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ETHACRYNIC ACID INFLUX AND EFFLUX IN KIDNEY CORTEX SLICES: DEPENDENCE ON SODIUM GRADIENT*

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Abstract—Ethacrynic acid (EA) accumulation in rat kidney cortex slices was inhibited by reduction of sodium concentration in the incubation medium. Preloading of slices with sodium reduced EA accumulation at medium sodium concentration of 30–140 mM. Ouabain (10^{-3} M) inhibited EA accumulation in kidney cortex slices of rabbit, guinea-pig, Psammomys obesus, rat, mouse and Acomys cahirinus in decreasing order. Ouabain inhibited the sodium pump in kidney cortex slices of these species in the same order. Ethacrynic acid efflux was faster from slices of rat kidney medulla than from kidney cortex. The efflux rate from cortical slices increased when sodium concentration in the incubation medium was lowered. Probenecid (10^{-3} M) in the medium enhanced the efflux rate of EA from kidney cortex slices. It was concluded that EA fluxes in kidney cortex slices fit the model of a sodium-activated carrier mechanism and that the sodium gradient across the cell membrane determines the direction of net EA transport.

It has recently been reported that Ethacrynic acid (EA) accumulates in rat kidney cortex slices, but not in slices of kidney medulla or papilla [1]. Different characteristics of EA accumulation were similar to those of organic acid transport. However, cardiac glycosides (ouabain) had no effect on the accumulation of EA [1]. Different reports on the effect of cardiac glycoides on EA binding in kidney were confusing, some finding no effect, others described definite inhibition of EA accumulation [2, 3]. Since cardiac glycosides would affect intracellular sodium concentration by inhibition of the sodium pump, we decided to investigate the effect of changes in intracellular sodium without the use of cardiac glycosides. Another approach to investigate further the role of sodium in EA transport was to study the efflux of EA, from preloaded kidney cortex slices, into media of various compositions. Finally, the action of cardiac glycosides on EA accumulation was studied in kidney cortex slices of different species. These various experimental approaches indicate that both EA efflux and influx are carrier-mediated and depend on the sodium gradient across the cell membrane.

MATERIALS AND METHODS

Most of the experiments were carried out on male albino rats of the Hebrew-University strain, weighing 180–250 g. Following decapitation, the kidneys were immediately removed and placed on ice. After saggittal section of the kidney, to expose the different regions, slices of cortex (outermost part) weighing 35–45 mg were prepared. Other rodent species studied were: rabbit, mouse, guinea-pig, Acomys cahirinus and Psammomys obesus. In all cases male animals were used and only outer cortex slices were prepared.

Ethacrynic acid accumulation in cortical slices. Accumulation of EA was studied in the following media: (1) 140 mM Na^+ medium consisted of: NaCl, 110 mM; $NaHCO_3$, 15 mM; Na-acetate, 9 mM; Na_2HPO_4, 2.4 mM; NaH_2PO_4, 0.6 mM; KCl, 5 mM; $MgSO_4$, 1.2 mM; Ca-gluconate, 1.0 mM; Na_2SO_4, 0.6 mM; glucose, 5 mM. The medium was aerated with 95% O_2/5% CO_2 to give a final pH of 7.5. (2) 120 mM Na^+ medium consisted of: NaCl, 120 mM; Tris–HCl, 18 mM; KCl, 5 mM; $MgSO_4$, 1.8 mM; Ca-gluconate, 1.0 mM; glucose 5 mM. The pH was adjusted to 7.5 with HCl. The medium was aerated with 100% O_2. (3) 30 mM Na^+ medium was of the same composition as medium (1) except that NaCl was replaced by 220 mM sucrose (equiosmotic to 110 mM NaCl). (4) Medium without Na^+. This medium was of the same composition as medium (2) except that NaCl was replaced by 240 mM of sucrose.

Kidney cortex slices were incubated in the appropriate medium with 2.5 μCi of [^{3}H]EA (final concentration 3.12×10^{-8} M) for 60 min at 25°. At the end of the experiment the slices were taken out, blotted to dryness, and then extracted in boiling water for 1 hr. Ten ml of the phosphor (prepared by dissolving 5 g PPO and 0.3 g POPOP in 1 l of toluene, 500 ml Triton × 100 and 150 ml of H_2O) were added to samples of the incubation medium and of the tissue extract, and counted in a Packard liquid scintillation spectrometer. Results are expressed as the ratio of EA concentration in the tissue (slice) to that in the medium (S/M).

Preloading of slices with sodium. Kidney cortex

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Abbreviations—PPO = 2,5-diphenyl-oxazole. POPOP = 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene.

slices were loaded with Na^+ by preincubating them anaerobically for 15 min at 37° in a medium consisting of: NaCl, 140 mM; $CaCl_2$, 3 mM; $MgSO_4$, 1.5 mM; sodium phosphate buffer 3.5 mM, pH 7.4 (according to Munday *et al.* [4]). Under these conditions the intracellular electrolyte composition approached the composition of the incubation medium.

Kinetics of [^{3}H]*EA accumulation in kidney cortical slices.* [^{3}H]EA binding to kidney slices was carried out at different concentrations of the drug in the medium (7.8×10^{-9} M, 3.1×10^{-8} M). The incubation was carried out for 30 min at 25°. Results are expressed as the reciprocal of EA concentration in the slice as a function of the reciprocal of EA concentration in the medium.

[^{3}H]*EA Efflux from kidney cortical slices.* In each experiment 12 slices (of total wt about 0.5 g) were loaded with [^{3}H]EA by incubation with 2.5 μCi [^{3}H]EA (final conc. 3.12×10^{-8} M) in medium (1) (as described in section 1 of Methods) for 60 min at 25°. After the preincubation, two slices were extracted to determine total amount of [^{3}H]EA accumulated. The other slices were transferred to 50 ml Erlenmeyer flasks containing 20 ml of unlabelled medium. The efflux of [^{3}H]EA was measured by taking samples of the medium (each sample 0.5 ml) at intervals, up to 90 min. At the end of the incubation, two slices were extracted to determine [^{3}H]EA in the slices at the end of the efflux experiment. The amount of [^{3}H]EA remaining in the slices at different intervals from beginning of efflux is expressed as the amount of [^{3}H]EA in slice at given time as per cent of amount of [^{3}H]EA in slice at zero time (beginning of efflux experiment).

Effect of ouabain on sodium efflux and on EA accumulation. Kidney cortex slices from various rodents were used. Preloading of slices with Na^+ was carried out as described in section 1 of Methods. The slices were then transferred to fresh medium (medium (1)) and incubated for 30 min in the presence or absence of ouabain (10^{-3} M) in the medium. Na^+ extrusion from the slices was calculated by subtracting the amount of Na^+ found in the slices at the end of incubation from the amount of Na^+ present in the slices after the preloading, as previously described (5). The extrusion of Na^+ in the presence of ouabain was calculated as per cent of the extrusion in the absence of ouabain.

For the study of the effect of ouabain on EA accumulation, kidney cortex slices of various rodents were incubated with [^{3}H]EA either in the presence or in the absence of ouabain (10^{-3} M). S/M for EA was calculated under both conditions.

Chemicals. Ouabain was purchased from Merck, Darmstadt, West Germany. [^{3}H]EA was prepared at the Nuclear Research Center, Negev, Israel. Ethacrynic acid was obtained from Assia Chemicals, Ramat Gan, Israel. Probenecid was purchased from Sigma Chemical Co., St. Louis, MO. E.A. concentration in slices was calculated per wt of slice *after* incubation.

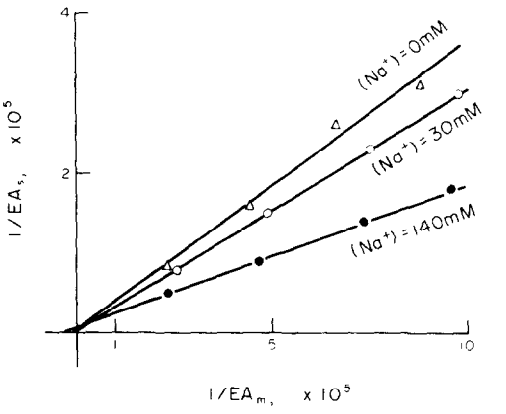

Fig. 1. Effect of Na^+ concentration in medium on EA accumulation in rat kidney cortex slices.
$1/EA_s$—Reciprocal of EA concentration in slices after 60 min incubation;
$1/EA_m$—Reciprocal of EA concentration in medium. Na^+ = 130, 40, zero mM concentration of Na^+ in incubation medium. Each point is mean of four to six experiments. Lines calculated according to the method of least squares.

RESULTS

Effect of sodium concentration in incubation medium on EA accumulation. Rat kidney cortex slices, incubated in media with decreasing concentrations of Na^+, showed also decreasing accumulation of [^{3}H]EA, as shown in Fig. 1. The effect on EA accumulation is plotted as reciprocal curve, i.e. reciprocal of EA concentration in slices versus reciprocal of EA concentration in medium. It is clear that reduction of Na^+ in the medium from 140 to 30 and to zero mM, respectively, caused a gradual reduction of EA accumulation in the slices. This indicates the role of extracellular Na^+ in EA accumulation, confirming our previous report [1]. However, it does not implicate intracellular Na^+

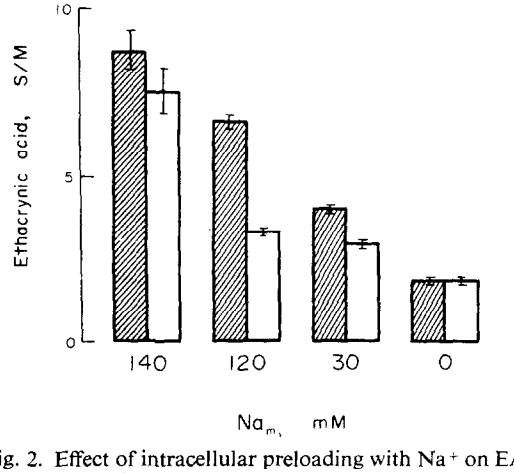

Fig. 2. Effect of intracellular preloading with Na^+ on EA accumulation in rat kidney cortex slices. Na_m concentration of Na^+ in incubation medium. S/M ratio of EA concentration in slice to EA concentration in medium. Vertical bars—S.E. Striped columns—untreated slices of rat kidney cortex. Open columns-slices preloaded with sodium, to increase intracellular Na^+ concentration. Each column is mean of eight experiments.

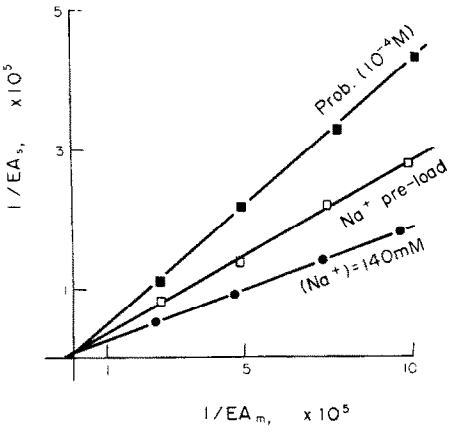

Fig. 3. Effect of Na^+ preloading and probenecid on EA accumulation in rat kidney cortex slices.
$1/EA_s$—Reciprocal of EA concentration in slices after 60 min incubation.
$1/EA_m$—Reciprocal of EA concentration in medium. Na^+—140 mM: control, untreated slices, incubated in medium of 140 mM Na^+. Sodium pre-load: slices pre-incubated in K^+-free, Na^+-medium, to cause increased intracellular Na^+ concentration, and then incubated with EA in medium of 140 mM Na^+, similar to control slices. Prob.—slices incubated in presence of probenecid (10^{-4} M). Each point is mean of four experiments. Lines calculated according to method of least squares.

concentration or the sodium gradient across the cell membrane in the process of EA accumulation. To evaluate this factor, the next experiment was performed.

Effect of sodium preloading on EA accumulation. Rat kidney cortex slices were preloaded with Na^+ and were then incubated with [^{3}H]EA in media of different Na^+ concentrations. Figure 2 shows that at each Na^+ concentration in the medium, intracellular preloading with Na^+ reduced EA accumulation. Only when Na^+ concentration in the medium was zero, no further reduction of EA accumulation could be observed by preloading of the slices.

This experiment indicates that, in addition to extracellular Na^+, intracellular Na^+ also affects EA accumulation. Thus, while increase of *extracellular* Na^+ enhances EA accumulation, the reverse holds true for *intracellular* Na^+—an increase of intracellular Na^+ diminishes EA accumulation. It should be noted that the magnitude of the observed effect of the increased intracellular Na^+ was blunted because sodium was actively extruded from the kidney slices during the incubation for EA accumulation, thus gradually increasing the Na^+ gradient towards the control state.

The effect of intracellular Na^+ on EA accumulation is also shown in Fig. 3, where 1/EA in slice is plotted against 1/EA in medium. Slices incubated in the same medium (140 mM Na) but preloaded with Na^+ showed decreased EA accumulation. For comparison the effect of probenecid on EA accumulation is also shown. Probenecid (at 10^{-4} M) increased the K_m of EA accumulation from 2.4×10^{-7} M to 1.1×10^{-6} M.

Inhibition of EA accumulation by ouabain in various species. The effect of ouabain on Na^+ extrusion in kidney cortex slices of various rodents was compared. As Fig. 4 shows, ouabain inhibited the active sodium extrusion to a different extent in kidney cortex of various rodents. For example in the rabbit, Na^+ efflux was completely abolished in the presence of ouabain (10^{-3} M), while in Acomys cahirinus, barely any inhibition of Na^+ efflux from kidney cortex was observed in the presence of ouabain. Thus, Na^+ transport in kidney cortex may result from two different mechanisms; one, dependent, on Na,K-ATPase and inhibited by ouabain, and another mechanism, independent of this enzyme, as shown for the guinea-pig kidney by Whittembury and Proverbio [6] and for the rat kidney by a recent report from our laboratory [5].

When the effect of ouabain on EA accumulation in kidney cortex slices was compared in these various rodents, we observed a correlation between the capacity of ouabain to inhibit Na^+ efflux (and thus to decrease the Na^+ gradient across the cell membrane) and the reduction in EA accumulation in the kidney cortex slices, as seen in Fig. 4.

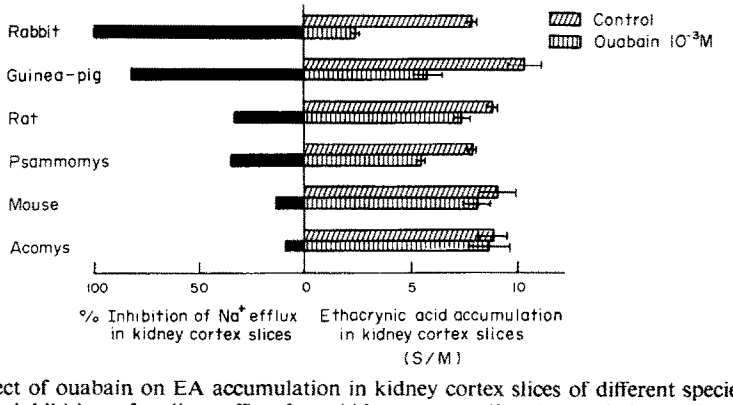

Fig. 4. Effect of ouabain on EA accumulation in kidney cortex slices of different species.
Left panel—inhibition of sodium efflux from kidney cortex slices by ouabain (10^{-3} M). Inhibition expressed as per cent of total Na^+ efflux from slices (in the absence of ouabain). Rabbit $N = 6$, guinea-pig $N = 6$, rat $N = 26$, Psammomys $N = 10$, mouse, $N = 14$, Acomys $N = 12$.
Right panel—EA accumulation in kidney cortex slices, in the absence (diagonally striped) and in the presence of 10^{-3} M ouabain (vertical stripes). S/M ratio of EA concentration in slices to EA concentration in medium. Vertical bars—S.E. Rabbit $N = 6$, guinea-pig $N = 6$, rat $N = 8$, Psammomys $N = 6$, mouse $N = 8$, Acomys $N = 6$.

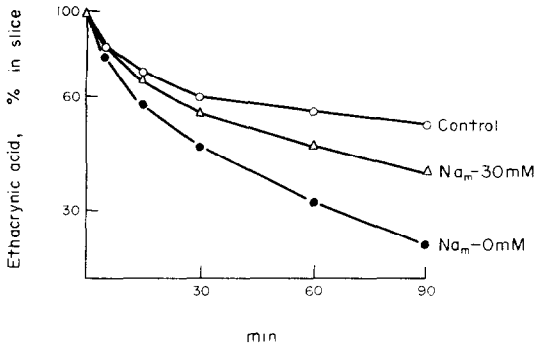

Fig. 5. Effect of Na^+ in medium on EA efflux from slices of rat kidney cortex.
Ordinate—EA remaining in slice as per cent of amount at beginning of efflux (zero time).
Control—Efflux of EA into medium containing 140 mM Na^+. Na_m, 30 mM: Efflux into medium containing 30 mM Na^+. Na_m, zero mM: Efflux into Na^+ free medium. Each point is mean of four experiments.

Effect of sodium concentration in medium on EA efflux from kidney slices. If EA transport in kidney cortex slices depends on a carrier mechanism, affected by Na^+, then one would expect efflux of EA to be subject to the same factors as influx. To study this effect, kidney cortex slices were loaded with [^{3}H]EA and then incubated in a medium free of EA. The rate of [^{3}H]EA efflux into the medium was followed. Figure 5 shows that when Na^+ concentration was decreased in the medium, [^{3}H]EA efflux was accelerated. This corroborates the assumption that EA movement across the membrane in kidney cortex involves a Na^+ activated carrier.

Further support for this assumption was found when efflux rate of EA was compared in kidney cortex and medulla slices. As we have previously reported, EA accumulation in kidney medulla was much smaller than in kidney cortex and was not affected by Na^+ concentration in the medium [1]. When slices of kidney medulla and of kidney cortex were first loaded with [^{3}H]EA and then efflux rate was followed, the efflux from the medullary slices

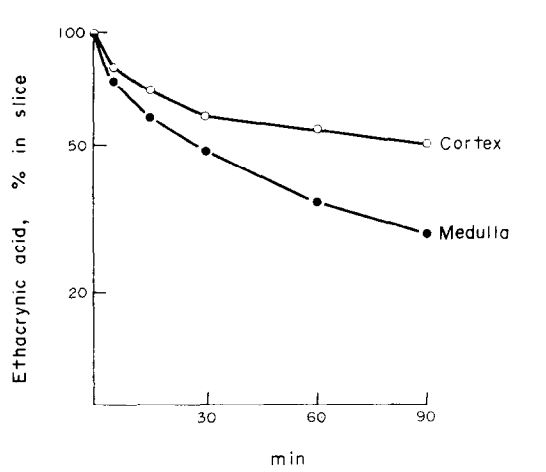

Fig. 6. Ethacrynic acid efflux from slices of rat kidney cortex and kidney medulla. Results expressed as in Fig. 5. Efflux into medium containing 140 mM Na^+. Each point is mean of four experiments.

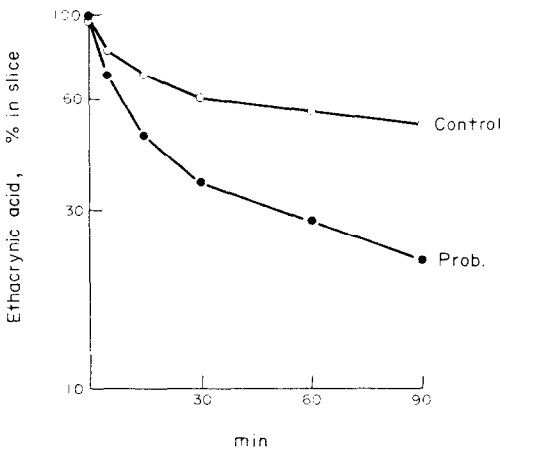

Fig. 7. Effect of probenecid on Ethacrynic acid efflux from rat kidney cortex slices. Results expressed as in Fig. 5. Efflux into medium containing 140 mM Na^+. Prob., efflux into medium with probenecid 10^{-3} M. Each point is mean of four experiments.

was faster than that from the cortical slices, as seen in Fig. 6. Furthermore, the efflux from medullary slices was independent of Na^+ concentration and was the same in a 30 mM Na^+ medium as in the 140 mM Na^+ medium (results not shown).

Effect of probenecid on EA efflux. Finally, the effect of probenecid on efflux of [^{3}H]EA from kidney cortical slices preloaded with [^{3}H]EA was studied. Figure 7 shows that probenecid, which inhibited EA accumulation, caused also acceleration of EA efflux from kidney cortex slices, similar to the effect of Na^+-free media.

DISCUSSION

The presence of Na^+ in the medium has been shown to be crucial for the transport of different classes of compounds; glucose and aminoacids in the intestinal mucosa [7], catecholamines in adrenergic nerve terminals [8], 5-hydroxytryptamine in platelets [9] and aminoacids in brain slices [10]. As an organic acid, EA seems to fall within the same pattern; reduction of sodium concentration in the medium inhibits EA transport into the slice (Fig. 1). The capacity to accumulate EA is limited to kidney cortex and is absent in the medulla [1], thus further suggesting that the organic acid transport mechanism is involved in EA uptake in kidney. Involvement of the organic acid transport mechanism is also supported by the finding that probenecid inhibited accumulation of EA in cortical slices (Fig. 3) but did not affect EA binding to medullary slices [1].

Furthermore, the transport of EA into kidney cortex slices showed saturation, as evidenced by the straight line in Lineweaver–Burke plotting (Figs 1 and 3), with a K_m of 2.4×10^{-7} M. Saturation kinetics would fit a carrier-mechanism. If the carrier-mechanism functions as in other cases of sodium-dependent transport of organic compounds [7] one would expect EA fluxes in both directions, i.e. efflux from slices as well as influx, to depend on sodium. This prediction was verified in the EA efflux experiments. Lowering of sodium concentration in the

medium increased the efflux rate of EA (Fig. 5). Therefore one can assume that the presence of sodium enhances EA binding to the carrier. When sodium is absent or low in the medium, EA binds poorly to the carrier and can not be transported into the cell. If EA has accumulated in the cell by pre-incubation, and its release into the medium is studied, high sodium concentration will prevent EA dissociation from the carrier and, therefore, will slow down EA efflux; lowering of sodium in the medium will enhance EA dissociation from the carrier and, therefore, will increase efflux rate (Fig. 5). A similar explanation can be given for the effect of probenecid; this compound acts as a competitive inhibitor of EA transport in the kidney cortex (Fig. 3). By displacing EA from the carrier, probenecid will enhance dissociation of the EA–carrier complex and, therefore, will increase efflux rate of EA (Fig. 7).

If the properties of the carrier do not differ when it faces the intracellular medium or the extracellular space, then one would expect intracellular sodium to affect the carrier–EA complex in the same way as extracellular sodium. An indication that this, in fact, is the case is seen in Fig. 2; an increase of intracellular sodium caused a reduction of EA accumulation, presumably because it slowed down the dissociation of EA–carrier complex on the intracellular surface of the membrane. A further support, though indirect one, for this explanation, was found in the effect of ouabain on EA accumulation in kidney cortex slices from different species; ouabain inhibition of EA accumulation varied widely from species to species but the degree of inhibition of EA accumulation was similar to the inhibition of the sodium pump in slices from kidneys of the same series of species (Fig. 4). Inhibition of the sodium pump results in increased intracellular sodium. Thus, EA accumulation was inhibited by ouabain mostly in the rabbit, where the sodium pump was the most sensitive to ouabain, while EA accumulation was not inhibited at all by ouabain in Acomys where ouabain barely affects sodium transport in the kidney cortex (Fig. 4).

The conflicting reports on the effect of ouabain on EA binding [1–3] can therefore reflect merely species differences in the sensitivity to ouabain or on the relative role of Na,K-ATPase vs other mechanism(s) of sodium transport in the kidney [5, 6].

Therefore, it seems that the direction of net EA transport, i.e. accumulation by slice or release into medium, depends on the relative concentration of sodium intracellularly and extracellularly, namely the sodium gradient.

In the intestinal mucosa it has been shown that the transport of glucose is not only dependent on the presence of sodium in the medium but also that sodium transport is enhanced when glucose is added to the medium [7, 11]. If a similar mechanism operates in the case of organic acid transport in the kidney, then part of the sodium accumulated in slices, incubated in the presence of EA, could be the result of co-transport of sodium and EA rather than a result of inhibition of a specific sodium pump in the cortex [5, 6]. Recent experiments in our laboratory support this assumption since we found sodium accumulation in kidney cortex slices incubated in the presence of another organic acid, probenecid (unpublished observations).

However, it should be noted that such accumulation of sodium developed only in the presence of relatively high concentrations of the organic acid (10^{-4}–10^{-3} M) and may reflect a different mechanism than that of the low concentrations of E.A. used in the accumulation studies (10^{-7}–10^{-8} M).

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