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RESEARCH ARTICLE

REGIONAL VARIATION OF ENERGY SOURCES IN INTESTINAL PHOSPHATE TRANSPORT USING EVERTED GUT SACS OF MICE

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

[1]Professor of Physiology, Annapoorna Medical College, Veerapandy, Salem-636308
[2]Professor of Physiology, American University of Antigua, College of Medicine, University Park, P.O.Box W- 1451, Coolidge, Antigua, West Indies

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ABSTRACT

Background & Aim: The transcellular transport of phosphate (Pi) from the intestinal lumen to the blood is documented as a secondary active transport requiring energy. In renal tubules the source of ATP generation for Pi transport seems to vary with site of transport. As studies are not available showing energy sources of intestinal Pi transport an attempt is done in this study to explore the metabolic energy source related to intestinal Pi transport.

Methods: Everted gut sacs of the proximal & distal intestine were prepared from Swiss male albino mice. The sacs were filled with 0. 5ml of serosal fluid and placed in a mucosal medium. Various metabolic blockers of glycolysis and oxidative phosphorylation like 2DG, monoiodoacetate and rotenone were added to the incubation medium to curtail the energy sources. Tricarboxylic acid intermediates, Succinate and fumarate were also used in the medium to stimulate ATP generation. After incubation of the filled sacs for an hour, the amount of phosphate removed from mucosal medium (Pi uptake) and serosal gain of phosphate (Pi release) were estimated according to Chen's method.

Results: In these experiments phosphate uptake remains unaffected in both proximal and distal everted gut sacs with varied metabolic blockers. However addition of 2DG and monoiodoacetate significantly (<.001) reduced Pi release from the proximal segments without affecting Pi release in distal segments. Rotenone significantly (<.001) lowered Pi release in distal segments only without affecting the proximal segments. Inhibitory effect of rotenone on Pi release of the distal segments was partly but significantly checked by succinate and fumarate.

Conclusion: Present studies indicate that the process of Pi extrusion/release from basolateral membrane only requires energy. For Pi release proximal intestine depend on glycolysis and distal part depend on oxidative phosphorylation for energy showing the regional variation.

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INTRODUCTION

Despite the plethora of knowledge on the mechanisms and regulation of renal phosphate (Pi) transport, less is known about the intestinal phosphate absorption. This is because, in contrast to the kidney, hormonal regulation of intestinal phosphate transport was thought to play only a minor role in phosphate homeostasis. But recently it is found that intestinal cells also may have a role in renal phosphate handling through elaboration of circulating phosphaturic substances in response to sensing a phosphate load (Wolf, 2012). It is also shown that the sodium-phosphate transporter, ie NaPi-IIb the key player involved in intestinal Pi transport present in the brush border membrane to be involved in the sensing of dietary phosphate composition to modulate renal phosphate reabsorption to achieve phosphate balance (Grace and Joanne, 2015).

Corresponding author: Mary Vincent Chirayath,
Professor of Physiology, Annapoorna Medical College, Veerapandy, Salem-636308.

Clinical relevance of intestinal phosphate transport

Phosphate consumption can vary significantly depending on food choice and ingestion of processed food containing high levels of Pi preservatives that may lead to supra-physiological postprandial spikes in blood Pi levels posing a long term cardiovascular risk (Takeda *et al*., 2012). Phosphate imbalance and its altered homeostasis can result in calcium phosphate deposition in blood vessels leading to stiffening of arteries and

myocardial dysfunction and an increased risk of cardiovascular diseases. Similar type of Pi toxicity is widely recognized to occur in patients with chronic kidney diseases (CKD). For a number of years there has been mounting interest in the concept of directly targeting intestinal phosphate transport to control hyperphosphatemia in conditions as in CKD (Marks *et al*., 2013). However, progress has been slow due to paucity of information on the mechanisms involved in intestinal Pi absorption. Hypophosphatemia is less common in adults but it may occur due to malnutrition, malabsorption or inherited disorders affecting phosphate homeostasis. Prolonged Pi deficiency is associated with bone demineralization leading to osteomelacia in adults and can increase the risk of nephrolithiasis, rhabdomyolysis, hemolysis , respiratory failure from muscle weakness, reduced myocardial contractility etc (Amanzadeh and Reilly, 2006). Hypophosphatemia got a high impact in pediatric patients due to high demand for Pi and calcium to support skeletal and somatic growth.

Role of Na dependent phosphate transporters in Pi transport

Phosphate absorption across the brush-border membrane (BBM) of the small intestine and kidney occurs by a Secondary Active Transport. The movement of phosphorus from the intestinal lumen to the blood consists of 3 steps. First step is the transport of Pi across the luminal brush-border membrane of the intestine followed by its transport through the cytoplasm and its extrusion process across the baso-lateral plasma membrane (BLM) of the epithelium. The rate-limiting step and the main driving force of absorption is the first step through luminal membrane (Lee *et al*., 1986; Loghman *et al*.,1993; Levine and Kleeman,1994). Phosphate and sodium ions cross the brush border membrane bound to a single bifunctional carrier (Harrison and Harrison 1963; Baumann *et al.*, 1975). These carriers are the Na dependent phosphate transporters present in the enterocytes. The reduced calcitriol decreases intestinal expression of these intestinal Pi transporters and phosphate absorption (Inoue *et al*., 2005). Polyvalent cations present in the diet such as Ca^{2+}, Mg^{2+}, and Al^{3+} bind to intestinal luminal phosphate and decrease its absorption. Hence phosphate binder is prescribed in the patients with renal failure along with meals.

Nature of Pi transporters and the clinical importance

Certain enterocytes during the process of maturation as they transit from the crypt of Lieberkuhn to villus tip express Na dependent phosphate transporters. These enterocytes with Na Pi transporters are responsible for phosphate uptake (Marks *et al.*, 2007). It is the type II transporters that are thought to play a key role in phosphate transport across the small intestine and kidney. NaPi-IIb is the key player in the intestinal transport of phosphate (Sabagh *et al*., 2009) and it is a N-linked glycoprotein that is glycosylated during the suckling/weaning transition, a process thought to be important for its plasma membrane expression (Arima *et al.*, 2002; Hayes *et al.*, 1994). In NaPi-IIb knockout mice, Na+ dependent phosphate transport was not evident in the small intestine, indicating that PiT1 and PiT2 transporters are not likely to play an important role in intestinal phosphate absorption (Sabbagh *et al.*, 2009). NaPi-11b also has been shown to play a major role during embryogenesis and ontogenesis (Shibasaki *et al*., 2009).There is a critical role for NaPi-11b in intestinal transport and Pi homeostasis during ontogenesis reflecting the higher Pi requirement for normal skeletal growth and development. Deletion of NaPi-IIb results in developmental arrest and fetal death (Ohi *et al*., 2011; Shibasaki *et al*., 2009).

Pi transporters of kidneys

NaPi-IIb is not expressed in the kidney (Hilfiker *et al.*,1998). Renal Pi transport is mediated by the Na+ dependent Pi transporter, NaPi-IIa and NaPi-IIc expressed at the BBM. The kidney specifically expresses NaPi-IIa at the apical membrane of proximal tubular cells, which is largely responsible for the maintenance of phosphate homeostasis (Murer *et al.*, 2000; Murer *et al.*, 2004; Tenenhouse and Murer, 2003). This corresponds to a high Na+ dependent phosphate transport in the proximal tubules, especially in the early segments S1 and S2 (Greger *et al.*, 1977; Kayne *et al.*, 1993; Baumann *et al.*, 1975).

The specific targeting of NaPi-11b and inhibitors of intestinal Pi transporters

NaPi-11b and inhibitors of intestinal Pi transporters were targeted to reduce Pi absorption and this has been a concept that received significant attention over recent years. Compounds such as nicotinamides and phosphonoformic acid (PFA) which competitively inhibit Na phosphate transporters in vitro have been tried out in hyperphosphatemic conditions. In one such study, nicotinamide administered daily to CKD rats as well as in CKD patients intestinal Pi uptake was found to be low associated with improvement in hyperphosphatemia (Eto *et al*., 2005). Hemodialysis patients receiving nicotinamide also exhibited emelioration of hyperphosphatemia but showed gastro-intestinal side effects (Takahashi *et al*., 2004).

Regional variation of intestinal Pi transporters

The formation of intestinal NaPi-IIb is regulated by the factors like level of 1,25 dihydroxy-cholecalciferol , dietary levels of phosphate uptake and age. When the age factor is concerned Na+ dependent phosphate transport and NaPi-IIb expression dramatically decreases with age (Xu *et al.*, 2002; Arima *et al.*, 2002). This regulation is both species- and region-specific in the small intestine, since different regions of the small intestine express distinct levels of NaPi-IIb across species (Huber *et al.*, 2002; Huber *et al.*, 2006; Marks *et al.*, 2006). So a regional variation of Pi transport is expected in the intestine.

Role of Sodium Potassium ATPase in Pi transport

The Na+ ion concentration within the intestinal cells is normally much lower than in the extracellular or luminal fluid. Therefore the sodium ions enter the cells by the facilitated diffusion down the concentration gradient. Simultaneously phosphate bound to the carrier also enters the cell. This process continues as long as the sodium gradient is kept up. So Na+ gradient is the rate-limiting step for intestinal and renal Na+ dependent phosphate transport according to the documented

theory. For the continued uptake of phosphate, it is necessary that the intracellular level of sodium is kept relatively low by the activity of the sodium/potassium pump situated in the baso-lateral membrane of the enterocyte. This sodium pump expels sodium ions from the inside of the cell in exchange for potassium ions from outside so that relatively a small concentration of sodium ions and a high concentration of potassium ions remains inside the cell relative to the external environment. The pump is closely associated with sodium-potassium stimulated ATPase activity. Intracellular metabolism provides the required ATP for the functioning of this pump. Thus Pi uptake initiated by Na+ dependent phosphate transporters present at the brush border membrane (BBM) utilize the Na+ gradient established by the Na+/K+-ATPase at the BLM (Forster *et al.*, 2006).

Energy requirement for Pi transport

As mentioned earlier Pi transport being a secondary active transport it needs energy in order to maintain a low intracellular concentration of sodium for the active transport of this ion by the Sodium Potassium ATPase. This pump is kept going by the ATP generated in the cell by various mechanisms. However, the source of ATP for any particular transport seems to vary with the substrate and the site of transport. Brazy *et al*. (1980) have shown that in proximal convoluted tubules from rabbit kidneys, the Pi transport derives energy from oxidative phosphorylation. Inhibition of oxidative phosphorylation seem to affect Pi transport much more than fluid and glucose transport in the tubules. Certain tricarboxylic acid intermediates (TCA intermediates) like succinate and maleate- which are capable of stimulating oxidative phosphorylation and gluconeogenesis are capable of increasing Pi transport. However the usage of 3-mercaptopicolinate, a blocker of gluconeogenesis failed to block the increase in Pi transport induced by TCA intermediates, eliminating the role of gluconeogenesis in the above action (Gullans *et al*., 1984). These authors also demonstrated a correlation between stimulation of Pi transport and mitochondrial respiration caused by succinate in the rabbit renal tubules.

Aim and objectives of the study

While studies are present showing energy sources related to Pi transport in kidneys, reports on energy sources for intestinal Pi transport are not available. Hence experiments are conducted to throw light in this area and is presented in this study. As regional variation is reported for intestinal Na dependent Pi transporters Pi transport is expected to vary in the different regions of the intestine. So regional Pi transport was carried out from the different segments of the intestine in order to choose the appropriate intestinal regions with maximal Pi transport . The present study is carried out with the following objectives.

Objectives

1. To check the regional variation in Pi transport in the different segments of the intestine using everted gut sacs of mice and to choose the appropriate segments with maximal Pi transport for the study.
2. Pi transport is known to be a secondary active transport depending on energy. So check the effects of curtailing the different energy sources on Pi transport in different regions of the intestine by the usage of metabolic blockers of glycolysis and oxidative phosphorylation
3. To check the effect of succinate and fumerate (TCA intermediates that stimulate oxidative phosporylation) on Pi transport in different parts of the intestine with and without the blocker of oxidative phosphorylation.

MATERIALS AND METHODS

This study is carried out using everted gut sacs of mice from male albino mice of 3 months old weighing 24 to 30 gm. Mice were fed on a standard laboratory diet obtained from Gold Mohur laboratory feeds, Bangalore (calcium 1%, phosphate 0.6%) for a week prior to experimentation. Everted gut sacs of 6cm length were prepared from the intestine after over night fasting and killing the mice under ether anesthesia. Method of Wilson and Wiseman (1954) was used for preparing the everted gut sacs.

Procedure for preparation of everted gut sacs of mice

For the preparation of the everted gut sacs, intestine extending from the pyloric end to the ileo-caecal junction was removed carefully from the mice killed under ether anesthesia. Eversion of the intestine was done with a steel rod with deep groove on the distal end (refer to Mary and J.Prakasa Rao, 2015b). To study Pi transport in the entire intestine, the intestine was segmented from the pyloric end to the ileocaecal junction and 6cm long gut sacs were prepared successively. Totally five gut sacs of 6cm length could be prepared from the entire intestine and they are numbered successively as 1, 2, 3, 4 and 5 from the pyloric end. On analysis of the data of Pi transport obtained from these different sacs, the first gut sac from the pyloric end and the last gut sac near the ileo-caecal junction (numbered 1 and 5 in Fig.1) were chosen for the further experiments as these showed Pi transport maximally.

These gut sacs are referred as proximal and the distal segments/sacs representing the initial and the terminal part of the small intestine. 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 the ligature was tightened. Everted gut sac was 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 metabolic shaker bath (Techno India Ltd, Pune, India) with constant shaking at a frequency of 90-100 oscillations/min. for 1 hr at 37^0 C. The incubation medium was of sodium phosphate buffer that contained (in mM) : NaCl, 135; KCl, 11 and $CaCl_2$, 0.04 dissolved in 2 mM phosphate buffer at 7.4 pH.

After 1hr of incubation, these sacs were removed from the flasks, blotted and weighed again. The serosal fluid was emptied from the sacs and this serosal fluid and the mucosal fluid from the flasks were used for the estimation of phosphate. The fall in phosphate content of the mucosal medium is considered as phosphate uptake by the sac while rise of Pi

content of the serosal fluid as phosphate release. The method of Chen *et al.* (1956) was employed to measure phosphate concentration in mucosal and serosal compartments.

Statistical comparisons were carried out using Student's t-test. All the values are expressed as mean ± S.E.M of six observations in each group. Uptake and release of Pi are expressed as µmol/gm tissue wet weight/hr. Plasma level of Pi in mice was found to be 2.14+0.02 mMol/L. For this blood was collected from the retro-orbital sinus of the eyes after giving light ether anesthesia.

Usage of metabolic blockers and tricarboxylic acid intermediates

Metabolic blockers were used to check the source of energy of the Na-K ATPase involved in Pi transport. Glycolytic blockers of 2-DG and monoiodo- acetate were used to curtail glycolysis to test the effect of blocking glycolysis. For knocking off the ATP production from oxidative phosphorylation rotenone was used. Similarly to enhance ATP production from oxidative phosphorylation TCA intermediates, succinate and fumerate were used. Details of the concentration and the addition of the metabolic blockers and TCA intermediates are given in the concerned legends to the figures and tables.

Viability test (refer Mary & J.Prakasa Rao, 2015b) of the mucosal cells were carried out using trypan blue according to Karsenty *et al*. (1985).

Chemicals

All chemicals were of analar grade. All the metabolites and metabolic blockers used in this study were purchased from Sigma Chemical Company, St. Louis, MO,USA.

Approval of the Ethical Committee of the institution was taken for carrying out this study

RESULTS

Preliminary study carried out on the segmented entire intestine clearly indicates the regional variation in intestinal Pi transport. Segments numbered 1 from the pyloric end and 5 from the ileo-caecal end showed the maximum Pi transport (Figure 1). Hence these segments were utilized for this study under the terms 'proximal segment' (the adjoining duodenal segment close to the stomach) and 'distal segment'(segment near to the ileo-caecal junction). The addition of succinate or fumerate, the TCA intermediates that enhance oxidative phosphorylation did not affect Pi uptake or release by the proximal segments of the intestine (refer to Tables 1a and 1b.).

However in the distal segments, the addition of these compounds led to an increase in Pi release (refer to Table 1b.) while the Pi uptake remained unaffected. Effects of glycolytic blockers 2-DG , monoiodo-acetate and that of rotenone, the blocker of oxidative phosphorylation are shown in Figures 2a and 2b.The results indicate that Pi uptake by either proximal or distal segments remained unaffected in presence of different metabolic blockers. However Pi release in the proximal segments is significantly reduced by the glycolytic blockers, 2-DG and monoiodo-acetate without affecting the Pi release in distal segment. But rotenone, the blocker of oxidative phosphorylation significantly lowered Pi release in distal segments only without affecting the Pi release in proximal segment ie various metabolic blockers have no influence on the process of Pi uptake in the different parts of the intestine but inhibit the process of Pi extrusion /Pi release selectively. On knocking off glycolysis Pi release process of proximal segment declined leaving this process uninterrupted in distal segments.

On the other hand in distal segments Pi release process was enhanced on stimulating oxidative phosphorylation and it was inhibited on blocking oxidative phosphorylation leaving the proximal segments intact. Rotenone also curtailed the rise in Pi release mediated by succinate &fumerate partly on the intestinal distal segment.

Table 1a. Effect of succinate and fumerate on Pi uptake by the mouse everted gut sacs of proximal and distal segments

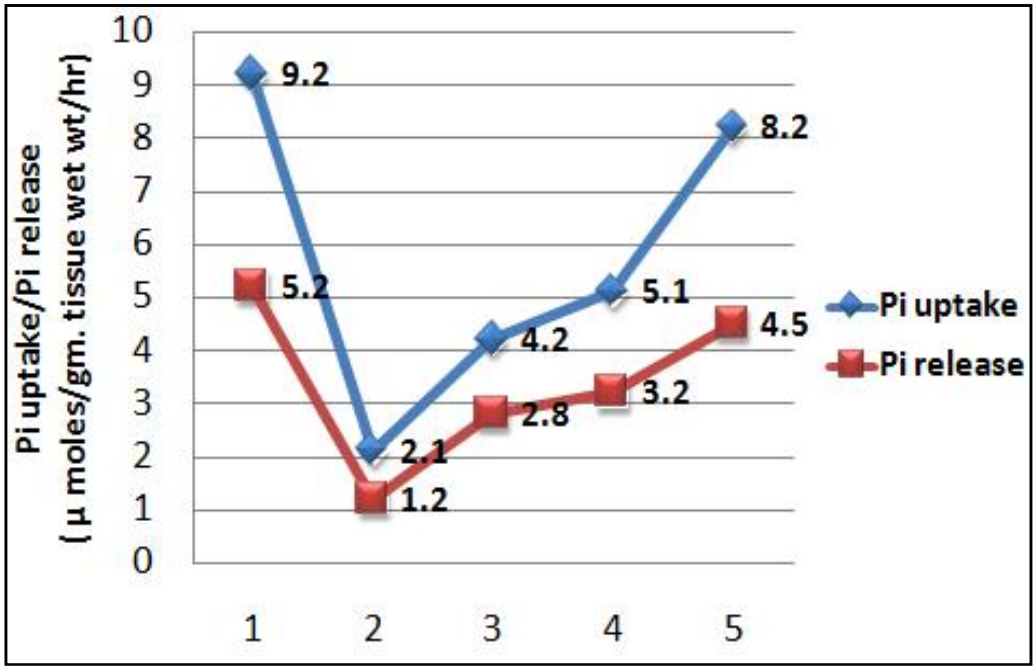
Additive to incubation medium	Pi uptake (Proximal segment) µmol/gm. tissue wet weight/hr	Pi uptake (Distal segment) µmol/gm. tissue wet weight/hr
None	9.3±0.1	8.2±0.1
Fumerate (10-3M)	9.3±0.4	8.4±0.2
Succinate (10-3 M)	9.2±0.3	8.3±0.2

No significant change is seen to proximal and distal segments in Pi uptake on addition of succinate and fumerate to the incubation medium

Table 1b. Effect of succinate and fumerate on Pi release by the mouse everted gut sacs of proximal and distal segments

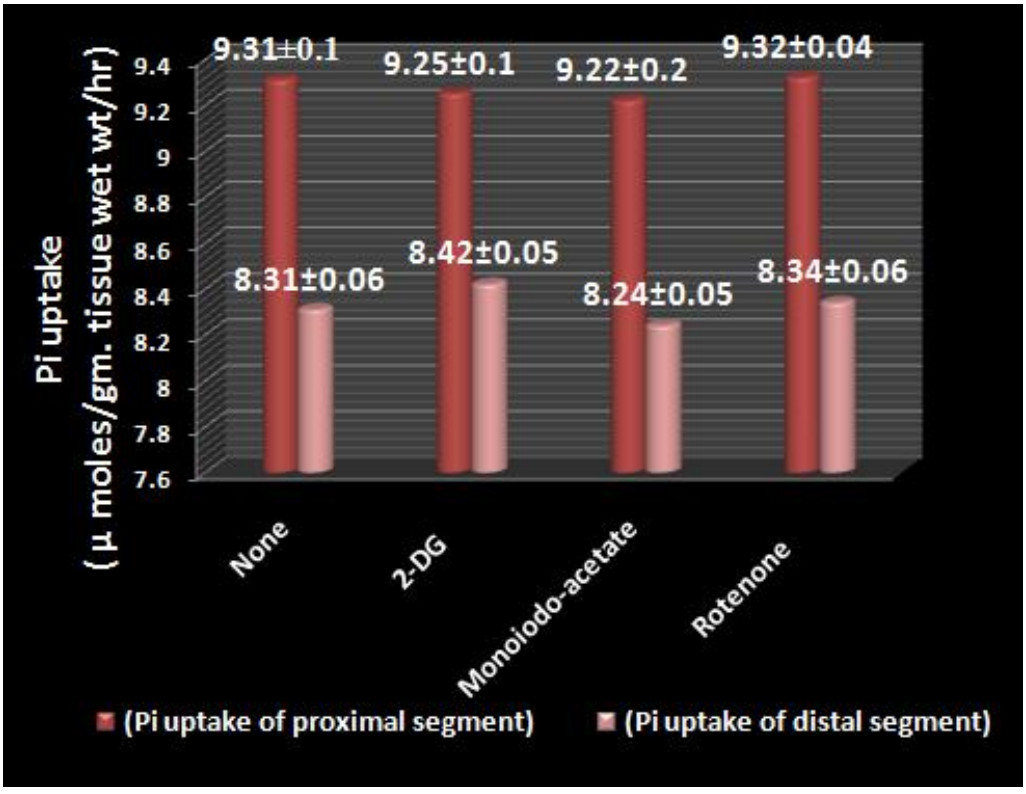
Additive to incubation medium	Pi Release of Proximal segment µmol/gm.tissue wet weight/hr	Pi Release of Distal segment (µmol/gm. tissue wet weight/hr)
None	5.3±0.1	4.5±0.1
Fumerate (10-3M)	5.2±0.3	* 5.4±0.1
Succinate (10-3 M)	5.3±0.2	* 5.5±0.2

Values marked *are significantly elevated (P<.001) from the topmost value in the same column. Each value is the mean ± SEM of six observations



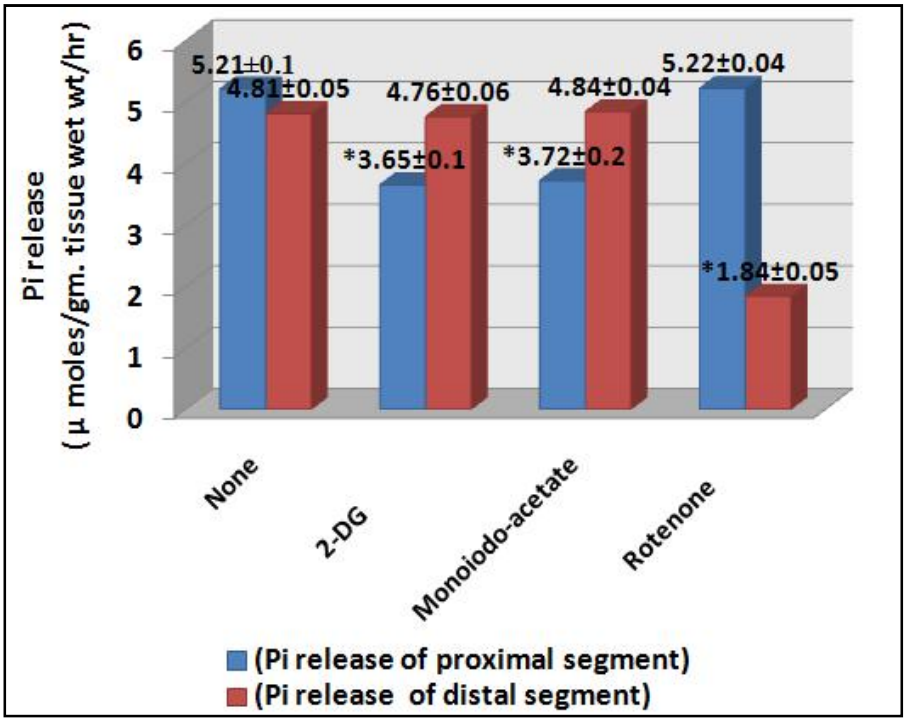
The animals used in this study are of 3months old male mice with maximum Pi transport. Everted gut sacs of 6 cm. were prepared successively from the pyloric end to the ileocaecal junction and the sacs are numbered from the pyloric end onwards (represented on x axis). Each value is the mean ± SEM of six observations.

Fig. 1. Segmental study of Pi transport in the everted mouse intestinal sacs



The Pi uptake values do not differ significantly from each other on usage of metabolic blockers. Each value is the mean ± SEM of six observations and are given at the top of the individual bars. Glycolytic blockers of 2DG (5mM) and monoiodo-acetate (10^{-4}M) were added to the incubation medium. Rotenone, blocker of oxidative phosphorylation was at 10^{-6}M in the incubation medium and it was first dissolved in acetone for its solubility.

Fig. 2a. Effect of metabolic blockers on Pi uptake by the mouse everted gut sacs



2-DG and monoiodo-acetate *significantly reduced (P<.001) Pi release in proximal segments. While rotenone lowered Pi release in the distal segments significantly*((P<.001).Mean values and ±SEM are given at the top of the respective bars.

Fig. 2b. Effect of metabolic blockers on Pi release by the mouse everted gut sacs

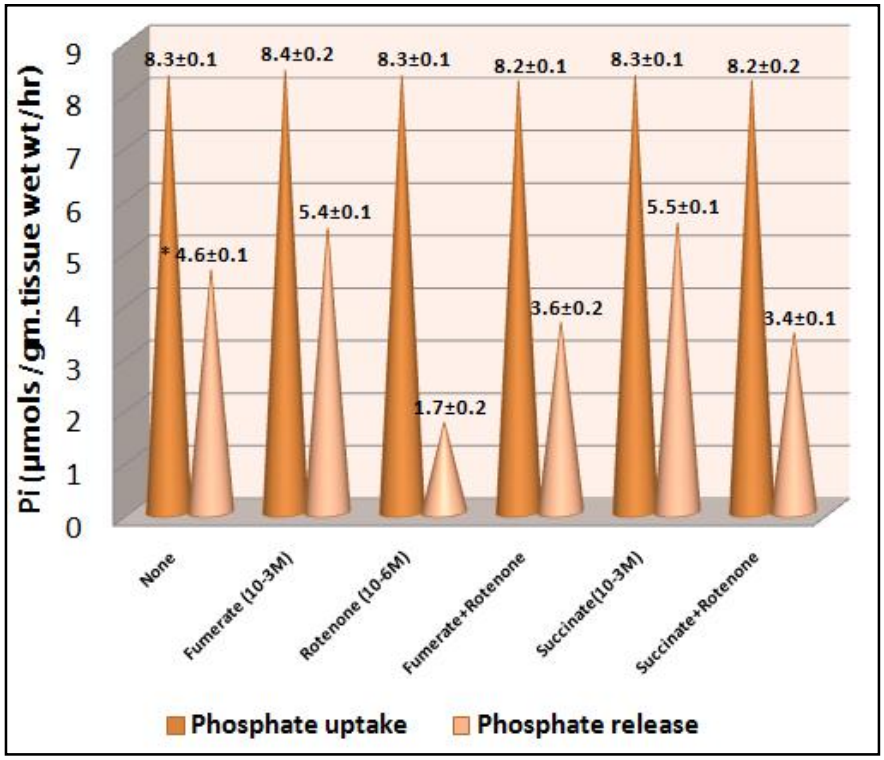


Fig 3. Mechanism of action of succinate and fumerate on phosphate transport of distal segments

Rotenone inhibited Pi release process significantly (P<.001) in distal segments without affecting Pi uptake. Mean and ±SEM are given as in other graphs. Each value is the mean ± SEM of six observations. Fumerate & succinate lifted the inhibitory effect of rotenone partly but significantly (P<.001)

DISCUSSION AND CONCLUSION

Regional variation of intestinal Pi transport

Phosphate absorption across the rat small intestine shows similar regional features to that of human small intestine in that the highest rate of phosphate transport is seen in the duodenum and jejunum and least in the ileal region (Walton and Gray 1979; Borowitz and Ghishan 1989; Marks *et al.,* 2006). A compartmental mathematical analysis based on an *in vivo* technique showed that although phosphate absorption in rats is generally greater in the duodenum, the ileum is responsible for the largest proportion of phosphate absorption, about 40% of total absorption, compared with other regions of the small intestine due to the increased segment length and thus longer transit time (Kayne *et al.*, 1993).In mice maximal phosphate absorption is reported in ileum with small amounts absorbed in the jejunum and duodenum (Marks *et al.*, 2006; Radanovic *et al.*, 2005). In our experiments too a regional variation is observed in Pi transport reflecting the regional variation of Na dependent Pi transporters. Though the first segment of the intestine showed the maximal transport of Pi in the segmental study ileum may be transporting the greater amount of Pi upon the total absorption due to the increased segment length. As it is generally believed that the uptake of Pi is driven by a sodium gradient established by the active extrusion of sodium, it is surprising to find no effect on Pi uptake by any of the metabolic blockers used either in proximal or distal segments of the intestine. Metabolic blockers are expected to inactivate the basolaterally located Sod.Pot.ATPase and hence to abolish the Na gradient across the brush border stopping the Pi uptake process. Similar observation has been made regarding glucose transport by Leese and Bronk (1972). It is possible that dissipation of sodium gradient achieved by the usage of the metabolic blockers was not sufficient to affect the uptake process as suggested in our earlier study (Mary and J. Prakasa Rao, 2015a).On the other hand Pi release process seems to be more sensitive to metabolic blockers.

While measuring Pi fluxes under anaerobic conditions of incubation, Peterlik and Wasserman (1978) noted that the energy state of the tissue might have some effect on the release of Pi. Present studies of Pi release clearly indicate that the release process is affected by glycolytic blockers, 2-DG and monoiodoacetate (Ellis and Beckett, 1954; Wick *et al*.,1957) in the proximal intestine and by inhibition of oxidative phosphorylation by rotenone (Gullans, 1982) in the distal intestine showing that glycolysis and oxidative phosphorylation are the chief sources of energy for Pi extrusion process in proximal and distal intestine respectively. A similar pattern in provision of energy for water transport has been identified by Detheridge *et al*. (1966).

Succinate and fumerate, the TCA intermediates are capable of entering mitochondria and stimulate oxidative phosphorylation (Coty and Pedersen, 1974; Brazy *et al*., 1984). In our study TCA intermediates are able to stimulate Pi release from the distal segments where oxidative phosphorylation seems to be the major source of energy for Pi release. The effect of these metabolites was curtailed by rotenone, an inhibitor of oxidative phosphorylation. Succinate and fumerate were without effect on Pi release in the proximal segment showing glycolysis to be the prime source of energy in this segment.

These studies clearly establish that curtailment of energy affects Pi release more seriously to that of Pi uptake. Our earlier studies too have indicated a role of Sod.Pot.ATPase in Pi extrusion process (Mary and Prakasa Rao, 2015a). It is possible that energy required for the activity of this pump comes from different sources in different tissues (Paul *et al*, 1979; Lynch and Paul , 1984; Takai *et al*.,1985). Kikuchi and Ghishan (1987) have also shown a beneficial effect of ATP on Pi transport across the basolateral vesicles. However, it is not clearly shown whether ATP effect is a direct one or indirectly mediated through Sod.Pot. ATPase.

Importance of understanding the mechanism of intestinal Pi transport

Clinically intestinal transport is targeted now in bringing out Pi homeostasis in hyperphosphatemic conditions as in Chronic Renal Disease and Familial Tumoral Calcinosis (Lisal and Erik, 2015). Current strategies for the treatment of hyperphosphatemia in dialysis patients include dietary Pi restriction and the usage of oral phosphate binders. So a better understanding of intestinal phosphate absorption is required as it has potentially wide reaching benefits in the clinical approach towards many diseases.

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