphates, we tested their sensitivity to hydroxylamine. The experiment shown in Table 1 demonstrates that 88% of the phosphorylation occurring in the presence of either Na or K is sensitive to incubation in hydroxylamine at 37 °C.

The Na/K-ATPase phosphoprotein lost 92% of its phosphate in the presence of hydroxylamine, while only 18% of the phosphate was lost under control conditions at 37 °C. In contrast, the potassium phosphoprotein released 68% of its phosphate under the control conditions.

Ouabain Binding

The Na/K-ATPase binds ouabain specifically according to the mass law equation $O + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} OR$ where O is the ouabain concentration, R is the receptor concentration, and OR is the ouabain receptor complex. The association and dissociation rate constants are k_{+1} and k_{-1} respectively. The ratio k_{-1}/k_{+1} is K_m, the Michaelis constant or dissociation constant.

The association rate follows second order kinetics, and the rate constant can be calculated if the initial receptor concentration (B) and the initial ouabain concentration (A) are known according to the equation:

$$k_{+1} = \frac{2.303}{(A-B)t} \log \frac{B(A-X)}{A(B-X)}$$

where X is the amount of ouabain bound to the receptor at time t (Erdmann & Hasse, 1975). The rate of ouabain binding was measured at a concentration of 6×10^{-7} M at 22 °C (Fig. 2). The half time for binding was 30 sec and the steady state binding amounted to 7 pmol/mg protein. The value calculated for k_{+1} is 1.3×10^3 M^{-1} sec^{-1} (Table 2).

The dissociation rate constant k_{-1} is a monomolecular reaction and can be calculated from the exponential decay of ouabain binding (Erdmann & Hasse, 1975). We determined the dissociation rate constant at a concentration of 6×10^{-7} M at 22 °C (Fig. 3 and Table 2). Within five min, with a half time of 20 sec, the amount of ouabain bound fell to the level of nonspecific binding and remained constant for at least 25 min. The calculated k_{-1} is 3.6×10^{-2} sec^{-1}.

The Michaelis constant K_m can be calculated from the ratio of k_{-1}/k_{+1} or can be measured directly from the equilibrium binding of ouabain as a function of ouabain concentration (Fig. 4 and Table 2). The binding of ouabain increased to a peak at 5×10^{-5} M and decreased below the maximum binding at 10^{-4} M. This peak in ouabain binding has been reported previously for rat heart by Allen and Schwartz (1969). As judged by the line of best fit, B_{max}, the maximum number of binding sites, and K_m for ouabain binding to rat basal lateral membranes were 175 pmol/mg protein and 1.5×10^{-5} M, respectively. The K_m obtained from the ratio of the rate constants (k_{-1}/k_{+1}) was 2.95×10^{-5} M.

[2] There was no significant "scrambling" of peptides between the brush border and basal lateral membranes of the isolated cells during a 10-min incubation at 37 °C (E.M. Wright, *manuscript in preparation*).

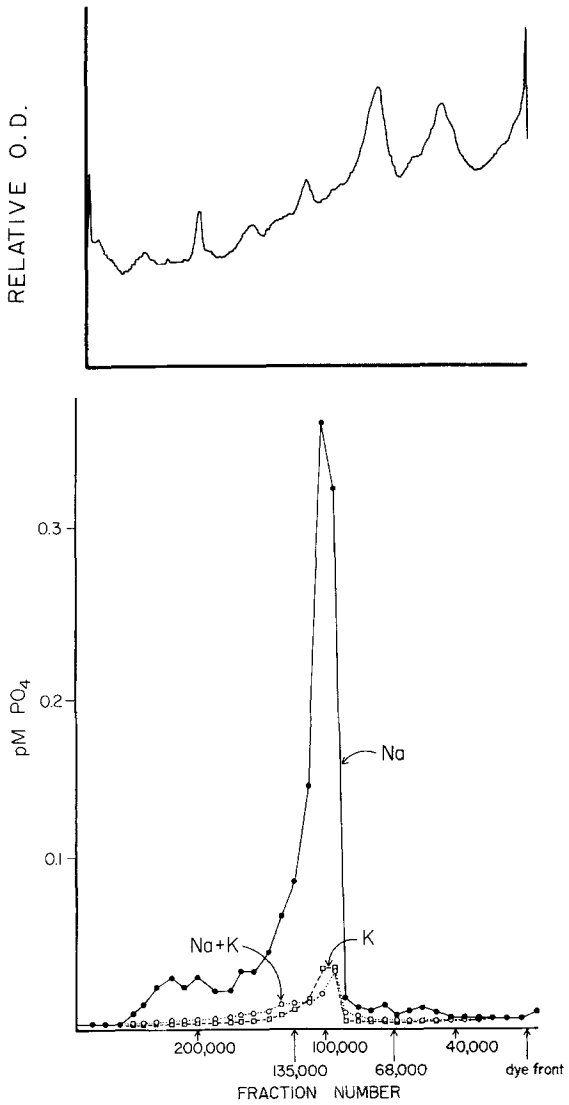


Fig. 1. Electrophoretic pattern of basal lateral membranes. Above is a densitometric tracing of basal lateral membranes purified 25-fold by digitonin density perturbation, electrophoresed, and stained with Coomassie Blue. Below is the pattern of ^{32}P incorporation in the presence of 50 mM NaCl (Na) or 50 mM NaCl, 10 mM KCl (Na-K) or 10 mM KCl, 40 mM choline chloride (K) and in all cases 0.5 μM γ ^{32}P ATP, 12 μM $MgCl_2$, and 5 mM Tris Hepes, pH 7.4. Molecular weights of standard proteins are indicated at the bottom. Note that the peak of phosphate incorporation is at 100,000 mol wt. 100 μg of membrane protein was run on slab gels of $4^1/_2$% acrylamide using Fairbanks buffers. ^{32}P was assayed by liquid scintillation counting of 3 mm slices

Table 1. Effect of hydroxylamine treatment on Na, K and Na/K phosphoproteins[a]

	Control	NaCl	Hydroxylamine
Na (pmol/mg)	4.36	2.34	0.49
	100%	54%	11%
K (pmol/mg)	2.46	0.78	0.33
	100%	32%	13%
Na/K-ATPase phosphoprotein (pmol/mg)	1.90	1.56	0.16
	100%	83%	8%

[a] Basal lateral membranes were phosphorylated in the presence of 2 μM γ ^{32}P ATP, 12 μM $MgCl_2$, 5 mM Tris-Hepes, pH 7.4, and either 50 mM NaCl (Na) or 10 mM KCl, 40 mM choline chloride (K) as described. The difference between phosphorylation in the presence of Na and K is the Na/K-ATPase phosphoprotein. The TCA precipitated membranes were resuspended in 0.1 N HCl and an aliquot was assayed for the control. The remaining sample was divided into three parts, one left on ice, and the remaining two incubated at 37 °C for 15 min in either 0.6 M NaCl or 0.6 M hydroxylamine. These samples were then precipitated with TCA and assayed for ^{32}P. Ninety % of the Na/K-ATPase phosphoprotein counts were recovered after incubation on ice in 0.1 N HCl

Table 2. Kinetic constants for the binding of ^3H-ouabain to the Na/K-ATPase of basal lateral membranes of rat intestine[a]

	Rat intestine	Human red cell[b]	Rabbit kidney[c]	Frog choroid plexus[d]
k_{+1} ($M^{-1}sec^{-1}$)	1.3×10^3	8×10^3	0.5×10^2	0.6×10^3
k_{-1} (sec^{-1})	3.6×10^{-2}	1.4×10^{-5}	1.3×10^{-4}	5×10^{-4}[e]
k_{-1}/k_{+1} (M)	2.95×10^{-5}	1.8×10^{-9}		
K_m (M)	1.5×10^{-5}		5.1×10^{-7}	8×10^{-7}

[a] The association rate constant was determined as described in the text. The initial number of ouabain receptors (B) was 1.4×10^{-11} M and the initial amount of ouabain (A) was 6×10^{-11} M, at a concentration of 6×10^{-7} M. The correlation coefficient for the line determining k_{+1} from 6 to 30 sec was 0.90. The dissociation rate constant k_{-1} was determined as described in Fig. 3 and the K_m is from Fig. 4. All values listed are for experiments at 22 °C.

[b] Erdmann & Hasse (1975). [c] Shaver & Stirling (1978).

[d] Wright (*in preparation*). [e] Determined from k_{+1} and K_m.

Na/K-ATPase Activity

In addition to ouabain binding, we have measured the sensitivity of the basolateral membrane Na/K-ATPase activity to ouabain (Fig. 5). At 10^{-6} M, there was a slight, but insignificant, stimulation of Na/K-ATPase activity as has been reported previously at low concentrations of ouabain (Bonting, 1970). The K_I for ouabain inhibition of the Na/K-ATPase was 3×10^{-5} M and this is similar to the K_m for ouabain binding.

The Na/K-ATPase accounted for between 26 and 55% (mean 44% ± 5 n=6) of the total ATPase activity at 22 °C and pH 7.5. The pH optimum of the Na/K-ATPase was 7.5–8.0, while the Mg ATPase had a pH optimum greater than 9.0. Attempts to selective-

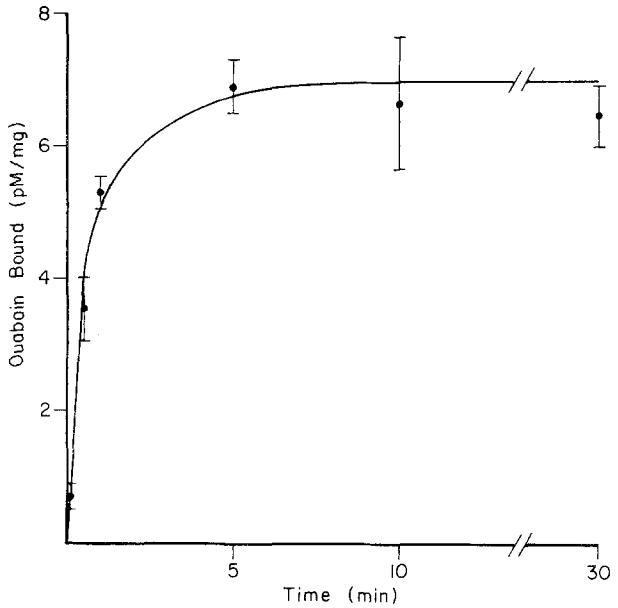


Fig. 2. Rate of ouabain binding at 22 °C. Fifty micrograms of basal lateral membranes were incubated in 6×10^{-7} M ^{3}H-ouabain, 100 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 3 mM ATP, 5 mM Tris Hepes pH 7.4 with tracer amounts of ^{14}C-sucrose for the time indicated, washed for one minute in buffer at 0 °C and filtered on 0.45 μ filters. Each point is the mean ± SE of 3 points. All ouabain samples were corrected for extravesicular space by subtraction of the ^{14}C sucrose space

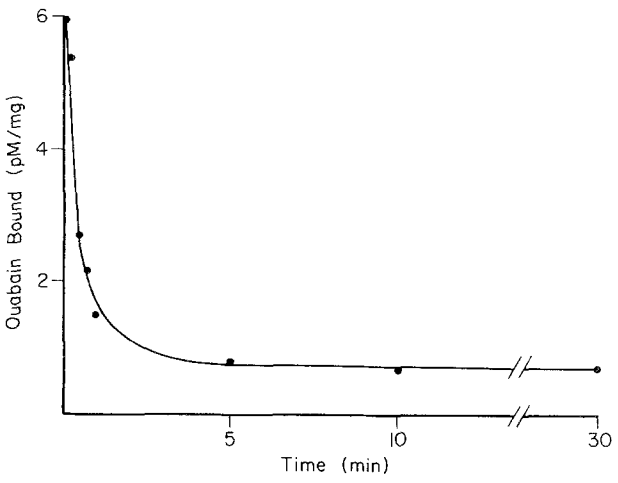


Fig. 3. Rate of ouabain release at 22 °C. Basal lateral membranes were incubated in the medium described in Fig. 2 for 5 min; an aliquot was diluted into 200 mM sorbitol, 5 mM Tris Hepes pH 7.4, 1 mM ouabain at 22 °C for the time indicated on the graph before filtration on 0.45 μ filters. The exponential decay of ouabain binding yields k_{-1} of 3.6×10^{-2} sec^{-1} with a correlation coefficient of 0.97. The sample has not been corrected for nonspecific binding. Each point represents the average of duplicate measurements

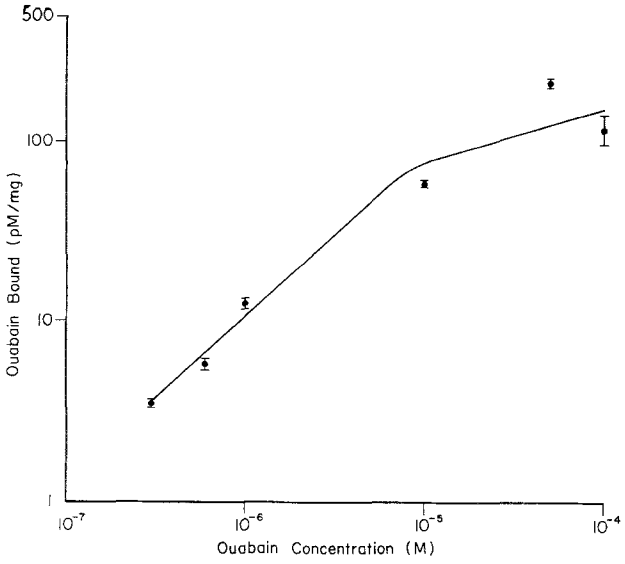


Fig. 4. Equilibrium binding of ouabain. Basal lateral membranes were incubated for five min, washed and filtered as in Fig. 2. The line was drawn for a B_{max} of 175 pM/mg and a K_m of 1.5×10^{-5} M calculated from a Scatchard analysis. Each concentration represents the mean ± SE of 3 measurements

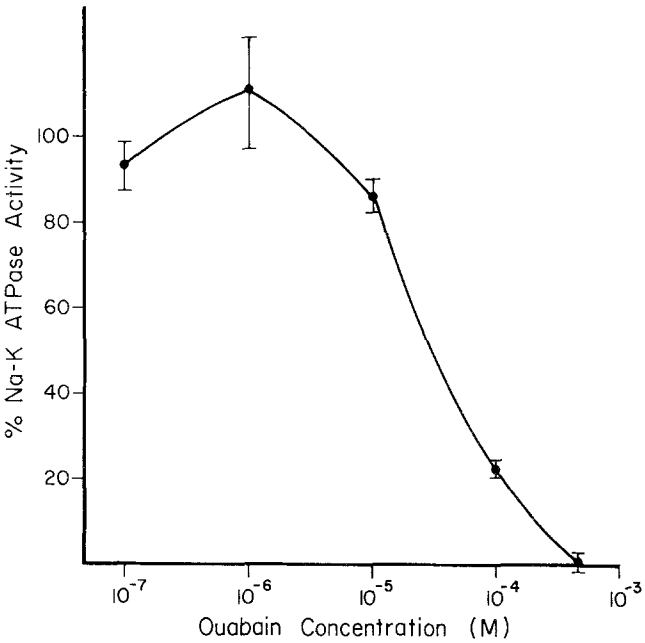
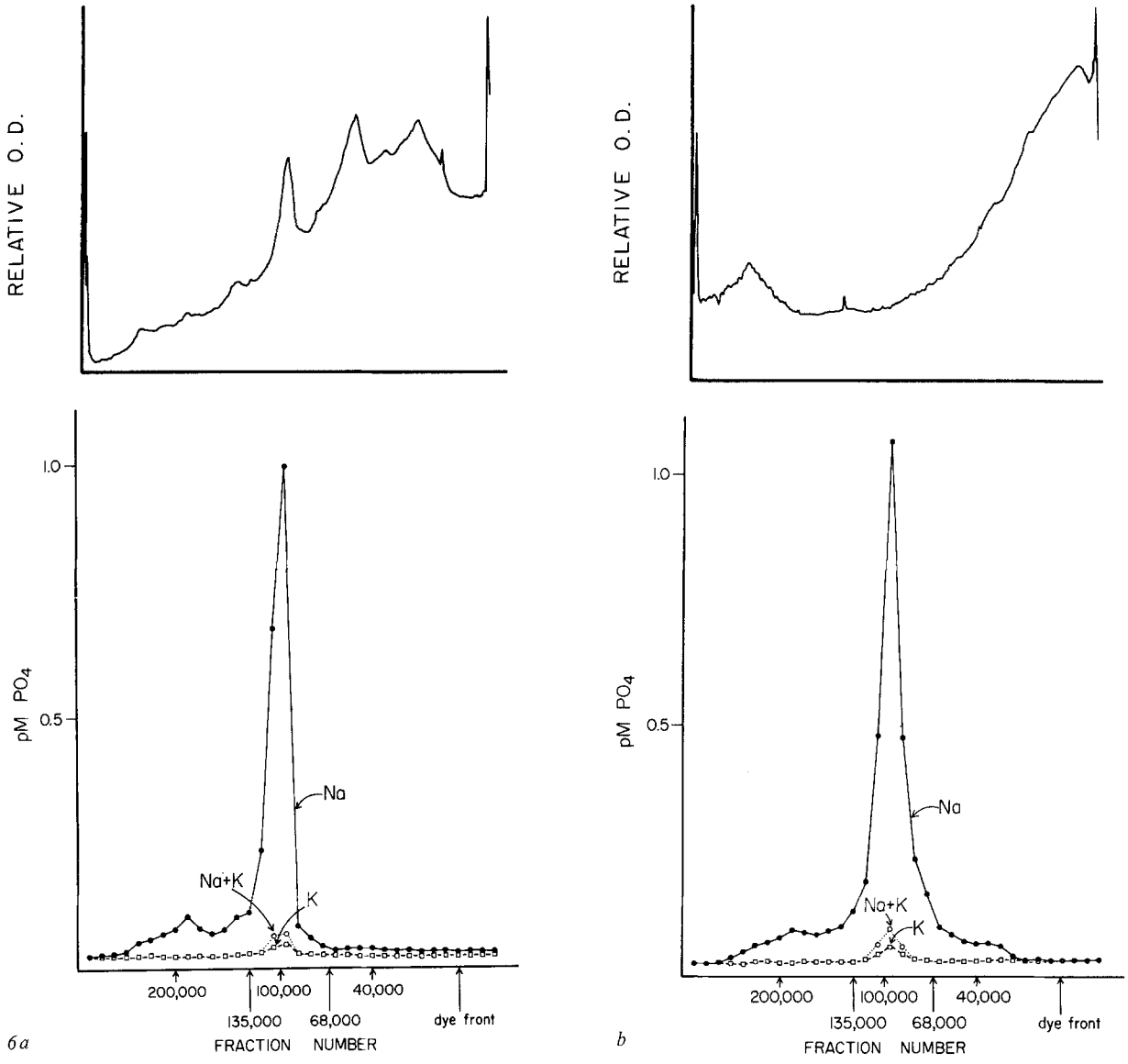


Fig. 5. Na/K-ATPase activity with increasing ouabain concentrations. No further decrease in activity was found by increasing ouabain concentration to 5×10^{-3} M. Na/K-ATPase activity represented 35% of the total ATPase activity in this preparation. The K_I for ouabain inhibition of Na/K-ATPase activity is 3×10^{-5} M. Each point represents the mean ± SE of 4 measurements



ly inactivate Mg ATPase with detergents, oligomycin, 1 phenylalanine, or NaI were unsuccessful.

Pronase Digestion

As demonstrated in Fig. 1, the phosphorylated intermediate of the Na/K-ATPase has a mol wt of 100,000. From a densitometric tracing of Coomassie Blue stained gels of basal lateral membranes purified 10 fold, 15% of the basal lateral membrane protein has an apparent mol wt of 100,000. To determine whether the Coomassie staining protein and the phosphorylated intermediate were identical, we followed the strategy of Knauf Proverbio, and Hoffman (1974) of digestion of the intact cells by pronase. Isolated cells were incubated at 37 °C for 10 min with and without pronase 25 μg/ml. The basal lateral membranes were isolated from these cells as described above.

To determine whether digestion had affected the mol wt of the Na/K-ATPase phosphoprotein, basal lateral membranes from control and pronase treated cells were phosphorylated and electrophoresed on polyacrylamide gels. As shown in Fig. 6*a*, incubation at 37 °C for 10 min without pronase had no effect on the mol wt of the phosphorylated intermediate (compare to Fig. 1). Phosphorylation in the presence of Na leads to the incorporation of phosphate into a protein of 100,000 MW; addition of K or substitution of K and choline for the Na decreased the amount of phosphate in this peak. Phosphorylation of basal lateral membranes from pronase treated cells (Fig. 6*b*), resulted in a similar phosphorylation of a

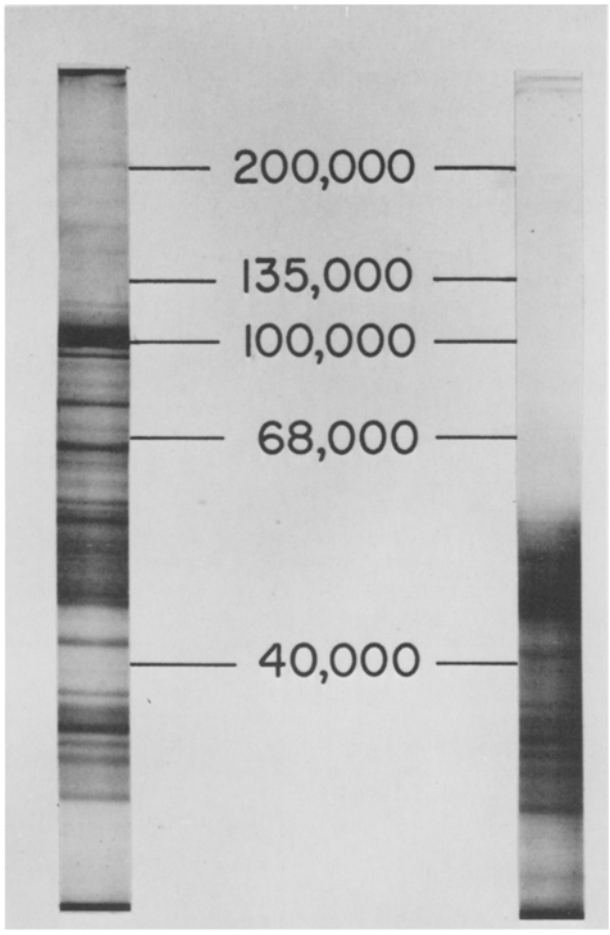


Fig. 6. Electrophoretic pattern of basal lateral membranes. (*a*): Above is a densitometric tracing of membranes purified 10-fold, electrophoresed and stained with Coomassie Blue. Below is the pattern of ^{32}P incorporation in the presence of 50 mM NaCl, or 50 mM Nacl, 10 mM KCl or 10 mM KCl, 40 mM choline chloride with 2 μM ^{32}P ATP, 12 μM $MgCl_2$ and 5 mM Tris Hepes, pH 7.5. Molecular weights of standard proteins are listed at the bottom. One hundred micrograms of membrane protein was electrophoresed on $4^1/_2$% acrylamide slab gels by the method of Fairbanks: ^{32}P was assayed by liquid scintillation counting of 3 mm slices. (*b*): Identical to *a* except that the cells from which the basal lateral membranes were isolated had been exposed to 25 μg/ml pronase at 37 °C for 15 min prior to homogenization. The basal lateral membranes are purified 10-fold (*c*): Basal lateral membranes from *a* (left) and *b* (right) electrophoresed on $7^1/_2$% gels by the method of Laemmli. Numbers indicate the positions and mol wt of standard proteins. Note that the membranes isolated from cells digested with pronase are devoid of protein of higher mol wt as already shown in the densitometric tracings of Fairbanks gels in *a* and *b*. In particular, notice the loss of the protein of 100,000 apparent mol wt while, as shown in *a* and *b* (bottom) the phosphorylated intermediate is still present at 100,000

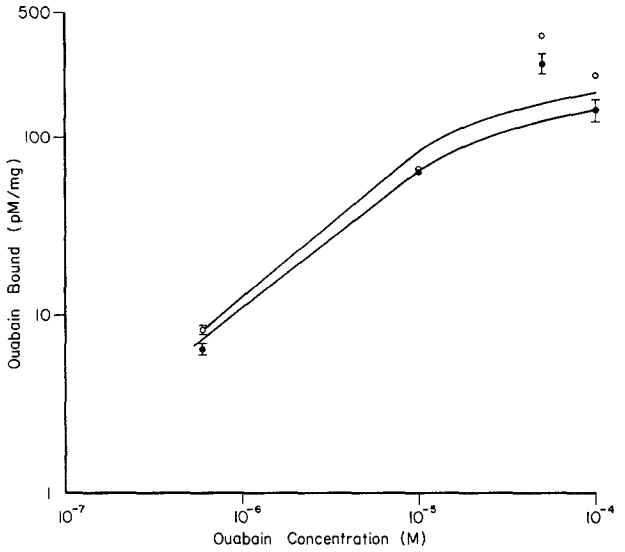


Fig. 7. Equilibrium binding of ouabain to membranes isolated from pronase treated cells and from the parallel control cells. Ouabain binding was performed as in Fig. 4. The lines are calculated from a B_{max} of 197 pM/mg and a K_m of 1.4×10^{-5} M for pronase treated cells (upper line) und a B_{max} of 155 pM/mg and K_m of 1.4×10^{-5} M for the parallel control (lower line). The K_m is similar to the 1.5×10^{-5} M reported in Fig. 4

protein of 100,000 MW. However, comparison of the Coomassie stained gels either by densitometric tracings of the Fairbanks gels (above in Fig. 6*a* and *b*) or directly in Laemmli gels (Fig. 6*c*) reveals that the major portion of the protein of 100,000 mol wt has been digested by pronase. Since both the amount of phosphate incorporated per unit of enzyme activity and the apparent mol wt of the phosphorylated intermediate were unchanged by pronase digestion, we must conclude that Na/K-ATPase represents a minor component of the protein comigrating at an apparent mol wt of 100,000.

To confirm that the pronase digestion of the intact cells had not digested the Na/K-ATPase, the dissociation constant of ouabain binding was measured in the basal lateral membranes from control and pronase treated cells (Fig. 7). The dissociation constants (K_m) calculated for ouabain binding from the membranes of control and pronase cells were both 1.4×10^{-5} M with B_{max} of 155 pmol/mg and 197 pmol/mg, respectively. The peak in ouabain binding at 5×10^{-5} M ouabain is also seen in these membranes. The similarity in ouabain binding curves again suggests that pronase at this concentration and digestion time has not affected the Na/K-ATPase. The Na/K-ATPase activity in the membranes from pronase-treated cells was not significantly enriched over the membranes from control cells, suggesting that insignificant amounts of protein have been lost from these membranes during pronase digestion.

Increasing the pronase concentration at which the cells were digested resulted in an increase in the specific activity of the membranes isolated from pronase-treated cells, and in Na-stimulated incorporation of

phosphate into a broad band of proteins of mol wt equal to and less than 100,000 Daltons. Presumably, the Na/K-ATPase retains its function while in the membrane, but solubilization in SDS and electrophoresis demonstrates its partial digestion. Preliminary experiments indicated that the transport of L-alanine into these vesicles was also unaffected by the pronase digestion.

Discussion

The Na/K-ATPase of the rat intestinal basal lateral membranes shows many of the characteristics of the Na/K-ATPase from other tissues. First, we have demonstrated that the phosphorylated intermediate of the Na/K-ATPase of intestinal basal lateral membranes has the same apparent mol wt as that identified in human red cells (Knauf, Proverbio & Hoffman, 1974) and as that isolated from dog kidney (Kyte, 1971), rabbit, pig, or sheep kidney (Jorgensen, 1975), brain (Uesugi et al., 1971), and shark rectal gland (Hokin et al., 1973). Secondly, we have shown that the phosphoprotein of the Na/K-ATPase of the basal lateral membrane is an acyl phosphate by its sensitivity to hydroxylamine. Thirdly, we have demonstrated that the basal lateral membranes bind ouabain specifically with a dissociation constant (1.5×10^{-5} M) similar to that required to inhibit the Na/K-ATPase activity in these membranes (3×10^{-5} M). This implies that the specific ouabain binding represents binding of the Na/K-ATPase. Comparison of the rate constants of ouabain binding in rat basal lateral membranes to the rate constants for ouabain binding in other tissues (Table 2) demonstrates that the association rate constant k_{+1} is similar among tissues and species ($\sim 10^{-3}$ M^{-1} sec^{-1}), but the dissociation rate constant and, therefore, the K_m's range over several orders of magnitude, ($k_{-1} 10^{-2} - 10^{-5}$ sec^{-1} and $K_m 10^{-5} - 10^{-9}$ M). Whether this difference in sensitivity to ouabain is a function of the lipids associated with the Na/K-ATPase or is an intrinsic characteristic of the Na/K-ATPase protein is not yet known. Finally, we have shown, through digestion of the intact cells with low concentrations of pronase, that the Na/K-ATPase represents a minor component (less than 1%) of the protein associated with the basal lateral membranes. This digestion also demonstrated that incubation at even low concentrations of protease (~ 25 μg/ml) for short periods results in extensive digestion of membrane proteins and suggests that use of proteases to isolate cells from tissues should be avoided.

Ouabain binding and phosphorylation can be used to determine the amount of ouabain or phosphate bound per unit of enzyme, the ratio of ouabain to phosphate bound, and the turnover of the Na/K-ATPase. These values calculated for intestinal basal lateral membranes are compared in Table 3 with values reported for some other tissues. The ouabain bound per unit of Na/K-ATPase is higher for rat intestinal basal lateral membranes than for other tissues. This binding reflects both the insensitivity of the rat to ouabain, making maximal ouabain binding difficult to measure, and the measurement of Na/K-ATPase activity, which was at 22 °C. The phosphorylation is similar to that measured for other tissues. The amount of ouabain binding per phosphoenzyme is very high for the rat. This value tends to increase as the sensitivity of the animal to ouabain decreases; guinea pig kidney, for instance, where the ratio of ouabain per phosphoenzyme is 4, has a K_m for ouabain binding of 2×10^{-7} M, compared to a ratio of ~ 1 and a K_m of 0.5×10^{-8} M for the beef enzymes. Whether the ratio of ouabain sites to enzyme molecules would continue to be greater than the ratio in enzymes purified from ouabain-insensitive species is unknown. The turnover number has been estimated from the enzyme activity per phosphoenzyme. Our

Table 3.

Preparation	Ouabain pmol/unit Na/K-ATPase	^{32}P (pmol/unit Na/K-ATPase)	Ouabain/ ^{32}P	Turnover ATP hydrolyzed/ min[a]
Red blood cell	12[b]	5.9[c]		2,800
Beef kidney[d]	6.5	4.9	1.3	3,430
Beef brain[d]	2.2	1.5	1.5	11,500
Beef heart[d]	2.2	2.0	1.1	8,550
Guinea pig kidney[d]	6.5	1.5	4.3	11,100
Rat kidney[e]	1.9	–	–	
Rat intestine[f]	32	2	16	8,300

[a] This calculation assumes that ^{32}P incorporation represents the total number of sites. The turnover is calculated as ATP hydrolyzed/enzyme/min.

[b] Joiner & Lauf (1978). Calculated from their pump turnover of 1400 ATPs/site/min and a ouabain/site ratio of 1. Since pump turnover was determined from K^+ fluxes in intact cells, and since the pump rate under these physiological conditions may be 30% of the rate measured in enzyme assays, the ouabain binding per unit of enzyme activity may be overestimated by a factor of 4.

[c] Knauf, Proverbio & Hoffman (1974). Calculated from the Na dependent phosphorylation of 0.55 pmol/mg ghost and Na/K-ATPase activity of 94 nmol/mg ghost/hr.

[d] Erdmann & Schoner (1973).

[e] Allen & Schwartz (1969).

[f] The unit enzyme activity (μM/hr) was measured at 22 °C rather than 37 °C in contrast to the other values in this table which are for enzyme activities measured at 37 °C. Therefore, the values of ouabain and ^{32}P/unit enzyme are overestimated for rat intestine.

value (8×10^3 min^{-1}) is similar to turnover numbers measured at 37 °C. An alternative method used to measure turnover is either ion transport per ouabain bound or Na/K-ATPase per ouabain bound, assuming that there is one ouabain bound per pump site. For measurements made at 22 °C this value is about 600/min (Landowne & Ritchie, 1970, and Wright, 1979). Our value of 32 pmol ouabain/μmol/hr of Na/K-ATPase activity yields a turnover of 580/min. These two methods of measuring turnover then represent the minimum and maximum values expected for turnover of Na/K-ATPase from rat intestinal basal lateral membranes.

Finally, considering the importance of the Na/K-ATPase in the transport of solutes across the intestine, there are several parameters that are interesting to calculate from our data on isolated basal lateral membranes: first, the number of pump sites per cell can be estimated from the maximum phosphorylation (10 pmol/mg protein) obtained for our isolated basal lateral membranes (purified 10-fold with respect to protein) and the amount of protein per cell (2.5×10^{-7} mg/cell, Rosselin, *personal communication*). Accordingly, there are about 1.5×10^5 pump sites per intestinal cell, which is in the same range as that reported for kidney, liver, and brain (Baker & Willis, 1972). Use of maximum ouabain binding to determine the number of pump sites yields an estimate an order of magnitude larger.

Second, we can estimate whether or not the Na/K-ATPase can account for the observed rates of sodium transport across the rat jejunum. Using the smaller number of pump sites per cell (1.5×10^{-5}), *see* above, the turnover number for the pump (8.3×10^3/min, *see* Table 3), and the assumption that there are 3 Na ions transported per cycle of the pump, we can calculate that there are 4.5×10^9 ions transported out of each epithelial cell per min. At this rate the pump could deplete the intracellular sodium in one to two min (number of intracellular Na ions = Na concentration (20 mM) × cell volume (5 μm × 5 μm × 25 μm) × Avogadro's number = 7×10^9 ions). This represents a maximum estimate at 22 °C. A minimum estimate has been determined for *Necturus* gallbladder using microelectrodes (Graf & Giebisch, 1979) and volume changes (Spring & Hope, 1979) to be 10–16% of the cell Na/min. In order to relate the rate of Na pumping out of one cell to the observed rates of intestinal sodium transport it is necessary to know the number of cells per cm^2 of serosal area. We have estimated that there are about 10^8 epithelial cells/cm^2 using (i) cell counts from photomicrographs of small intestine and (ii) epithelial cell dimensions and the amplification in area caused by the intestinal villi (× 8, Wilson, 1962). Based on the Na transport rate per cell and the number of cells per cm^2 of intestine, the predicted rate of Na transport across the intestine is 48 μ-equivalents/cm^2/hr. The rate of sodium transported by the rat jejunum amounts to only 8 μ-equivalents/cm^2/hr (Barry, Smyth & Wright, 1965). Our estimates of active sodium transport should be considered underestimates in view of the fact the turnover number of the pump at 37 °C should be substantially higher than that observed at 22 °C. These calculations suggest that the Na/K-ATPase in the tissues is more than adequate to account for active sodium absorption.

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Note Added in Proof

Since submission of this manuscript, a paper (U.A. Liberman, Y. Asano, C.S. Lo and I.S. Edelman, 1979, *Biophys. J.* **27**:127) has appeared which measures phosphorylation and ouabain binding in crude membranes from rat intestinal cells. Values obtained at 37 °C are not inconsistent with those reported here.