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A STUDY ON MECHANISM OF INTESTINAL PHOSPHATE TRANSPORT USING EVERTED GUT SACS OF MICE

[1,*]Mary Vincent Chirayath and [2]Prakasa Rao J.

[1]Department of Physiology, Annapoorna medical college, Veerapandy, Salem-636308, India
[2]Department of Physiology, American University of Antigua, College of medicine, University Park, P.O. Box W- 1451, Coolidge, Antigua, West Indies

***Corresponding Author**

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Abstract

Background & objective: The transcellular transport of phosphate from the intestinal lumen to the blood requires

- Phosphate uptake across the brush border of the enterocyte
- Its sojourn through the cytoplasm and
- Its exit across the basolateral membrane.

The *rate-limiting step* and the *main energy driving part* of absorption is first step. Extensive studies are carried out on the luminal phosphate uptake but not on the exit process of phosphate from the cell. The documented theory is that sodium and phosphate cross the brush border bound to a single bifunctional carrier according to the gradient created for sodium by the activity of Na^+-K^+ ATPase present on the baso-lateral membrane. So the present study is carried out on exit of phosphate from the baso-lateral membrane and its relationship to the functioning of Sodium Potassium ATPase and to sodium gradient.

Materials and methods: Everted gut sacs of 6cm of the proximal intestine were prepared from Swiss male albino mice. The sacs were filled with 0. 5ml of serosal fluid and placed in a mucosal medium. After incubation of an hour, the amount of phosphate removed from mucosal medium (mucosal uptake) and serosal gain of phosphate (phosphate release) into the serosal compartment are estimated according to Chen's method. Sodium substitution study was done on replacing sodium chloride by choline chloride to different degrees and the manipulation of the Sod. Pot. ATPase was done to check its role in phosphate transport with the addition of ouabain in serosal compartment, altering the potassium content of the serosal compartment and by the addition of adenosine.

Results: Shows that the uptake of phosphate remains unaffected until 90% of sodium was replaced in the incubation medium. However release of phosphate was impaired significantly at 75% of the normal sodium content ($p<0.001$). Ouabain inhibits the release of phosphate significantly and this inhibitory effect of ouabain is partly removed on stimulating Sod. Pot. ATP ase with adenosine and with excess potassium inside the serosal compartment.

Conclusion: Rather than phosphate uptake, it is the process of phosphate release from the enterocyte that requires sodium-potassium ATPase. A small gradient of sodium is only required for the brush border uptake of phosphate.

Keywords: Phosphate Transport, Ouabain, Intestine, Sodium-Potassium ATP Ase, Brush Border Membrane, Everted Gut sacs

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INTRODUCTION

Inorganic phosphorus is vital for various processes such as cell signaling, skeletal mineralization, energy metabolism, membrane function and nucleic acid synthesis (Berndt and Kumar, 2007; Tenenhouse, 2007). The small intestine and kidney are the two main organs primarily involved in phosphate homeostasis. The small intestine absorbs phosphate in a regulated manner from the chyme passed from the stomach. Despite the importance of extracellular phosphate, the mechanism and control of intestinal phosphate transport remain unclear.

Mechanism of intestinal phosphate transport

Phosphate absorption across the brush-border membrane (BBM) of the small intestine and kidney occurs against an electrochemical potential difference. The transport of phosphate from the intestinal lumen to the blood requires 3 steps.

- Phosphate uptake across the brush border of the enterocyte
- Its sojourn through the cytoplasm and
- Its exit across the baso-lateral membrane.

The active process of transfer of the phosphate anion from intestinal lumen to blood is inhibited by metabolic inhibitors (Berner *et al*., 1976; Gullans *et al*., 1982). The first step of phosphate uptake across the brush border is considered to be secondary active as the transport of phosphate into the enterocyte is coupled to the flux of sodium ions and is driven by the Na+ gradient (Baumann *et al*., 1975; Harrison and Harrison, 1963). Therefore Na+ gradient seems to be the rate-limiting step for intestinal brush border uptake and in renal proximal tubular phosphate (Pi) transport. The mucosal uptake of phosphate in small intestine and kidney is clearly documented to be sodium dependent by using brush border membrane vesicles (BBM) (Berner *et al*., 1976; Lee *et al*., 1986; Loghman-Adham *et al*., 1993). Phosphate absorption in various species such as chick, mice, rainbow trout and ruminants had been also studied. The Na+ dependent phosphate transporters present at BBM utilise the Na+ gradient established by the Na+/K+ ATPase at the baso-lateral membrane (BLM) (Lee *et al*., 1986; Loghman-Adham *et al*., 1993). The Pi that enters through the mucosal brush border along with Na dependent Pi transporters has to sojourn through the cell cytoplasm towards BLM as a second step. This is followed by the extrusion Pi across the BLM of the cell to get entry into the extracellular space and this is considered as release process (Matsumoto *et al*., 1980).

Objectives of the study

To summarize the mechanism of intestinal Pi transport, sodium and phosphate cross the brush border bound to a single bifunctional carrier according to the gradient created for sodium by the activity of Na^+-K^+ ATPase present on BLM. But no satisfactory explanation is available about Pi release mechanism from the serosal side. So phosphate efflux across the BLM of absorptive cells in the small intestine has to be characterized. Therefore the present study is carried out with the following objectives.

- To reinvestigate the documented theory of sodium dependent phosphate uptake and to check the role of sodium potassium ATP ase pump in this.
- To explore the nature of the exit process of Pi across the serosal side.

The time honored everted gut sacs of the proximal intestine prepared according to the method of Wilson and Wiseman, 1954, from Swiss male albino mice were used for this study. To check the sodium dependency of mucosal Pi uptake sodium chloride is replaced by choline chloride in the incubation medium to different degrees. Evaluation of the role of Sodium Potassium ATPase (Sod.pot.ATP ase) in phosphate transport was carried out by manipulating its activity through inhibition studies using ouabain and by stimulation studies altering the level of potassium chloride in the serosal compartment.

MATERIALS AND METHODS

Male Swiss albino mice of 3 months old (23-28 gm weight) obtained from institutional animal house were used for this study. Approval of the Ethical Committee of the institution was taken for carrying out this study. The animals were fed a standard laboratory diet (calcium 1%, phosphate 0.6%) for a week prior to experimentation.

After over night fasting, the mice were killed under ether anesthesia and everted gut sacs of 6cm length were prepared from the proximal part of the intestine according to the method of Wilson and Wiseman (1954). The sacs were filled with 0.5 ml of the incubation medium (serosal fluid) using a micro-syringe (Gastight syringe 1750, Hamilton Co: USA) and were placed in 25 ml Erlenmayer flasks with 5 ml of mucosal fluid. After oxygenation of the flasks with 100% O_2 for 1 minute they were tightly closed and incubated in a shaker bath (90-100 oscillations/min.) for 1 hr at 37^0 C. The medium used by Wasserman (Wasserman, 1960) to measure calcium transport was utilized for this study after slight modification. The incubation medium was of sodium phosphate buffer that contained (mM): NaCl, 135; KCl, 11 and $CaCl_2$, 0.04 dissolved in 2 mM phosphate buffer at 7.4 pH. After incubation, these sacs were emptied and the serosal fluid from the sacs and the mucosal fluid from the flasks were used for the estimation of phosphate.

Volume changes were taken into consideration in the chemical estimation. The initial serosal fluid content was determined as the difference between the weight of the empty sac and filled everted sac before incubation. The final serosal fluid content was calculated by subtracting the weight of the empty sac from that of the filled sac after incubation. The initial and final weights of empty sacs did not differ significantly. The amount of phosphate removed from the mucosal compartment is characterized as mucosal "uptake" while the serosal gain of the substance is considered as the "release". Uptake and release are expressed as µmol/gm tissue weight/hr. The method of Chen *et al* was employed to measure phosphate concentration in mucosal and serosal compartments.

Microdetermination of Pi by Chen *et al*. (1956): This method utilizes the greater sensitivity of the ascorbic acid for the determination of phosphorus in small quantity of whole blood, plasma, serum, urine and other samples. This method depends on the following principle. The phosphate is allowed to react with acid molybdate to form phosphomolybdic acid which is then reduced to molybdenum blue by ascorbic acid, the reducing agent. The color developed is stable for about 2 hours. The absorbance was read at 820 mµ using a spectrophotometer (Zeiss, model PMQ 11).

Trypan blue test: (Karsenty *et al*., 1985**)** Enterocytes were isolated mechanically by vibrating the drained gut sacs. These cells were then incubated with 0.2% trypan blue solution at 37^o to check the viability.80 to 90% of the mucosal cells showed the exclusion of the stain showing their viability.

Ouabain, theophylline and adenosine were obtained from Sigma Chemical Co.(St Louis, MO,USA).They were selectively used in the serosal compartments by dissolving them in the incubation medium at the following concentrations: ouabain (0.1and 1mmol/L), theophylline and adenosine (0.1mmol/L) as given in the legend. In experiments of sodium replacement studies, sodium chloride was replaced by choline chloride. In certain experiments potassium concentration in the serosal medium was altered at the expense of sodium. In experiments with ouabain to check if ouabain has the ability of metabolic inhibition lactate was analysed. Analysis was carried out using the method of Strom (Strom, 1949).This method is

based on the oxidation of lactic acid to acetaldyhyde by con.H_2SO_4. From acetaldyhyde, a violet colour is obtained on reaction with p-hydroxydiphenyl in the presence of the optimal concentration of cupric ions. The color developed was measured in a spectrophotometer at 560mμ.The original lactate concentration was calculated from a standard curve.

Statistical evaluation: Student's t – test was used for statistical evaluation. Values given in the results are the means ± SEM of six observations.

RESULTS

In brief when sodium chloride was replaced by choline chloride in both mucosal and serosal compartments, mucosal Pi uptake seems to be resistant. Significant decline is indicated in the uptake process only with very low NaCl concentration of 10 %. Further decrease in sodium level led to a steep fall in the uptake to nearly 10% of the normal value, on 100% replacement**.** But Pi release seems to be more sensitive to show a perceptible decline at 25% NaCl concentration and this was dropped to 10% of the control value on 100% replacement. The mucosal uptake of Pi from the medium remained unaltered in the above observations on addition of ouabain in the serosal compartment. But presence of ouabain showed a significant reduction in Pi extrusion process (by about 75%) into the serosal compartment showing the inhibitory effect of ouabain on the release process of Pi only. The lactate production was not altered by ouabain. Therefore the attenuating effect of ouabain on Pi release process is not due to metabolic inhibition as the lactic acid output from the sacs did not show any change. The magnitude of inhibitory response remained the same when the dose of ouabain was stepped up from 10^{-4}M to 10^{-3}M.Therefore ouabain of 10^{-4}M is utilized in the following experiments thereafter.

Table 2 shows the effect of changing KCl concentration of the serosal medium on the Pi transport by the everted sacs of mouse intestine. Alteration of potassium level in serosal compartment could not bring about any change in the mucosal uptake of Pi. However reduction or elevation of potassium chloride of the medium filling the serosal compartment are attended by similar changes in the release of Pi from the gut wall into the serosal compartment ie when the lack of potassium caused a reduction in the release of Pi into the serosal compartment the reverse caused a stimulatory effect on the release of Pi.

Table 3 shows the ability of the glycoside, ouabain to restrict the release process of Pi significantly to that of the control value in the 1st row. This inhibition of ouabain was reduced remarkably by increasing the potassium level of the serosal fluid as shown by the 4th row of observation in Table 3. Such an evidence clearly points out that the action of ouabain in the Pi release is mediated through inhibition of Sod. Pot. pump, since potassium can overcome the action of ouabain. Studies of sodium chloride replacement with choline chloride shows that Pi release process is much more sensitive to sodium lack than the uptake process. It must be remembered that these maneuvers also affect the functioning of Sod.Pot. ATPase by altering the availability of the cations at their respective high affinity site of the enzyme. Stimulation of the pump by increasing the availability of potassium at the serosal site enhanced the phosphate release. Reduction of sodium chloride to 25% of the normal (5th row) and the presence of ouabain with the normal sodium chloride (2nd row) leaves mucosal Pi uptake unaltered. But ouabain was effective in inhibiting Pi uptake process in combination with reduced sodium chloride of 25%.Ouabain's inhibitory action on release process also becomes pronounced under this condition. On addition of adenosine to the serosal compartment, an agent capable of increasing ATP level in the cell has promoted the exit of phosphate from the gut sacs. This action is not blocked by theophylline, a blocker of purinergic receptors.

DISCUSSION

Reasons for the usage of everted gut sacs

It is true that the usage of isolated membrane vesicles has vastly improved our knowledge regarding the brush border uptake of many substances from intestine and renal proximal convoluted tubules. But some investigators (Hammerman, 1986; Murer and Hildmann, 1981) feel that studies with disintegrated systems in artificial environments should be revalidated with systems that are close to physiological conditions. The technology for the isolation of uniform population of tightly sealed, right side out brush border vesicles (BBM Vesicles) has been perfected but similar success has not been achieved with baso-lateral membranes which are needed to study the release process from the serosal side (Gmaj *et al*., 1984). Moreover, the membrane vesicles are not of much use to study the actions of agents that are mediated through intracellular mechanisms.

These above considerations and the extreme simplicity of the technique of everted gut sacs enabling one to measure mucosal uptake of a substrate and its release into the serosal compartment led to choose the method of everted intestinal sacs for this study of phosphate transport in small intestine. The kinetics of phosphate transfer by the everted gut sacs in certain studies were also comparable to the results obtained with isolated membrane vesicles (Fuchs and Peterlik, 1980; Peterlik *et al*., 1981). Viability test was carried out for the isolated enterocytes with trypan blue after the incubation of the everted gut sacs and the viability of the cells after incubation was confirmed. There are other studies too to prove the viability of the everted gut sacs for up to 120 minutes (Bridges *et al*., 1978; Barthe *et al*., 1998).

Sodium dependent phosphate transporters of BBM

Enterocytes undergo a process of maturation during transit from the crypt of Lieberkuhn to villus tip. Only those enterocytes located in the mid to upper region of the villi are responsible for phosphate uptake (Marks *et al*., 2007). These are the only cells that express sodium phosphate transporters (Berndt *et al*., 2007; Marks *et al*., 2006; Marks *et al*., 2007) on BBM. The Pi transporters of BBM function with the Na+ gradient established by the Na+/K+-ATPase at the baso-lateral membrane (BLM) (Forster *et al*., 2006). So far, three different Na+ dependent phosphate transporter families have been identified. These transporters differ in their amino acid sequence, affinity for phosphate and mechanisms controlling their activity and tissue expression. These Pi transporters are

designated as NaPi-11b, PiT1 and PiT2 (Prasad and Bhadauria, 2013). NaPi-IIb is the key player in the intestinal transport of phosphate (Sabbagh *et al*., 2009) and 1, 25 dihydroxy-cholecalciferol enhances intestinal Pi absorption via increased NaPi-11b expression (Ganong, 2010). In mice NaPi-IIb is responsible for over 90% of total active phosphate absorption (Sabbagh *et al*., 2009).

Requirement of sodium and sodium gradient for Pi transport

If Na+ gradient is likely to be the rate-limiting step for intestinal phosphate transport deficiency of sodium or decrement of sodium gradient across the brush border should be able to curtail the mucosal uptake of phosphate in intestine. ie lowering of sodium chloride in the medium or inhibiting the Sod.Pot.Pump by ouabain responsible for maintaining low intracellular sodium level and thereby the sodium gradient , is expected to cause a fall in Pi uptake.

But the substitution studies of sodium chloride with choline chloride in these experiments (Graph-1) shows no affect on Pi uptake process significantly .Only when sodium chloride was brought down to 10% of the normal value in the incubation medium mucosal Pi uptake was affected. So the effort to alter the sodium gradient by substitution with choline shows that phosphate uptake is remarkably resistant even when sodium level drops substantially to 25% of the normal.

However at 25% of the normal level of sodium, if ouabain is used a significant fall in uptake of Pi could be demonstrated (refer to 6th row of Table-3). But ouabain with normal incubation medium fails to affect the Pi uptake process (refer to Table - 1). These results indicate that while phosphate uptake is sodium dependent, the cation gradient needed to drive it is indeed very small!.

Table 1. Effect of ouabain on phosphate transport and lactate release

Ouabain (dose)	(Pi uptake) µmol/ gm tissue wet weight/ hr	(Pi release) µmol/ gm tissue wet weight/ hr	(Lacatate release) µmol/gm tissue wet weight/hr
None	9.31±0.09	5.31± 0.04	0.86± 0.02
10 -4M	9.22 ± 0.11	**1.56± 0.05	0.91± 0.06
10 -3M	9.11± 0.08	**1.51± 0.03	0.81 ± 0.02

Table 1. Shows the effect of adding ouabain to the serosal compartment of the everted gut sacs prepared from the proximal part of the intestine. Each value is the mean of six observations. ** marked values are significantly less than the top values of the control group. (P< 0.001, t-test)

Table 2. Effect of altering the concentration of potassium chloride in the serosal compartment of everted gut sacs of mice (keeping 11mM of normal KCl in mucosal compartment)

Concentration of KCl (mM) in serosal fluid	(Pi uptake) µmol/gm tissue wet weight / hr	(Pi release) µmol /gm tissue wet weight /hr
(Normal) 11	9.34 +0.06	5.30 +0.03
0	9.28 +0.08	**2.45 +0.02
33	9.41 +0.06	**6.31 +0.07

Table 2. Each value is the mean of six observations. ** marked values are significantly less than the top control value (P< 0.001, t-test).

Table 3. Effect of ouabain with altered level of sodium and potassium chloride concentration of the incubation medium.

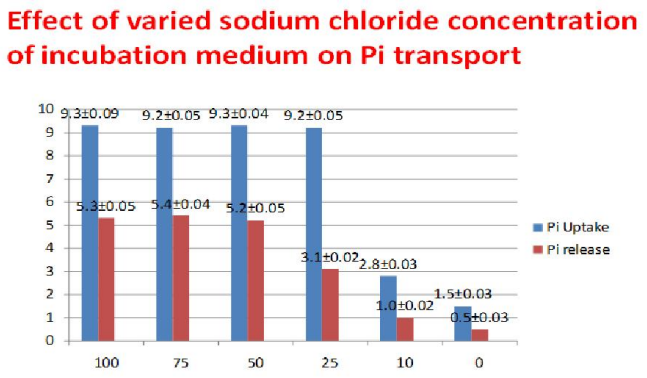
Nature of the incubation medium	Presence(+) or absence (-) of ouabain	(Pi uptake) µmol gm / tissue wet weight/ hr	(Pi release) µmol/ gm tissue wet weight/ hr
1. Normal (in both compartments) - control	-	9.31 + 0.09	5.31 + 0.04
2. Normal in both compartments	+	9.14 +0.08	**1.52 + 0.03
3.33mM KCl (in serosal side only)	-	9.32 + 0.06	**6.31 + 0.07
4. 33mM KCl (in serosal side only)	+	9.21 + 0.08	**4.09 + 0.03
5. 25 % Sodium Chloride (In both compartments)	-	9.20 +0.05	**3.10 + 0.04
6. 25 % Sodium Chloride (in both compartments)	+	**1.45 + 0.01	**0.65 +0.02

Presence of ouabain (10 -4M) in serosal comprtment is indicated by + sign in the second column .Concentration of sodium and potassium chloride is altered in the mucosal and serosal compartments as mentioned under the nature of the incubation medium in the 1st column. Each value is the mean of six observations. ** marked values are significantly different from that of the top control value (P< 0.001, t-test). Pi release value of 4th row is significantly elevated to that of Pi release of 2nd row with ouabain (P< 0.001, t-test) . Last row shows the significant inhibitory effect of oubain on Pi uptake and on Pi release at 25% sodium chloride concentration of the incubation medium (P< 0.001, t-test)

Table 4. Effect of adenosine on Pi transport of everted gut sacs of mice

Additive to serosal content	(Pi uptake) µmol /gm/ tissue wet weight /hr	(Pi release) µmol/ gm/tissue wet weight /hr
None	9.41 +0.15	5.31 + 0.06
Adenosine (10 -4M)	9.35 +0.08	**6.42 + 0.05
Theophylline (10 -4M)	9.42 +0.10	5.24 +0.03
Adenosine (10 -4M) + Theophylline (10 -4M)	9.34 + 0.04	**6.34 +0.06

In Table 4, each value is the mean of six observations. ** marked values are significantly different from that of the top control Value (P< 0.001, t-test).



Graph 1.

Graph 1 shows the results of the substitution studies of sodium chloride with choline chloride. 'X' axis shows the % of NaCl in incubation medium on replacement with choline chloride. 135 mM of NaCl is taken as 100% .'Y' axis gives Pi uptake (blue) and Pi release (red) as µmol/gm tissue wet weight/hr along with ± SEM. Each value is the mean of six observations. Analysis of Pi in the mucosal and serosal compartments showed mucosal uptake of phosphate (Pi) to be 9.3 ±0.09 and Pi release as 5.3±0.05 µmoles /gm.tissue wet weight/hr with normal incubation medium. Mucosal Pi uptake values with sodium chloride of 10% and 0% in incubation medium are significantly less to the control value (P< 0.001, t-test). Similarly Pi release values are significantly less to the control values on reducing the NaCl concentration to 25% onwards in the medium. (P< 0.001, t-test) .

This also point out the possibility of the contribution of Na+ independent paracellular transport involved in intestinal phosphate absorption apart from the transcellular transport.
Certain studies on sodium dependent transport of other substrates show similar findings. Robinson, 1970 failed to observe an attenuating action of ouabain on sodium dependent transports of phenyl alanine and galactose at a dose which almost completely inhibited Sod. Pot. ATP ase. Several explanations have been suggested to account for the failure of ouabain to restrict sodium dependent transports.

Possible existence of some other pump responsible for sodium extrusion other than Sod.Pot. ATP ase to maintain the sodium gradient; ability of a small sodium gradient that remained even in the presence of ouabain to drive the transport; metabolic inhibition by ouabain on certain substrates thereby affecting the transport of other substrates are some of the explanations provided (Farber *et al*., 1989; Newey *et al*., 1968; Proverbio *et al*., 1970). The affinity of different carriers (of glucose, galactose, aminoacids etc.) for sodium may be different, so that the carrier with a low affinity for sodium will be affected first by ouabain.

Pi release phenomenon into the serosal compartment seems to be more sensitive to sodium gradient (refer to Graph-1) in these studies**.** Presence of 25% sodium chloride attenuated the release process of Pi into the serosal compartment. It is interesting to note that Taylor (Taylor, 1974) employing everted sacs of chick intestine noted that ouabain did not affect the uptake of phosphate at a normal sodium concentration. But he did not try to study the effect of glycoside at lesser concentration of sodium. He felt that lack of effect of ouabain may be due to incomplete inhibition of Sod.Pot.ATPase. However Taylor reported an interesting finding that ouabain, while leaving the Pi uptake unaffected decreased the release of Pi by the gut sacs of the chick.
The studies shown in Table 3 (refer to 3rd and 4th rows) shows that the inhibition of ouabain on Pi release was reduced remarkably by increasing the potassium concentration of the serosal compartment. Potassium has been shown to displace ouabain from its receptors and thereby reactivate Sodium-Potassium ATP ase (Dunham and Glynn, 1961). Therefore, it is suggested that ouabain induced inhibition of Pi release may be due to the direct inhibition of Sod. Pot.ATPase, indicating the role of the pump in the release process. So it is the stimulation of this enzyme by increasing the availability of potassium at the serosal side enhanced the phosphate release. Similarly on blocking Sod.Pot ATPase with strophanthidin could bring significant effect in phosphate efflux of non-myelinated nerve fibres (Ritchie and Straub, 1979). It is not due to metabolic inhibition of ouabain if any, because the lactic acid output from the sacs did not show any change (refer to Table-1).

Adenosine, an agent capable of increasing ATP level in the cell (Lund *et al*., 1975; Weinberg *et al*., 1988) has promoted the exit of phosphate from the enterocyte. It is possible that ATP so generated has activated the Sod.Pot.ATP ase resulting in an increase in the extrusion of phosphate from the enterocytes. This points out to a possible role of energy requirement for the Pi release process. This effect of adenosine is not mediated through the purinergic receptors, as theophylline, the receptor blocker (Rall, 1980) has failed to block the action of adenosine.

Conclusion

- Intestinal Pi uptake process requires only a small amount of sodium and Pi release process from BLM seems to more sensitive to sodium deficiency in comparison to Pi uptake.
- Though the usage of cardiac glycoside, ouabain knocks off Na-K ATP ase mucosal Pi uptake continuous to be normal. This suggests that the small gradient of sodium present in the presence of ouabain drives the uptake process.
- Pi release from the serosal surface is easily altered on manipulating the activity of Sod.Pot.ATP ase with the usage of ouabain, on raising the potassium concentration of the serosal medium and with the usage of adenosine. This points to the possible involvement of Sod.Pot.Pump in the extrusion of Pi from the intestine. But the exact nature of this role is not clear yet.

Therefore the last two steps of phosphate transport across intestinal enterocytes ie phosphate sojourn to the serosal side of the cell, followed by phosphate extrusion across the basolateral membrane has yet to be characterized (Matsumoto et al., 1980).The theories so far proposed for the Pi extrusion process are such as: an anion exchange mechanism, a type 3 transporter and an "unspecific" phosphate leak pathway (Tenenhouse, 2007).
Importance of this study: Chronic renal failure does not affect the intestinal handling of phosphate (Douard *et al*., 2010; Marks *et al*., 2007). But when dietary phosphate intake is restricted in chronic renal failure rats, circulating PTH level is lowered. These studies show no change in intestinal phosphate absorption or NaPi- IIb mRNA level (Douard *et al*., 2010; Marks *et al*., 2007). So small intestine is a promising target in the prevention and treatment of hyperphosphataemia. For this, our knowledge has to be widened out about the process involved in phosphate absorption across the intestinal tract.

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