Interactions between the gastrointestinal tract, kidney, and liver in the regulation of body phosphate balance

by

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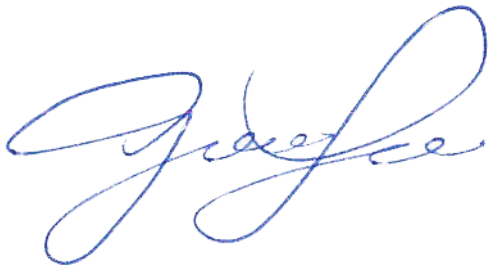
I, Grace Lee, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I verify that this has been indicated in the thesis.



Abstract

Inorganic phosphate (Pi) is an essential element that fuels vital processes in the body. To date, there is discord regarding the mechanisms of Pi regulation and the proportion of transport attributed to active (sodium-driven), paracellular, or uncharacterized transcellular pathways. The present studies address this by comparing Pi transport in different segments of the intestine using *in vitro, in situ,* and *ex vivo* techniques. Potential Pi signalling between the intestine, kidney, and liver was also investigated using intestinal perfusion and *in vivo* renal clearance surgeries.

Regional differences in intestinal Pi transport were investigated using the *in situ* closed-loop, *in vitro* everted sleeve, and *ex vivo* intestinal perfusion techniques. These studies highlighted measured Pi transport discrepancies between methods, confirmed the jejunum as the site of highest Pi transport ability in the GI tract, and also revealed that the distal colon transported a significant amount of Pi both *in vitro* and *in situ.* An intestinal perfusion technique never applied to studies of Pi transport also exposed a concentrated amount of Pi transported directly across the rat intestinal epithelium.

Renal Pi clearance surgeries investigated a proposed Pi sensing mechanism between the small intestine and the kidney in which a high duodenal Pi load triggered rapid phosphaturia. Present data show no phosphaturia after a physiological 10mM Pi duodenal instillation. In contrast with previously published data, a high Pi load into the

duodenum increased plasma Pi and parathyroid hormone (PTH) levels resulting in correlated phosphaturia.

The role of the liver in Pi transport was investigated by removing the liver following instillation of 1, 5, 10, and 15mM ^{32}P coupled Pi buffer into the jejunum. Data show a steady increase of Pi accumulated in the liver, which correlated with increased Pi concentration instilled into the jejunum. Between 10 and 15mM however, the Pi in the liver reached saturation, suggesting that the liver may only store physiological concentrations of Pi. Sodium-dependency of Pi uptake by the liver was also not apparent until 15mM Pi, in contrast with sodium-dependent Pi transport by the intestine at all four concentrations. This finding suggests a separate mechanism of liver Pi transport at this supraphysiological Pi concentration.

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ABBREVIATIONS, ACRONYMS, and ALTERNATE NAMES

ADHR ---------- Autosomal dominant hypophosphataemic rickets

ASARM ---------- Acidic serine-aspartate-rich MEPE-associated motif

ATP ---------- Adenosine triphosphate

BBM ---------- Brush border membrane

BLM ----------Basolateral membrane

cAMP ----------Cyclic adenosine monophosphate

Calcifediol----------25-hydroxyvitamin D_3 (unhydroxylated vitamin D)

Cholecalciferol---------7-Dehydrocholesterol (unhydroxylated calcifediol)

CaSR ---------- Calcium-sensing receptor

CKD ----------Chronic kidney disease

EGF ---------- Epidermal growth factor

EM ----------Electron microscopy

FGF-23 ----------Fibroblast growth factor – 23

FGF-7---------- Fibroblast growth factor – 7

GFR ----------Glomerular filtration rate

HHRH ---------- Hereditary hypophosphatemic rickets with hypercalciuria

HPO_4^{2-} ---------- Divalent phosphate anion

HPO_4^{-}----------Monovalent phosphate anion

IP ---------- Intraperitoneal

IV---------- Intravenous

KO ----------Knock out (mouse)

LXR ---------- Liver X receptor

MAP---------- Mean arterial (blood) pressure

min----------Minutes

NAD ----------Nicotinamide adenine dinucleotide

NaPi ---------- Sodium phosphate cotransporter

Pi ---------- Inorganic phosphate

PFA ---------- Phosphonoformic acid

PTH ---------- Parathyroid hormone

SD ---------- Sprague dawley (rat)

sFRP-4 ---------- Secreted frazzle-related protein – 4

TJ---------- Tight junction

TMD ---------- Transmembrane domain

TIO ---------- Tumor-induced osteomalacia

VDR ---------- Vitamin D receptor

Vitamin D ---------- 1, 25 dihydroxyvitamin D_3, (active vitamin D)

VSMC ---------- Vascular smooth muscle cells

XLH ---------- X-linked hypophosphatemia

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Introduction to the physiological importance of phosphate and its regulation

Phosphate is an important element that is required for vital pathways necessary to sustain life, pathways which include DNA regeneration, bone formation, and energy currency in the form of adenosine triphosphate (ATP). Phosphate is mostly obtained through the diet, and the regulation from the postprandial state to excretion is tightly maintained through a complex network of transporters and endocrine factors. Shedding light on the links between dietary phosphate and the functions of these circulating factors in the small intestine, kidney, and potentially the liver, is particularly important in the context of chronic kidney disease (CKD), as phosphate homeostasis is disturbed and can lead to conditions exhibiting a high mortality rate.

1.1 Physiological phosphate homeostasis

Inorganic phosphate (Pi) is the form of phosphate obtained from the diet and differs from the organic compound found in nucleic acids, bioactive signaling proteins and cell membranes [1-3]. Pi is regulated according to the amount consumed, with male adult humans ingesting and storing a slightly higher amount than women [4]. As food enters the small intestine, Pi is absorbed in the duodenum and jejunum and transported to the extracellular fluid where it is then freely filtered by the kidney. Approximately 70% of ingested Pi is absorbed in the small intestine, which constitutes more than what is biologically required [5]. Following that, about 80% of the Pi in the blood is reabsorbed by the kidney proximal tubules [6]. Skeletal Pi requirements involve roughly 70-80% of

total Pi in the blood, with Pi contained and stored in the bone and a small amount of Pi making a steady and equal Pi exchange with the extracellular fluid as needed [7]. The main concept in this homeostasis model is that excreted Pi via feces and urine is approximately equivalent to the amount ingested by the individual – this mechanism is essential in maintaining Pi balance **(Figure 1.1).**

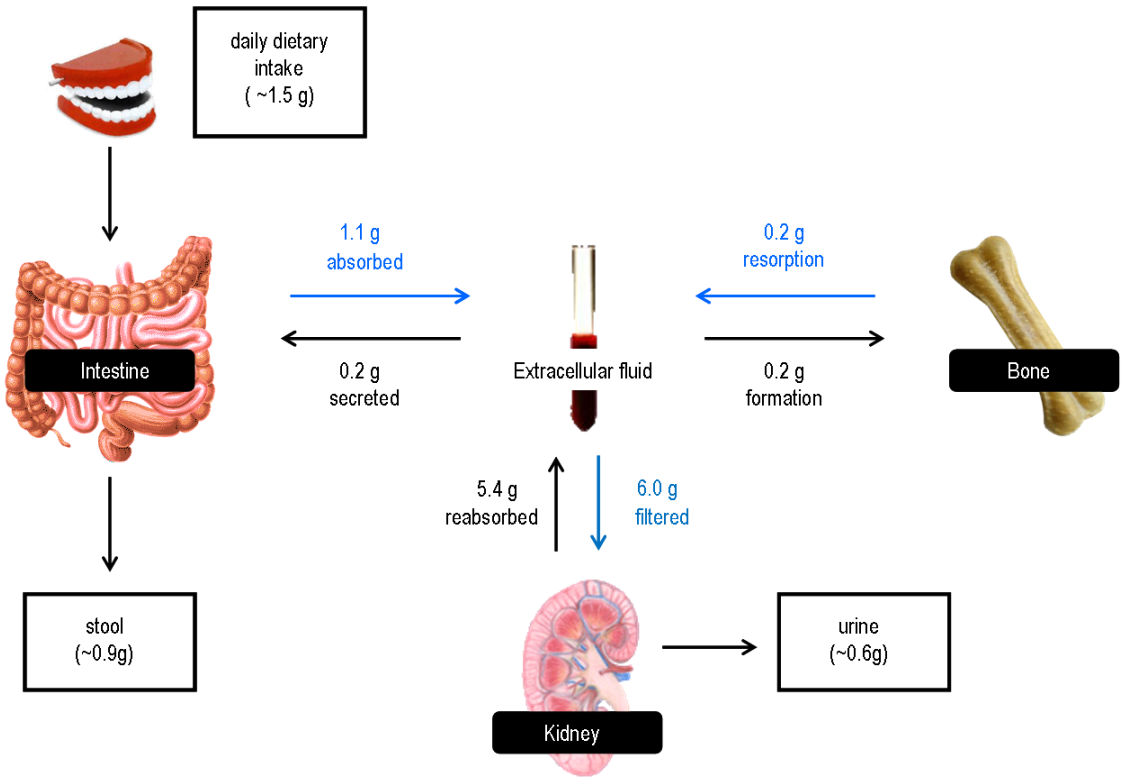


Figure 1.1. Distribution of Pi in the human body from dietary source to excretion (adapted from [7])
The amount of Pi excreted via stool and urine is equivalent to the amount of Pi ingested, while there is an equal and constant exchange of Pi between extracellular stores and the bone.

1.2. Active Pi transport

1.2.1 Sodium phosphate cotransporters – Type II (NaPis)

The type II sodium-dependent phosphate cotransporters, part of the solute carrier family (SLC34), are the main Pi handlers in the body and are expressed primarily in the small intestine and the kidney. Their expression at the brush border membrane (BBM) of intestinal enterocytes (NaPi-IIb) and renal proximal tubules (NaPi-IIa and IIc) is the rate limiting step for Pi transport [8, 9].

1.2.1.1 NaPi distribution and protein expression

NaPi-IIa is expressed in the S1-S3 [10] segments of kidney proximal tubules (highest in S1), while NaPi-IIc is confined to the S1 region [11]. NaPi-IIa and NaPi-IIc are expressed on the BBM of the kidney proximal tubule across all species; however, segmental distribution of NaPi-IIb in the small intestine is species-specific. In mice the highest expression occurs on the BBM of the ileum whereas in rats and humans, NaPi-IIb expression and the majority of Pi transport takes place in the jejunum [12]. This difference in intestinal Pi handling is one of the main reasons behind using rat models when performing *in vivo* experiments relating to Pi uptake and regulation, as rats serve to be more physiologically relevant to humans. More recently, NaPi-IIb has been shown to be expressed in the liver, supplementing previous studies showing that the only other tissue specific expression for the protein was the lung [13, 14]. The original proposed role of NaPi-IIb in the lung was to transport Pi to alveolar epithelial type II cells for surfactant synthesis which is important for lung function [15].

Expression of intestinal NaPi-IIb and renal NaPi-IIc decreases with age while renal NaPi-IIa expression seems to remain stable throughout life [16, 17]. This age-related decrease in expression of NaPi-IIb has been attributed to fact that Pi absorption from the diet/maternal circulation is essential during early development to promote skeletal growth [16]. Indeed the crucial role of intestinal NaPi-IIb in embryonic development has been shown by the phenotype of a targeted deletion mouse NaPi-IIb-/- model, which dies between week 8 and 9 of embryogenesis [18]. It has been shown that NaPi-IIb mRNA is present in the placenta [19], however protein expression and quantitative measurements of Pi absorption from placenta to embryo have not currently been investigated.

1.2.1.2. NaPi structure and function

All three isoforms of NaPi (IIa, IIb, and IIc) have recently been determined to possess a 12-pass transmembrane domain (TMD) [20], replacing the original model of an 8 TMD protein established soon after the rat and human isoforms of these transporters were first cloned [21]. With regards to structure, NaPi proteins share no homology with other protein families and they exists as functional monomers with both the C and N terminus residing intracellularly, making them important target sites for protein-protein interactions and hormonal control [22]. For example, the Lys-Arg (KR) motif located in an intracellular linker region between TMD10 and TMD11 in NaPi-IIa is critical for parathyroid hormone (PTH) sensitivity and subsequent retrieval of transporter from the BBM [23] Another example is the TRL motif in the C-terminal region which forms the

basis for a PDZ-binding motif, a crucial protein sequence for NaPi function and retrieval [24] (in depth discussion in section 1.2.1.4).

The process of shuttling fully functional NaPi proteins to and from the apical membrane does not involve recycling. Instead, transporters which are targeted for internalization are partially detected by clathrin-coated pits, trafficked to early endosomes and targeted by lysosomes for degradation [25, 26]. Retrieval of NaPi-IIc is slower than NaPi-IIa, does not entail lysosomal degradation, and involves interactions with other cell structures such as microtubules [27].

1.2.1.3 Kinetic properties of NaPis

NaPi proteins utilize the free energy produced by the Na^+ gradient across the cell membrane to actively transport Na^+ and Pi at a 3:1 and 2:1 ratio (NaPi-IIa and NaPi-IIb, and NaPi-IIc respectively) [28] **(Figure 1.2).** The only other cation that has been shown to be able to partially replace sodium in driving Pi transport has been Li^+, which is able to replace at least one of the 3 Na^+ ions utilized by NaPi-IIa and NaPi-IIb [29]. The NaPis can also be distinguished by their electrogenicity; NaPi-IIa and NaPi-IIb are electrogenic, whereas NaPi-IIc is electroneutral owing to the difference in Na^+ and Pi transport ratio. Once the transporters are translocated to the BBM, the only factors that influence transport kinetics are membrane potential and extracellular pH [28, 30].

All three NaPi isoforms have a divalent phosphate (HPO_4^{2-}) preference, thus pH is a strong determinant of Pi transport capacity as it affects the monovalent/divalent Pi

distribution in the lumen of the proximal tubule and the small intestine; divalent Pi anions are in abundance at a neutral to basic pH [31]. pH also affects transport kinetics in a very direct manner as the proton concentration within a solution directly modulates kinetics [31]. At pH 7.4, NaPi proteins share similar substrate affinities of 100uM for divalent Pi and 40mM for Na^+ - these values are far below normal concentrations in the proximal tubule, therefore it is likely that these transporters function at close to their maximum rates in the kidney [32].

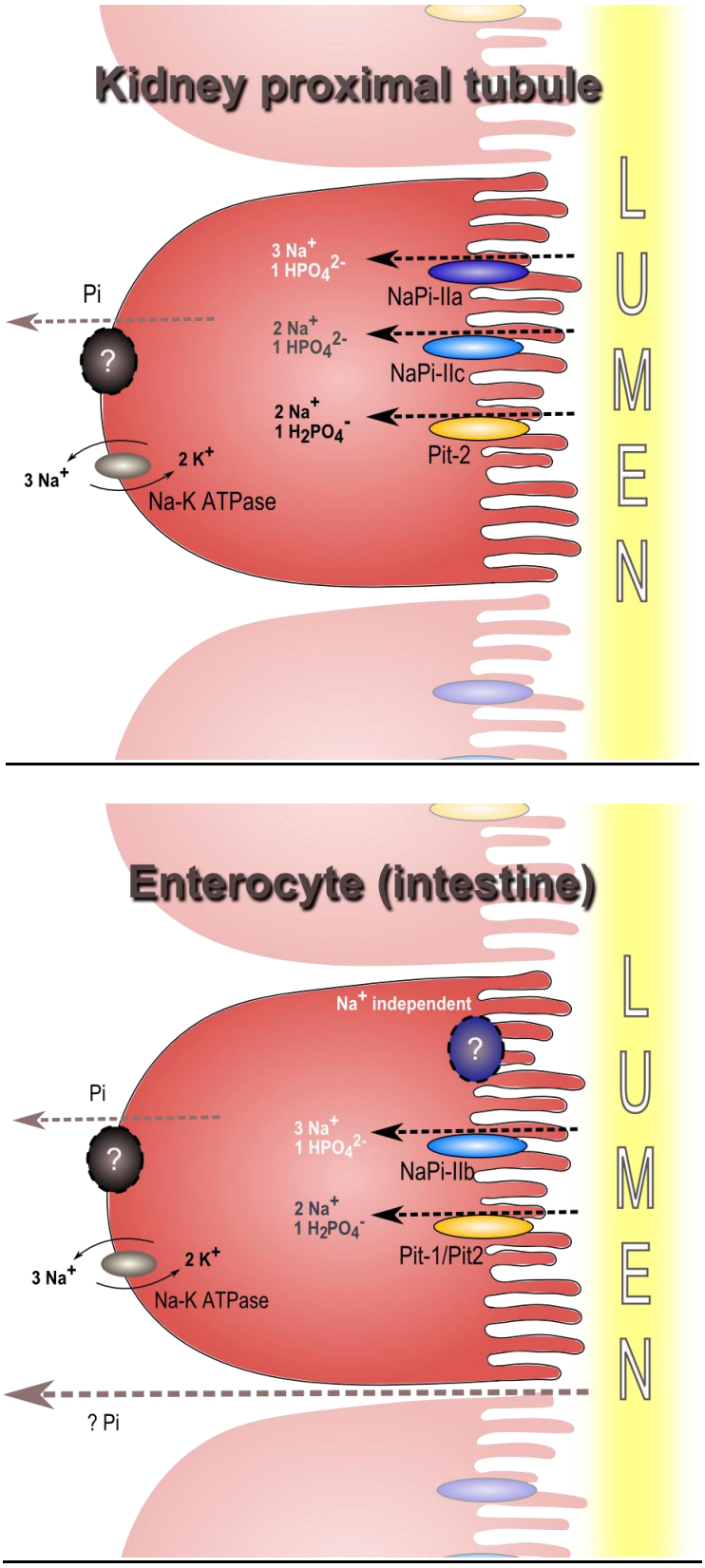


Figure 1.2. Properties of kidney and intestinal Pi transporters

1.2.1.4. Regulation of NaPis

The main regulators of renal NaPi-IIa and IIc are parathyroid hormone (PTH), dietary Pi, dopamine, 1,25 dihydroxyvitamin D_3 (vitamin D) and FGF-23 (a phosphatonin which will be discussed in section 1.3).

In mice and rats, PTH stimulation of renal PTH1 receptor, which is located on both the apical and basolateral side of the proximal tubule [32] results in a decrease of NaPi-IIa expression within minutes – NaPi-IIc expression alterations occur after a few hours [33]. It is unknown if this same response occurs in humans. Interestingly, PTH activates both protein kinase A and C pathways [34], which indirectly results in phosphorylation of a PDZ-containing protein NHERF1 at Ser77 and decreases the domain's affinity for NaPi-IIa [35]. NaPi-IIa binds to several PDZ domain-containing proteins (NHERF1, NHERF3) [36] and as demonstrated through the use of NHERF1-null mice, the interaction of NaPi-IIa with NHERF1 is crucial for apical stability and retention of this transporter [37]. These mice exhibited hypophosphatemia and phosphaturia as well as a reduction in apical NaPi-IIa. In contrast, NHERF3 ablated mice show no signs of alterations of NaPi-IIa expression or Pi excretion [38]. Recently, NHERF1 has also been shown to interact with intestinal NaPi-IIb through the use of NHERF1 knockout mice as well as confocal microscopy [39]. The lack of NHERF1 however, only seems to impair NaPi-IIb adaptation to low Pi diet conditions and thus its interaction with NaPi-IIb may not be as crucial for Pi transport in the intestine.

Dopamine has been shown to regulate NaPi-IIa by a mechanism similar to PTH with experiments utilizing mouse renal proximal tubule cells. Protein kinase A and the cyclic AMP (cAMP) pathway are activated by dopamine, which then stimulates protein kinase C. This results in the aforementioned phosphorylation of NHERF1 at Ser77 and the subsequent disassociation of the NaPI-IIa/NHERF1 complex [40].

Diet-induced, high serum Pi triggers an increase in urinary Pi excretion in a relatively acute manner (~1 hour), a mechanism which seems to be indistinguishable from PTH [41]. The signalling mechanisms attributed to acute changes in renal NaPis in response to dietary Pi are independent of the known factors that are directly affected by changes in dietary Pi (calcium, PTH, vitamin D, FGF-23); hence it is implied that the kidney may detect changes in Pi concentration in the plasma by an unknown sensing mechanism [42] (a more thorough review of renal Pi regulation and the gastro-renal Pi axis will be discussed in section 1.6). Conversely, a low Pi diet leads to a more chronic adaptation of NaPi transporters since hypophosphatemia first increases plasma calcium concentration. Increased plasma calcium then triggers feedback inhibition of PTH release and induces maximal renal Pi reabsorption by upregulating NaPi-IIa and IIc expression [25].

Vitamin D induces genomic reduction of NaPi-IIa but increases NaPi-IIb protein in adult rats directly, post-transcription [43, 44]. This process occurs via an alteration of FGF-23 levels (a phosphatonin discussed in section 1.3) [45]. In suckling rats, vitamin D

increases NaPi-IIb mRNA only, thus it can be concluded that the effect of Vitamin D on NaPi-IIb is age dependent [46]

Very recent experiments have shown that NaPi expression can be altered by protein kinases that regulate insulin-like growth factor-1 (IGF-1) [47]. In these studies, serine/threonine protein kinase B-RAF was shown to upregulate both renal and intestinal NaPi-s expressed in oocytes. When oocytes where co-expressed with NaPi and B-RAF or a water control and placed in a Pi bath, a larger intracellular current was generated and thus higher Pi uptake capability was produced in the oocytes co-expressed with NaPi and B-RAF. Thus far, only *in vitro* experiments have been conducted utilizing B-RAF, so its significance in regulating NaPis in humans has still not been determined.

The mode of activation and regulation of intestinal NaPi-IIb is much less understood and great focus has been placed on investigating Pi absorption in this area. As mentioned previously, NaPi-IIb is regulated mainly by vitamin D and serum Pi [44] with only 30% of intestinal Pi regulation occurring through the influence of vitamin D [48]. In depth regulation of NaPi-IIb (intestinal Pi absorption) will be discussed in section 1.5**.**

1.2.1.5. Knockout studies

Segawa et al [49, 50] and Tenenhouse et al [49, 50] have conducted numerous studies using transgenic mouse models expressing various degrees of gene knockouts. Renal NaPi-IIa -/- mice exhibit hypophosphatemia, hypercalcemia and hypercalciuria while

there is also a compensatory upregulation of NaPi-IIc protein expression [49, 50]. NaPi-IIc knockout mice possess normal levels of serum Pi and excrete urinal Pi and the uptake of Pi into renal BBM is indistinguishable from WT mice [50] **(Figure 1.3).** The expression of NaPi-IIa remains the same in these NaPi-IIc -/- mice as well, which implies that the normal contribution of NaPi-IIc to Pi homeostasis is minor but may be particularly important under severe circumstances.

Although NaPi-IIc is thought to only contribute a negligible role in overall Pi homeostasis from observations of the aforementioned mouse models, the transporter may have a more significant role in Pi transport in humans. Mutations in NaPi-IIc in humans have been implicated as the cause of hereditary hypophosphatemic rickets with hypercalcuria (HHRH) [51-53]. This condition is characterised by renal Pi wasting which leads to hypophosphatemia and secondary rickets or osteomalacia. As a result, studies focusing on this specific genetic condition suggest that NaPi-IIc may have more of an impact on Pi balance in humans.

In the case of NaPi-IIb knockout mice, early embryonic lethality was observed, likely due to an impaired Pi absorption from maternal circulation. By bypassing this phenotype and generating a tamoxifen inducible NaPi-IIb deficient model, groups have shown increased fecal Pi excretion and a 90% reduction in sodium-dependent Pi transport in the small intestine. Despite this reduced intestinal Pi absorption, a ~40-45% decrease compared to WT mice, the knockouts maintained a normal level of serum Pi possibly due to a compensatory upregulation of NaPi-IIa [54] **(Figure 1.3)**.

Age dependent variations of the role of NaPi-IIb were also investigated by generating heterozygous knockout mice. At 4 weeks, NaPi-IIb +/- mice exhibited a significant decrease in of NaPi-IIb mRNA and protein levels, along with impaired intestinal Pi transport and hypophosphatemia. Compensatory upregulation of NaPi-IIa and IIc and increased circulating levels of FGF-23 were noted, but these changes were not enough to prevent hypophosphatemia [55]. In contrast, mice at 20 weeks maintained normophosphatemia, which highlights and reaffirms the importance of intestinal Pi transport during ontogenesies (44, 45). Interestingly, these heterozygous mice at 20 weeks did not show any changes in the expression of renal NaPi transporters or FGF23, yet still maintained a persistent hypophosphaturia. These phenotypes mimic those of patients with human pulmonary alveolar microlithiasis, a disorder involving inactivating mutations in NaPi-IIb. Those affected do not exhibit changes in plasma Pi concentration [56] yet concurrently have hypophosphaturia and low levels of FGF-23 [57] **(Figure 1.3).**

	NaPi-IIa -/-	NaPi-IIc -/-	NaPi-IIb +/- 4 weeks	NaPi-IIb +/- 20 weeks	NaPi-IIb -/- inducible
Serum Pi	Hypo-phosphatemia	Normal	Hypo-phosphatemia	Normal	Normal
Urinary Pi	Normal	Normal	Low Pi excretion	Low Pi excretion	Normal, increased fecal Pi excretion
Effect on other transporters	Compensatory upregulation of NaPi-IIc	No change in expression of NaPi-IIa	Compensatory upregulation of NaPi-IIa + IIc	No change in expression	Compensatory upregulation of NaPi-IIa

Figure 1.3. Summary of NaPi knockout mice phenotypes

1.2.1.6. Inhibitors

Pi transport by all isoforms of NaPi is blocked by nicotinamide and phosphonoformic acid (PFA) as they act as competitive inhibitors. Nicotinamide is a compound which stimulates biosynthesis and halts catabolism of nicotinamide adenine dinucleotide (NAD) – NAD is a component cAMP generation which initiates a main pathway of NaPi-IIa/IIc retrieval. However, nephrotoxicity and gastrointestinal side-effects prevent their use as targets for Pi control in CKD patients [58] (further discussion of these blockers (specifically for intestinal NaPi-IIb) and their use in therapy will be discussed in section 1.8.1.3).

1.2.2. Sodium phosphate cotransporters – Type III (PiTs)

Type III cotransporters (SLC20), PiT1 and PiT2, are similar in structure to the NaPis, however their roles were initially speculated to involve support of the actin cytoskeletal

network [59]. Unlike the type II NaPis, they have a preference for transporting monovalent phosphate ($H_2PO_4^-$) with a 2:1 Na+:Pi transport stoichiometry **(Figure 1.2).** This difference in Pi preference leads to the fact that the PiTs function with a greater capacity at a lower pH as this alters the Pi composition to greatly increase the monovalent forms of circulating Pi [60, 61]. The affinities for Pi and Na+ are similar to those of Type II NaPis ($\leq$ 100uM and ~50mM respectively), however PiTs are significantly less sensitive to pH; PiT1 in particular has been shown to have stable transport rates across a 3 pH unit range [32]. Also, unlike type II NaPis which are almost exclusively driven by Na+, Li+ has the ability to fully replace sodium in driving Pi transport in type III PiTs [61]. However, a result of this cation replacement is significantly reduced Pi transport rates – the serum concentration of Li is also relatively low, thus this phenomenon may have little impact on Pi homeostasis in general.

Although mRNA for both PiT transporters are found across many cell types, detectable levels of protein expression for PiT1 is found throughout the small intestine and PiT 2 protein on the BBM of kidney proximal tubules [62], [60]. Similar to NaPi transporters, inter-organ PiT expression differs between species; in mice there does seem to be protein expression of PiT2 strictly in the ileum [63] [64] whereas rats, minimal PiT2 protein is found in small intestine [62].

In associated knockout studies, it was shown that PiT2 was responsible for carrying out residual renal Pi transport in NaPi-IIa and IIc double knockout mice [50]. However, due to the fact that these cotransporters do not show rapid Pi-dependent regulation between

apical and basolateral membranes of these epithelial cells, it can be concluded that their expression is dependent on functions other than Pi homeostasis [20]. Some of these functions have been revealed to include roles in early liver development, bone matrix calcification, and cell proliferation [65-67]

There is a strong interest in the role of PiT transporters in vascular calcification as they are more highly expressed in vascular smooth muscle cells (VSMCs) than Type II sodium phosphate cotransporters [68, 69] (more detailed discussion in section 1.8.1.2) . PiT1 is also important for normal liver growth since disruption of the PiT1 gene in mice leads to mid-gestation lethality due to severe defects in liver development [65].

1.2.2.1. PiT regulation

The abundance of PiT2 protein at the renal BBM is regulated by dietary Pi with experiments showing an upregulation of transporter protein following a chronic low Pi diet in rats [60]. According to the group's time course studies, this adaptation took approximately 8 hours, much longer than changes seen to the renal NaPi transporters. Thus the signalling associated with this regulation can be deduced to involve NaPi-independent pathways. In contrast, protein levels of PiT1 do not seem to be altered by dietary Pi load [62]. PiT2 mRNA is also upregulated following vitamin D treatment *in vitro* [70], however the significance of this effect is unknown.

1.2.3. Sodium phosphate cotransporters – Type I

The type I transporters also belong to the solute carrier family (SLC17) and are currently known to play a much smaller role in Pi homeostasis in the body.

Although 9 separate homologues of the type I cotransporters have been characterized previously, recent discoveries place one of the NaPi homologues (SLC17a4) as an organic cation transporter in the small intestine and another (SLC17a7) an inorganic Pi transporter in the brain [71, 72]. Proposed theories for their expression in these regions involve intestinal urate and drug metabolite extrusion and mineral transport for neuronal function respectively. The fact that type I transporters only share about 20% structural homology with the NaPis, [73] suggests that their mechanisms are not crucial for Pi handling and absorption in most organs.

1.3. Phosphatonins

A group of proteins known as phosphatonins have come into the foreground as primary regulators of Pi homeostasis due to their rapid phosphaturic effect. The term phosphatonin was coined to refer to an endocrine substance that acts to increase Pi excretion via a cAMP-independent pathway and reduces the function of 1a-hydroxylase to convert 25 dihydroxyvitamin D_3 (calcifediol) to active vitamin D [7]. Fibroblast growth factor 23 (FGF-23) and its co-receptor αKlotho are the most well described factors, as well as matrix extracellular phopshoglycoprotein (MEPE), frizzled-related protein 4 (sFRP-4), and fibroblast growth factor 7 (FGF-7). All four of these Pi-regulating factors were found and isolated from Pi-wasting tumors associated with tumor-induced osteomalacia (TIO) in various clinical disorders and serve to increase Pi excretion in the urine by directly acting on the number of sodium phosphate cotransporters expressed at the brush border membrane of the proximal tubule [7].

1.3.1. FGF-23

FGF-23 is an established and well-studied factor involved in Pi regulation which acts mainly at the renal proximal tubule. FGF-23 is a 32 kDa protein that is expressed mostly in osteoblasts and osteocytes while structurally, it lacks a heparin sulfate binding motif which is characteristic of others in the FGF family – this enables it to act in an endocrine manner [74]. Gain of function mutations in *FGF-23* cause autosomal dominant hypophosphatemic rickets (ADHR) and FGF-23 itself has been shown to reduce expression of renal NaPi-IIa and NaPi-IIc at the proximal tubule BBM [75] while

also decreasing circulating levels of vitamin D. This discovery was made using transgenic mice overexpressing FGF-23 as well as wild-type mice treated with FGF-23, both of which showed a decrease in serum vitamin D [76, 77]. In a complementary fashion, FGF-23 null mice exhibited elevated levels of vitamin D [78], which was later discovered to be the result of the effect of FGF-23 inhibiting renal 24-αhydroxylase activity, which catabolises vitamin D [79]. The reduction in vitamin D in turn, also serves to reduce intestinal Pi transport and decrease NaPi-IIb expression [80]. In order for FGF-23 to become biologically active, it requires another co-receptor; membrane bound αKlotho (described in the following section). αKlotho first binds to an FGF receptor (FGFR) then forms a ternary structure with the C terminus of FGF-23 while the FGFR binds to its N terminus [75]. The location of action in which FGF-23 downregulates NaPi expression is still debated since αKlotho has been shown to be expressed in the renal distal tubules whereas almost all renal NaPi protein is located in the proximal tubules as previously discussed. This observation suggests that there may be some signalling factor which originates from the FGF-23-αKlotho complex in the proximal tubule [81]. Using FGF-23 as a biomarker for CKD has been a topic of discussion since elevated levels are seen before altered levels of PTH (an established indicator of CKD), but whether FGF-23 is the cause or effect of increased PTH is still to be determined.

1.3.2. αKlotho

The importance of the αKlotho protein in Pi metabolism was established in the last few years with the observation that the phenotypes of αKlotho-deficient mice were almost

identical to mice lacking FGF-23 [78], [82]. It has now been confirmed that in order for FGF-23 to fully form an active complex and induce the aforementioned modifications in renal Pi transport and vitamin D levels, it must interact with a co-receptor known as αKlotho. The membrane-bound form of αKlotho first establishes a binary structure by binding with certain isoforms of FGFR (1C, 3C, 4) in order to enhance its affinity for FGF-23. This initial interaction with αKlotho and FGFR is the main event which determines the specific tissue target of FGF-23 [83]. Recent discoveries show that αKlotho exists in both membrane bound (68kDa) and a secreted, circulating form (130kDa) [84, 85] with the circulating type acting on tangential pathways such as increasing cell surface expression of certain renal ion channels (e.g. TRPV5 and ROMK) (reviewed extensively in [86]). The role of αKlotho in Pi handling has been a relatively recent finding with data suggesting a strong relationship. Utilizing a renal free-flow micropuncture method, Hu and colleagues discovered that αKlotho protein is found in proximal tubular fluid and directly inactivates BBM expressed NaPi-IIa resulting in phosphaturia [87]. The method of action was found to occur via αKlotho's glucuronidase activity and is completetly independent of interaction with FGF-23. Moreover, another isoform of Klotho, ßKlotho, has been shown to interact with human FGF-19. ßKlotho protein is also expressed in the small intestine and is known to inhibit sodium-dependent bile acid transport [88]. Whether ßKlotho has any relevance to Pi handling similar to those of αKlotho is still under investigation.

1.3.3. MEPE

MEPE belongs to the group of small integrin-binding ligand interacting glycoprotein family (SIBLING) and is one of the most overexpressed proteins in diseases related to renal Pi wasting. Although MEPE is derived from the cellular components of the bone, its mRNA is present in both the small intestine and kidney [17] while protein expression and bioactivity are most highly concentrated in the proximal tubule of the kidney; these findings point to the fact that MEPE may play a significant role in Pi homeostasis [89]. Through studies conducted utilizing an *in-situ* gut loop technique in rats, MEPE is currently the only phosphatonin known to directly influence intestinal Pi absorption [90]. Following an intravenous (IV) MEPE infusion, rats exhibited a decrease in jejunal Pi absorption which was independent of changes in PTH, vitamin D (unlike following FGF-23 infusion), or FGF-23 itself [90, 91]. It has also been confirmed that MEPE inhibits proximal tubular Pi reabsorption in rats following an IV infusion and single-nephron micropuncture and [91]. In these experiments, no changes were seen in glomerular or single-nephron filtration rate or filtered Pi load, which suggests that the primary target organ of MEPE is the kidney. However, under certain physiological constraints (i.e. renal insufficiency), adaptation of the location of action of MEPE and its potential effect on intestinal Pi transport is still unknown.

1.3.4. sFRP-4

sFRP-4 is a phosphatonin that is highly expressed in tumors associated with renal Pi wasting and osteomalacia. sFRP-4 was thought to play a role in the long-term regulation of renal Pi reabsorption following a high dietary Pi intake since sFRP-4

protein levels were upregulated in rat kidneys following a chronic high Pi diet [45] although levels of sFRP-4 were unaltered in animals on a low Pi diet. It has been proposed that sFRP-4 decreases renal NaPi-IIa expression by inhibiting wnt pathways which serve to activate frizzled protein receptors. [92]. Its affect on other phosphate transporters has not been determined.

1.3.5. FGF-7

FGF-7 is also known as keratinocyte growth factor and was isolated from tumors of TIO patients [93]. As its alternative name suggests, FGF-7 is highly expressed in keratinocytes and its primary role is to stimulate wound healing [93]. In experiments on opossum kidney cells, it was shown that FGF-7 inhibits sodium-dependent Pi transport in opossum kidney cells [93] while also causing phosphaturia in rats *in vivo* [94]. The pathways involved in this method of action still remain unknown.

1.4. The kidney and Pi handling

Pi homeostasis in healthy mammals is largely maintained through glomerular filtration followed by reabsorption of Pi across the kidney proximal tubule. Approximately 80-90% of total Pi in the plasma is reabsorbed at the proximal tubules, with only 15-20% taking place in the proximal straight tubule and the remainder in the proximal convoluted tubule [95].

1.4.1. Pi reabsorption at the proximal tubule

Expression of NaPi-IIa at the apical membrane of the proximal tubule is largely responsible for Pi regulation, with a relatively lesser contribution by NaPi-IIc and type III sodium phosphate cotransporter, PiT2 [96]. Indeed, early kidney micropuncture and cell culture studies showed that sodium-dependent Pi transport is higher in the proximal convoluted tubules compared with proximal straight tubules and distal sections of the nephron [97]. This observation was later attributed to the higher V_{max} of the sodium-dependent Pi transport mechanism which directly correlates with the expression distribution of NaPi-IIa, specifically the early S1 and S2 segments [10].

1.4.2. Regulation of renal Pi reabsorption

Short term regulation of renal Pi resabsorption is carried out by the shuttling of NaPi-IIa and IIc within vesicles between the cytoplasm and the BBM of the proximal tubule [27, 33]. The main regulators of renal NaPi-IIa and IIc are dietary Pi, PTH, vitamin D.

1.4.2.1. Renal Pi regulation by dietary Pi

A low Pi diet has historically been shown to increase renal Pi reabsorption to its maximum [98], with experiments using mice on a chronic low Pi diet showing an increase in sodium-dependent Pi transport in the proximal tubules with a correlating increase in NaPi-IIa protein but not mRNA [99]. However, expression of NaPi-IIc protein at the BBM and its mRNA expression was increased which suggests a compensatory mechanism under abnormal Pi conditions. Rats given a similar low Pi diet however,

showed an increase in both NaPi-IIa protein and mRNA which highlights the discrepancies seen between species [60].

Interestingly, an *in vitro* study which examined the effect of low Pi concentrations on cultured proximal tubule cells confirmed a rapid upregulation of Pi transport, suggesting that the cells were able to sense a change in Pi concentration independent of other circulating factors such as PTH [100].. This proposed Pi-sensing mechanism by proximal tubule cells may also be linked to the calcium-sensing receptor (CaSR) since serum Pi and calcium levels are closely dependent on each other [96].

1.4.2.2. Renal Pi regulation by PTH

PTH levels in the body are regulated by two separate processes – first is the release of preformed PTH stored in the secretory cells of the parathyroid, and the second is the *de novo* synthesis of PTH [101, 102]. In accordance to these two processes, rapid changes in PTH result from the secretion of already fully formed PTH which is directly controlled by the binding of calcium to CaSR in the parathyroid gland [103, 104]. PTH release can also occur independently of calcium signals through the vitamin D pathway which inhibits PTH gene transcription and secretion in the parathyroid glands [105]. An increase in vitamin D is thought to act on the PTH gene by increasing the corresponding vitamin D receptor mRNA in the parathyroid gland [106]. Subsequently, the receptor forms a heterodimer with retinol X receptor and binds to vitamin D response elements within the PTH gene which represses its transcription. Long-term adaptations resulting in altered PTH levels usually involve the synthesis of new protein.

PTH induces phosphaturia by directly acting on renal NaPi proteins at the BBM; serum levels are then acutely modified, with some changes reported within minutes [96]. This regulation was demonstrated following a low and high Pi diet gavage in rats, which caused changes in plasma PTH levels within 10-15 minutes [107]. PTH has also been shown to decrease activity of the Na/K ATPase at the basolateral membrane (BLM) of the proximal tubule which may indirectly affect Pi transport by altering the electrochemical gradient across the BBM [108]. For the specific pathways and mechanisms in which PTH regulates renal Pi transporters, please refer to section 1.2.1.4.

1.4.2.3. Renal Pi regulation by vitamin D

Calcifediol is hydroxylated and converted to active vitamin D in the kidney proximal tubule – subsequently, treatment of animals with vitamin D also stimulates Pi absorption in this tubule region [109]. In vitamin D deficient rats, protein and mRNA levels of NaPi-IIa are significantly reduced in the kidney cortex. Consequently, after a vitamin D dose, both NaPi-IIa protein and mRNA as well as sodium-dependent transport increase [43].

The role of Vitamin D in renal Pi transport regulation is also intricately linked with factors such as levels of PTH and FGF-23. The enzyme 25-hydroxyvitamin D_3 - 1αhydroxylase (aka 1α-hydroxylase) converts calcifediol to active vitamin D – this enzyme is also stimulated by PTH through protein kinase A and C pathways [110, 111]. Interestingly, vitamin D has been shown to inhibit renal Pi reabsorption in rats which have undergone

thyroparathyroidectomy [112]. This result was later discovered to be a consequence of vitamin D acting on serum levels of FGF-23 [45]. For a detailed overview on FGF-23 and other phosphatonins, please refer to section 1.3

1.4.2.4.Other factors regulating renal Pi transport

See phosphatonins, section 1.3.

Metabolic acidosis is a condition in which blood pH is drastically lowered, resulting in an increase in renal excretion of Pi and ammonia to preserve bicarbonate and to excrete excess protons [113, 114]. Pi plays an essential role in correcting metabolic acidosis by acting as a buffer in extracellular fluid and more importantly, in urine [115]. Phosphaturia is mediated by inhibition of renal Pi reabsorption, supported by the data that NaPi-IIa protein and mRNA as well as sodium-dependent Pi transport in BBM vesicles is decreased during this condition [115, 116]. This effect is independent of PTH, extracellular fluid volume, natriuresis, or plasma Pi concentrations [116]

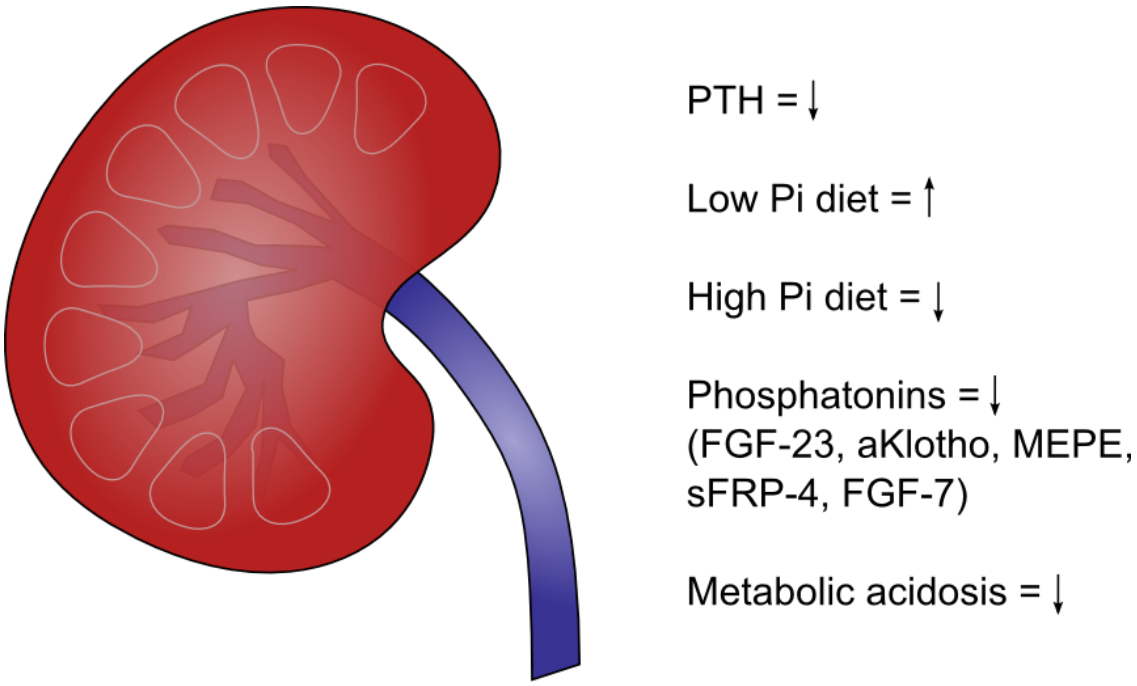


Figure 1.4. Summary of factors influencing kidney PI reabsorption

1.5. The intestine and Pi handling

Although the kidney is the main player in body Pi homeostasis, there is increasing interest in the role of the small intestine in overall Pi handling. Through recent advances in investigating Pi absorption, intestinal Pi absorption is thought to have a greater role in the maintenance of Pi balance than previously recognized. There has also been an established connection between the intestine and the kidney to maintain postprandial Pi balance.

1.5.1. Pi absorption across the intestine

Enterocytes mature during their transit from the crypt of Lieberkühn to the villus tip, thus only those enterocytes located in the mid to upper region of the villi express NaPi-IIb and are responsible for Pi uptake [117]. Studies have shown that instillation of Pi directly into the small intestine, specifically the duodenum, results in an acute increase in serum Pi concentration [54, 62] mediated by both NaPi-IIb and subsequent sodium-independent pathways (both paracellular and transcelluar, discussed in section 1.5.3) [54]. Immunohistochemistry has revealed that type III transporter PiT1 protein is also located at the rat small intestinal BBM, however its role still remains unclear [62]. Sodium-dependent Pi absorption across the intestinal BBM decreases with age in several mammalian species with a corresponding decrease in NaPi-IIb gene and protein expression [46, 118].

1.5.2. Intestinal Pi regulation

It is widely accepted that the type II transporter, NaPi-IIb, is responsible for Pi transport across the enterocyte BBM and provides the route for sodium-dependent transepithelial Pi absorption. Dietary Pi and vitamin D are considered to be the most important physiological regulators of this transporter with early experiments looking at NaPi-IIb adaptations to a low Pi diet.

1.5.2.1. Intestinal Pi regulation by dietary Pi

The foundations of what we know to be physiological luminal Pi concentrations along the human gastrointestinal tract result from early studies showing the range to be about

0.7-12.7mM, varying according to the content and timing of the last meal [119]. Pi, used as preservatives in processed foods, has been shown to possess a much higher bioavailability, resulting > 90% absorption in the intestine, compared with only 40-60% for naturally occurring dietary Pi [120].

Hattenhauer and colleagues showed that NaPi-IIb protein expression increases at the BBM of the mouse intestine following a chronic low Pi diet [44], in order to maximize Pi absorption in this primary location. Increased expression of NaPi-IIb can also be induced by injection of cholecalciferol (unhydroxylated vitamin D) intraperitoneally 24 hours before tissue collection. This supports previous studies showing that vitamin D increases the rate of Pi transport across the intestinal BBM vesicles [121]. Low Pi diet and vitamin D-induced increases in intestinal Pi transport also occur independently of one another, as shown by experiments utilizing vitamin D receptor knockout mice. These mice exhibited an increase in NaPi-IIb expression in response to a low Pi diet, which was comparable to wild-type mice [122].

1.5.2.2. Intestinal Pi regulation by vitamin D

Extensive studies have revealed the effects of vitamin D on intestinal Pi transport and NaPi-IIb (section 1.2.1.4), however vitamin D deficiency does not have a major effect on total intestinal Pi absorption in adult rats [123]. This may suggest that vitamin D plays a minor role in directly affecting Pi handling in the intestine, but its effects on other Pi regulatory factors such as FGF-23 still makes it an important factor in maintaining overall Pi homeostasis.

1.5.2.3. Intestinal Pi regulation by other factors

Epidermal growth factor (EGF) [124] , glucocorticoids [118], estrogens [125], and metabolic acidosis [115] also have an effect on NaPi-IIb in more rare and clinically less relevant instances. This section will focus on a brief summary of each.

EGF is an important factor in the maintenance of gut morphology [126] and corresponding, high affinity EGF receptors are expressed along the entire GI tract [124]. EGF treatment in rats decreases NaPi-IIb mRNA levels by approximately 50%. by reducing the NaPi-IIb gene promoter activity [124]. In rare clinical cases such as intestinal ischemia or injury-induced hyperphosphatemia, circulating EGF levels are elevated as well as endogenous EGF requirement in tissues [127], suggesting that EGF plays a part in intestinal Pi transport.

Glucocorticoids are important regulators of overall enterocyte maturation and plasma levels of glucocorticoids are age-dependent [128]. Glucocorticoids inhibit intestinal Pi transport in an age-dependent manner with the highest level of inhibition occurring in suckling rats in contrast to the effect on renal Pi reabsorption which only occurs in adult rats [118]. The inhibitory effect of glucocorticoids on intestinal Pi uptake is mediated through changes in genomic and protein expression of NaPi-IIb.

Estrogen is a main regular of calcium homeostasis since it affects calcium absorption, bone density, and vitamin D synthesis [125]. Estrogen stimulates intestinal sodium-

dependent Pi transport by altering genomic and protein expression of NaPi-IIb [125], which may occur through estrogen receptors which are expressed along the intestinal epithelium [129].

The effect of metabolic acidosis on intestinal Pi transport is not as well characterized as in renal Pi reabsorption. Gafter et al showed that metabolic acidosis in rats increased intestinal sodium-dependent Pi uptake with a parallel increase in expression of NaPi-IIb protein at the intestinal BBM [130]. However, in both rat and mouse animal models, Pi transport adaptations to metabolic acidosis does not extend to changes in genomic expression of NaPi-IIb, thus the importance of intestinal Pi absorption during metabolic acidosis is questionable. An increase in intestinal Pi absorption would theoretically be helpful in buffering extracellular protons and compensate for loss of Pi from the bone during acidosis.

Although dietary Pi and vitamin D have well-defined roles in altering intestinal Pi transport, phosphatonins have yet to become fully understood in this context. Further research is crucial in obtaining knowledge of the intestinal actions of phosphatonins in order to increase the available targets for regulating Pi absorption across the intestine.

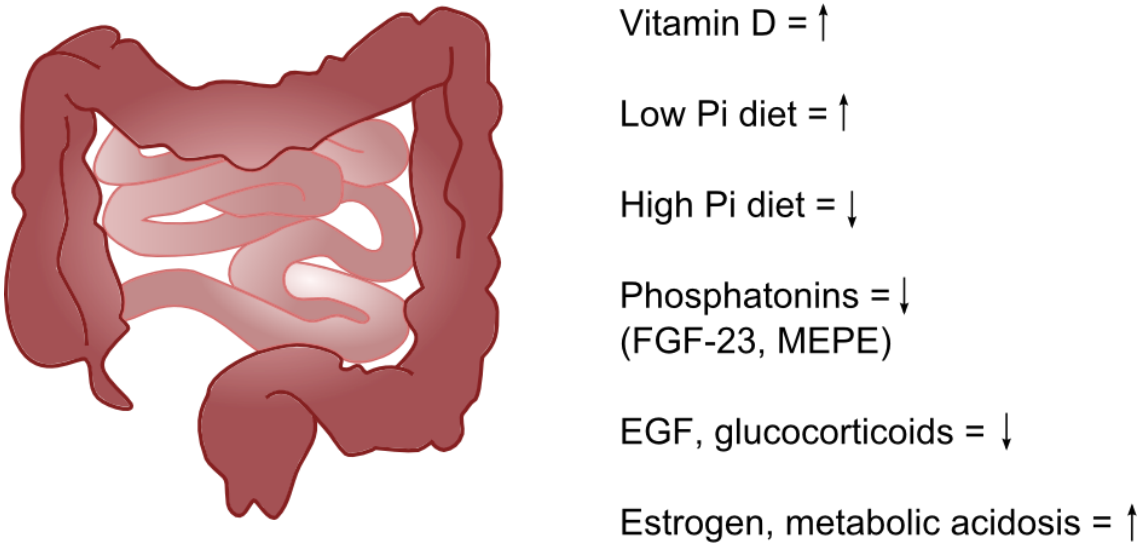


Figure 1.5. Summary of factors influencing intestinal Pi absorption

1.5.3. Passive paracellular and unknown transcellular Pi transport

There is ongoing debate as to the specific proportion of intestinal Pi transport which is attributed to sodium-independent paracellular and transcellular routes. Intestinal Pi absorption can be divided into a saturable, sodium-dependent transcellular pathway and a non-saturable sodium-independent pathway – the luminal concentration of Pi and the specific bioavailability of Pi in luminal contents determine which transport system is dominant.

Sodium-independent Pi transport was revealed with very early studies using intestinal brush border membrane vesicles (BBMV) demonstrating that this transport pathway contributed to as much as 40-50% of total Pi transport [131, 132]. Thus, unlike the

kidney where sodium-independent Pi transport is negligible, this pathway seems to comprise a significant proportion of Pi transport which has been reported in both rat [44, 70] and human small intestine [133]. This pathway is also not regulated by the classical modulators of intestinal Pi transport (e.g. vitamin D, dietary Pi), however experiments reveal that young animals and those treated with glucocorticoids have increased sodium-independent intestinal permeability [134, 135]. These results point to the fact that there may be an unidentified Pi transporter located at the BBM which is responsible for sodium-independent transcellular Pi transport. Conversely, sodium-independent transport measured in BBMV may be an artifact of the technique and the measured Pi transport in that scenario could have occurred via an unregulated route.

In depth *in vivo* examination of different routes of intestinal Pi transport arose from early studies in man which indicated that Pi absorption was mediated by active, sodium-dependent transport at luminal concentrations below 2mM and by passive diffusion when over 2mM Pi [119]. Further studies using the conditional tamoxifen-induced NaPi-IIb$^{-/-}$ KO mouse showed that NaPi-IIb is indeed accountable for 90% of Pi transport across the mice ileum. However, only 50% of the total amount of Pi absorbed from the diet could be attributed to sodium-dependent, transepithelial Pi transport [54]. This conclusion reaffirms previous observations of a significant sodium-independent component of intestinal Pi transport, although whether this transport pathway occurs via a paracellular or transcellular route is still unidentified [133, 136]

Interestingly, tight junction proteins such as claudins and occludins, which provide a regulated and selective route for passive paracellular ion flow as well as epithelial cell adhesion [137] show subtle changes in expression under the stress of renal damage and CKD. Studies show alterations in protein levels of claudin-1 and occludin, key constituents of tight junctions in intestinal epithelia, in patients with CKD [138]. Since tight junctions may regulate the still uncharacterized sodium-independent Pi transport pathway, these changes need to be investigated further to elucidate their consequence on intestinal Pi transport in CKD. These data further support the notion that there is interplay between the small intestine and the kidney in Pi transport

To summarize, it is probable that during fasting and low dietary Pi concentrations, Pi absorption is mediated mostly by NaPi-IIb. However, when luminal Pi levels are elevated post-prandially, transport could also occur via a sodium-independent transcellular or paracellular pathway. It is significant to note that both pathways have the potential to contribute to Pi imbalance, and whilst the NaPi-IIb knockout mouse model has advanced our understanding of the role of this protein in intestinal Pi absorption, further studies are necessary to define the mechanisms of sodium-independent Pi transport.

1.6. Kidney and GI tract: a potential signalling axis

In a publication by Berndt et al (2007), an 'intestinal mucosal factor' that directly influences renal Pi absorption was hypothesized [139, 140] . Their experimental work

showed a rapid increase in Pi excretion < 30 min. after duodenal instillation of 1.3M Pi [140]. This acute and specific phosphaturic effect, unlike the long-term adaptation to dietary Pi, was found to be independent of PTH and occurred without changes in plasma concentrations of Pi or other known phosphatonins such as FGF23 and sFRP4. The high concentration of Pi used in these experiments was an issue of concern as it may also alter physiological responses – the effects of a Pi solution with such high osmolarity on the intestinal lumen are unknown. MEPE has been proposed as a potential candidate for this unknown phosphaturic factor since its mRNA is expressed in the small intestine and treatment of rats with MEPE increased Pi uptake at the intestinal BBM [90] (see section 1.3.3); however the specific mode of action has not yet been investigated.

Interestingly, studies using NaPi IIb$^{+/-}$ and NaPi-IIb$^{-/-}$ KO mice have shown a role for NaPi-IIb in this potential cross-talk mechanism. Thus targeted deletion of NaPi-IIb is associated with decreased FGF-23 levels and decreased urinary Pi excretion in order to maintain normophophatemia [55, 141]. In support of these findings, data from previous studies have shown that in contrast to the well documented association between FGF-23 and dietary Pi intake [142], acute non-dietary modulation of serum Pi levels within the normal range does not induce changes in FGF-23 [143, 144]. Therefore it appears that changes in intestinal Pi concentrations and/or intestinal Pi sensing may be the primary regulator of FGF-23 and that only supra-physiological changes in serum Pi concentration, such as during CKD, are associated with increased FGF-23 levels.

1.7. Potential role of the liver in Pi handling

The liver is an important metabolic organ well known for its function as a filtering unit for the body [145] The liver weighs between 1200 and 1500g and, with exception of the skin, is the largest organ in the human body (approx 4-5% of body weight in newborn and 2-5% in an adult). The blood supply to the liver also has two sources; the portal vein contributes approximately 75% of total circulating blood to the liver, the hepatic artery contributes the other 25%. The liver is strategically situated in the circulatory system, receiving the portal blood that drains the stomach, small intestine, large intestine, pancreas, and spleen. In this position, the liver plays a key role in processing nutrients from postprandial contents assimilated by the small intestine **(Figure 1.7).**

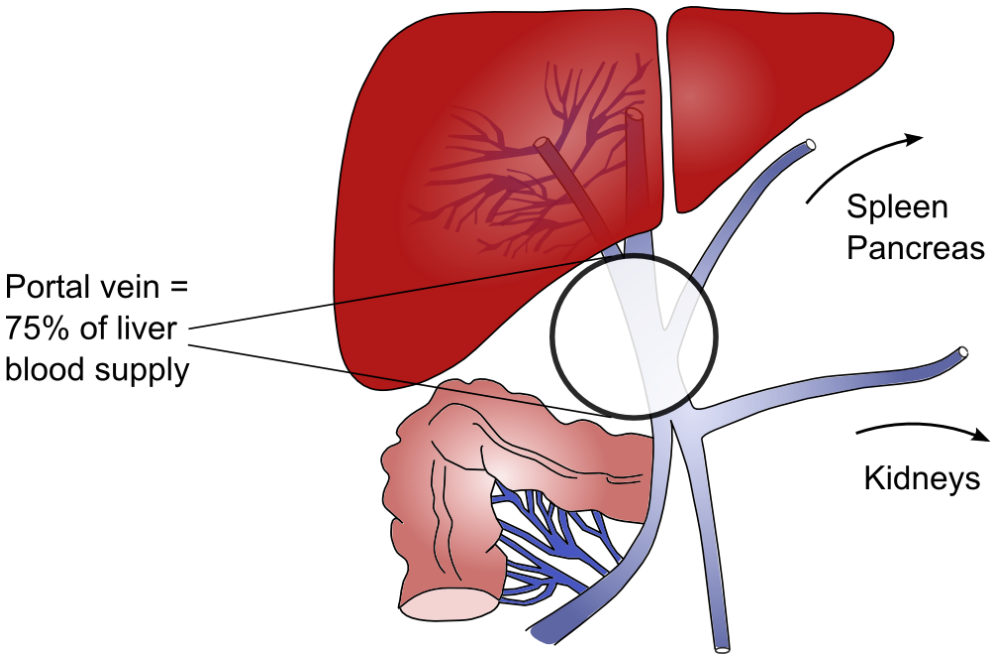


Figure 1.6. Schematic of hepatic portal vein location relative to the liver and small intestine

The liver metabolizes an extensive variety of compounds that are delivered by the portal and systemic circulations which include endogenous molecules (bile salts and bilirubin, key ingredients of bile) and exogenous molecules such as drugs and toxins. The liver also has the capacity to convert important hormones and vitamins into a more active form. Pi homeostasis relevant examples include the initial hydroxylation of vitamin D. One of the first steps of conversion of 7-dehydrocholesterol (cholecalciferol) and ergosterol into the active form of vitamin D also occurs in the liver through cholecalciferal 25-hydroxlyase in the mitochondria and microsomes [146]. Apart from this function of activating certain hormones, the liver also functions as a secretory organ through bile.

Bile is a complex secretory product produced by the liver which eliminates many endogenous and exogenous waste products from the body, such as bilirubin and cholesterol [145] and potentially, Pi. The composition of the bile is modified significantly as a result of the absorptive and secretory properties of epithelial cells that line the intrahepatic and extrahepatic bile ducts.

The Na-K pump present at the BLM of hepatocytes maintains a low cytosolic Na and high K concentration, as in most cells, while a basolateral ATP dependent Ca+ pump maintains Ca+ at an extremely low level, approx 100nM. Hepatocytes use the inwardly directed Na gradient to fuel a variety of active transporters, such as the Na-H exchanger, Na/HCO_3 cotransporter, and Na driven amino acid transporters (e.g.

SNAT4, LAT1/2 [147, 148]). Hepatocytes also take up glucose via the GLUT2 facilitated diffusion mechanism, which is insensitive to regulation by insulin.

The idea that the liver plays a role in the context of Pi transport and homeostasis stems from the longstanding observation that serum Pi fluctuations occur following hepatectomy and in patients with liver diseases. The initial consensus was that hypophosphatemia was always seen immediately following hepatectomy and proposed theories include the liver requiring extra metabolic needs, and Pi, from the blood for regeneration [149]. However, the magnitude of hypophosphatemia seen could not be accounted for by the demands of extra Pi uptake, thus other factors were investigated to explain this clinical observation. Nafidi et al as well as Salem et al observed an early post-operative increase in fractional excretion of Pi post-hepatectomy which was independent of FGF23, FGF7, sFRP-4 and PTH (after day one) [150, 151].

In 2004, type II and III sodium phosphate cotransporters (NaPi-IIb and PiT1 and 2) were indentified in hepatocytes and cholangiocytes in rat liver. Their possible roles in this organ involve regulating biliary Pi concentration since hepatocytes and cholangiocytes release ATP into bile which degrades to adenosine and Pi [13]. Excess Pi in the bile could potentially be taken up by these transporters to maintain Pi homeostasis.

Another example of a functional process performed by the liver which is relevant to Pi regulation involves cathepsin B, the protease that cleaves a recently characterized phosphatonin MEPE into ASARM, another biologically-active and protease-resistant

phosphaturic factor that is also produced in the liver [152]. These findings combined with the knowledge that there is a PTH receptor in the liver that is also downregulated in a similar fashion to the renal PTH receptor under the constraints of CKD [153] further supports the notion that the liver may be a key player in Pi regulation and may be an intermediary in the proposed intestinal-renal axis of Pi homeostasis.

Recent preliminary studies have shown that a phosphaturic factor may be excreted by the liver [154]. Using a rat model of partial hepatectomy, which show similar hypophosphatemia and phosphaturia as seen in patients, this group then utilized BBMVs prepared from the intestine and the kidneys of these animals. These BBMVs showed a decrease in NaPi expression as well as a decrease in overall Pi transport. Utilizing a DNA microarray, this group revealed that this abnormal Pi transport was due to a defect in metabolism of nicotinamide, a known blocker of NaPi transport. Further studies seeing if this pathway could be blocked were conducted on cell lines, so the application of this work to intact tissue is yet to be determined.

1.8. Pi imbalances and current treatment

1.8.1 Hyperphosphatemia

Hyperphosphatemia is a serious condition commonly associated with renal insufficiency and CKD. In CKD, glomerular filtration rate (GFR) gradually decreases as time progresses, and renal Pi handling is altered by the progressive decline in the fraction of

filtered Pi that is reabsorbed - normally about 70% (see figure 1.1). In patients with CKD, PTH is secreted in response to a decline in ionised serum calcium concentrations due to higher than normal Pi levels binding with serum calcium [155]. High PTH levels in turn, prompt calcium release from the bone and also increase urinary Pi excretion to normal serum Pi concentration. Due to a decline in the number of functional nephrons in later stages of CKD, the phosphaturic actions of PTH become less effective.

Patients with CKD also exhibit suppression of renal 1α-hydroxylase activity which results in lowered plasma levels of active vitamin D [156]. This could be due to a decreasing functional renal mass and lower baseline 1α-hydroxylase levels in general, a declining GFR which limits the distribution of calcifediol (precursor to active vitamin D), hyperphosphatemia itself [157], and increasing levels of FGF-23 seen in patients (see phosphatonins 1.3) [76, 158].

Hyperphosphatemia stimulates PTH release, and over secretion of PTH eventually leads to secondary hyperparathyroidism [159]. Furthermore, a decrease in CaSR expression in parathyroid glands of CKD patients may also be a contributing factor to secondary hyperparathyroidism [160], secondary hyperparathyroidism now also contributes to hyperphosphatemia by a feed forward mechanism by continuously stimulating the release of calcium phosphate from bone. Although PTH increases renal Pi excretion, in late CKD this proves to be inefficient as there is a decrease number of functional nephrons.

The development of high blood Pi contributes to secondary hyperparathyroidism and cardiovascular (CV) complications, conditions which drastically increase mortality rates among CKD patients [161] **(Figure 1.7).** There is a long established correlation between hyperphosphatemia and CV complications in end-stage CKD patients receiving dialysis, however hyperphosphatemia is also a risk factor for CV disease in patients with early stages of CKD [8]. In individuals possessing normal renal function, there is data proposing that even small changes in serum Pi may also be associated with an increase in age-related CV disease [162, 163]. Although there are no studies in man concerning the direct effects of Pi toxicity and CV disease, there has been recent findings exploring this causal link. Finch et al investigated dietary Pi intervention on degree of calcification of the aorta of surgically induced CKD rats. One group of rats with CKD were put on high Pi diets and another on Pi binders with or without additional low Pi diets. A marked difference in aortic calcification was seen, with untreated CKD rats showing the most calcification, rats on Pi binders had a lowered calcification, and those on both Pi binders and Pi-controlled diet had the least calification. Furthermore, kidney tissue histology from animals on Pi binders and both Pi binders and diet control also showed the most normal ultrastructure, with minimal interstitial fibrosis and damage [164].

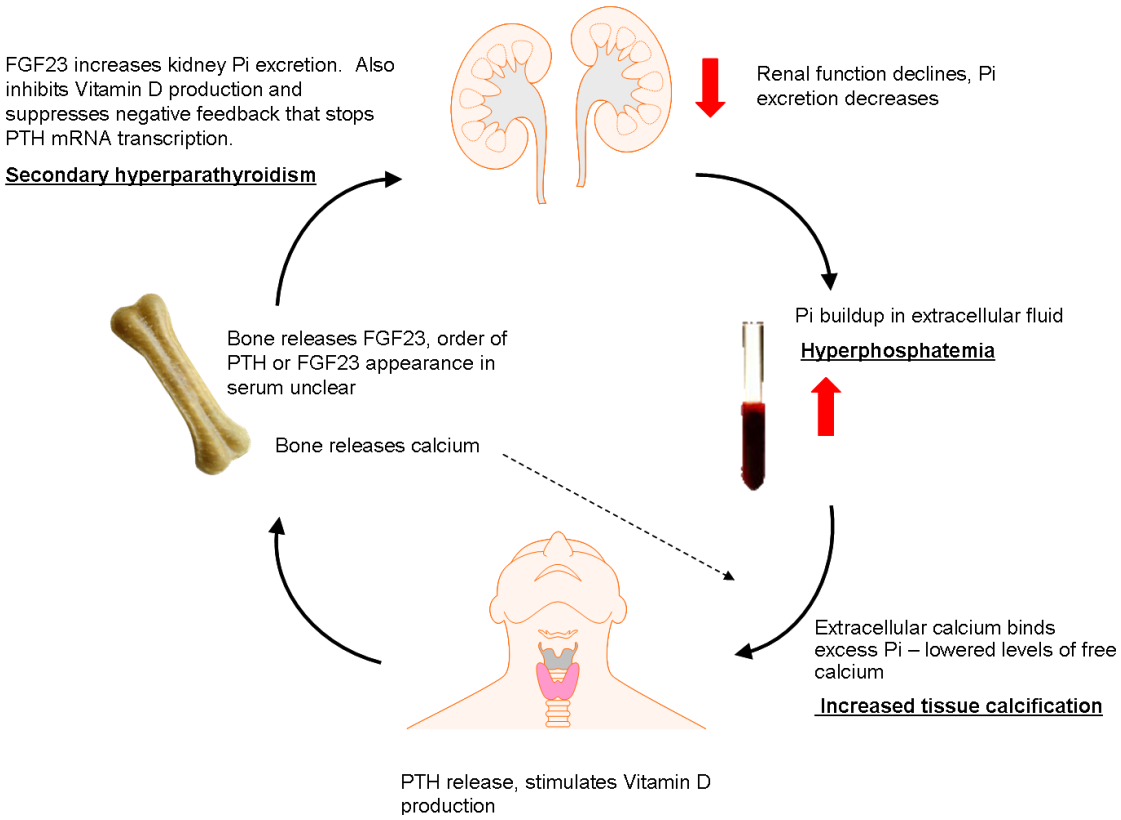


Figure 1.7. Sequence of hyperphosphatemia-related conditions in CKD

In early CKD, serum Pi may sometimes be within the normal, accepted range and thus may not be a reliable first marker for altered Pi handling. This fact is emphasized with the findings that increased serum FGF-23 (with or without combined low urinary Pi excretion) and low serum αKlotho all correlate with an increase vascular calcification and high morbidity in CKD patients with normal serum Pi levels [165-167].

1.8.1.2 Vascular calcification

Vascular calcification is a serious condition associated with CKD and lends to the high mortality rates in this patient group as mentioned in the above section. The condition involes ossification (deposition of calcium phosphate precipitate products) within arterial

vessel walls which causes them to harden and restricts blood flow. Calcification of vascular smooth muscle cells (VSMC) is stimulated by elevated extracellular levels of Pi, calcium and PTH which are all commonly observed in patients with CKD [168]. The actual process of calcification during hyperphosphatemia involves further differentiation of VSMCs into osteoblast-like cells which is promoted by transcription factors Runx2 and CBfa1 [169]. This leads to expression of a broad range of genes related to the osteoblast phenotype (Gla protein and alkaline phosphatase)[169, 170].

Pi uptake by VSMCs involve both sodium-dependent and independent pathways [69]. The saturable, sodium-dependent Pi transport mechanism is mediated by type III transporters PiT1 and PiT2 [68], whereas expression and function of the type II NaPi transporters are minimal [69]. Increased expression of both PiT1 and PiT2 is thought to mediate vascular calcification during CKD which would result in an increase in Pi absorption into VSMCs and trigger the osteogenic differentiation [171]. Although both PiT1 and PiT2 are upregulated in VSMCs during CKD, it has been recently shown in mice that the role of PiT1 and PiT2 in VSMCs is redundant in the specific context of vascular calcification [172]. In this study, a VSMC-specific, PiT1 knockout mouse model of CKD showed upregulated PiT2 protein with an equivalent amount of vascular calcification as wild type mice following dietary Pi loading.

1.8.1.3. Treatment for hyperphosphatemia

Current treatments for high blood Pi include dietary Pi control as well as oral Pi binders, however these therapies prove to be substandard in regulating serum Pi in and may

even contribute to malnutrition and acceleration of calcification in soft tissues as well as a spectrum of unpleasant side effects such as gastrointestinal discomfort [173].

Novel Pi binders are frequently studied with the goal of optimizing Pi binding capacity and minimizing side effects. One compound being investigated in stage III clinical trials is ferric citrate hydrate (FC). Experiments conducted in normal rats have shown that FC significantly decreases Pi absorption across the intestine whilst increasing fecal Pi excretion to a degree comparable to calcium carbonate [174]. When FC is administered to adenine-induced CKD rats, there is a reduction in aortic calcification, serum PTH levels and the severity of parathyroid hyperplasia [174]. In patients, FC treatment is effective at reducing serum Pi concentrations and the results of early studies suggest that FC may also increase levels of iron and iron storage capacity in the blood; this finding could imply that CKD treatment costs involving additional anemia could be reduced with the use of FC [175].

Another treatment strategy currently under investigation is the idea to potentially target the process of intestinal Pi absorption to reduce CKD-induced hyperphosphatemia Data from NaPi-IIb KO mice with adenine induced CKD have confirmed positive results from this approach by showing that the absence of NaPi-IIb partially attenuates hyperphosphatemia [141]. However, combined with the known Pi binder, Sevelamer, hyperphosphatemia is completely normalized; this finding emphasizes that both NaPi-IIb dependent and independent processes contribute to elevated Pi serum levels in CKD [141]. Future treatment options are envisaged to revolve around targeting NaPi-

IIb dependent Pi transport combined with using Pi binders to patients on dialysis. Known inhibitors of NaPi-IIb, nicotinamide and phosphonoformic acid (PFA), which competitively inhibit NaPi transporters *in vitro* have varying effects on transport in *in vivo* CKD animal models as well as CKD patients [123, 176, 177]. Daily administration of nicotinamide to CKD rats decreased Pi uptake across the jejunal BBM and was associated with improved blood Pi levels [178]. Treatment of hemodialysis patients with nicotinamide also resulted in amelioration of hyperphosphatemia, although this was also coupled with varying gastrointestinal side effects which may prevent its clinical use [179]. More alarmingly, thrombocytopenia has also been reported in certain dialysis patients who were receiving nicotinamide treatment for 3 months or more [180]. Whilst PFA administered to CKD induced rats show an increase in phosphaturia [177], it does not appear to fully attenuate hyperphosphatemia. [177, 181].

1.8.2. Hypophosphatemia

Hypophosphatemia may be attributed to poor diet, malabsorption of Pi, or inherited conditions affecting Pi reabsorption in the kidney such as hypophosphatemic rickets and X-linked hypophosphatemia (XLH) [182]. When hypophosphatemia is allowed to go untreated, further complications occur in the form of respiratory failure as a result of muscle weakness and other conditions involving the muscles such as rhabdomyolysis [183].

Hypophosphatemia in pediatric patients leads to much more severe consequences due to the high demand for Pi and calcium to support both skeletal and somatic growth and

development. Prolonged hypophosphatemia in children can occur as a consequence of insufficient dietary intake or vitamin D deficiency, leading to calcipenic rickets. The reduction of available calcium in the intestine leads to elevated levels of serum PTH (stimulating release of calcium from the bone) which then triggers the internalization of renal NaPi-IIa and NaPi-IIc. This results in renal Pi wasting and hypophosphatemia [184].

Renal Pi wasting can also be a consequence of phosphopenic rickets, but can also develop as a result of disorders which directly affect Pi homeostasis such as excessive secretion of FGF-23 (in TIO), mutations in genes encoding FGF-23 (ADHR), PHEX (XLH), and DMP-1 (autosomal-recessive hypophosphatemic rickets) or inactivating mutations in renal Pi transporter NaP-IIc (hereditary hypophosphatemic rickets with hypercalcuria) [185]. PHEX and DMP-1 are known factors upstream of FGF-23 that contribute to its synthesis in the bone [186].

Skeletal growth abnormalities associated with hypophosphatemia in pediatric patients depend on the severity of hypophosphatemia and the age of onset - young infants develop deformities in their weight bearing limbs with crawling infants' forearms being particularly affected, while bow-legged (genu varum) phenotypes appear in toddlers. Older children and adolescents generally show different conditions such as muscle weakness and pain in the lower limbs which are magnified by exercise. Other clinical features consist of growth retardations, dental deformities leading to abscesses, skulll deformities, spinal curvature, and joint swelling and pain [185].

1.8.2.1. Treatment for hypophosphatemia

Current treatment for calcipenic rickets involves oral loading of vitamin D, its synthetic analogue 1α-hydroxyvitamin D_3, and calcium supplementation. Individuals with phosphopenic rickets are treated with Pi supplements combined with vitamin D or 1α-hydroxyvitamin D_3. Although these treatments address the issue of hypophosphatemia, their success in minimizing bone growth defects in later life are variable [187].

Through recent advantages in the understanding of the pathogenesis of familial hypophosphatemic rickets, novel therapeutic options for the management of these disorders are under investigation. The latest studies involving a cohort of patients with XLH show promise of increasing serum Pi levels through treatment with calcitonin [188]. This hormone acts via its receptor on osteocytes [189] to inhibit the release of FGF-23 and reduce the renal Pi wasting characteristics of this disease. Studies using the *Hyp* mouse (a murine homologue of XLH), show that treatment with an anti-FGF-23 neutralizing antibody results in improved Pi balance, bone growth, and reduced muscle weakness [190]. More recently, further studies using the *Hyp* mouse and also the Dmp-1 null mouse show that a newly described selective, pan-specific FGFR inhibitor also corrects hypophosphatemia as well as enhances bone growth, increases mineralization, and restores growth plate organization [191]. An important note is that inhibition of FGFR did not influence renal NaPi-IIa expression or the fractional excretion of Pi, signifying that the amelioration of hypophosphatemia may have been mediated via another route such as intestinal absorption of Pi [191]. Recombinant human growth

hormone (rhGH) has also been suggested as a standalone or adjunct to standard therapy for XLH, however this treatment still remains debatable as data show variability in benefits in terms of bone growth. Some studies present convincing results showing increases in truncal height leading to a disproportion throughout the body, while other studies actually point to an increase in mortality – the current benefit to risk ratio is currently under review [192]

Another potential treatment for hypophosphatemia in early stages of study is the potential to directly stimulate the intestinal Pi transport process in order to increase dietary Pi absorption, increase serum Pi levels, and promote bone growth in phosphopenic rickets. However, regardless of the treatment combination chosen for the patient-specific needs, the need for early diagnosis and promptness of treatment is crucial and can result in steering children with XLH towards normal growth [193].

1.9. Project aims

Sprague Dawley (SD) rats were used in these studies for a variety of reasons. Rats are a good animal model to study body Pi balance as their regional expression of sodium-phosphate transporters is most similar to humans. Thus, the results from these studies may have a more direct, translational aspect than using mice. Organs that are manipulated in the experiments (intestine, kidney and vessels/veins) are also more easily handled in rats compared to mice, as the larger overall size of these tissues in

rats leads to a decrease in potential damage from utilization. Intestinal and renal brush border tissue collected for analysis is also more abundant in rats compared with mice.

The aims of the project were to:

1. Define the reasons for discrepancies in intestinal Pi transport previously reported using different techniques for measuring Pi transport. Specifically, Pi uptake using the *in vitro* everted sleeve was compared to the *in situ* intestinal loop methods.

2. Elucidate components of active/passive Pi transport by using an *ex vivo* intestinal perfusion model.

3. Replicate *in vivo* experiments conducted by Berndt et al (2007) with a more physiological intestinal luminal concentration of Pi (10mM) as well as the 1.3M concentration that Berndt et al utilized [139, 140]. To determine whether or not there is a duodenal phosphaturic factor working following an elevated level of Pi in intestinal luminal fluid, and to also examine NaPi-IIa and NaPi-IIc expression at the renal BBM under these experimental conditions.

4. Conduct *in vivo* experiments to make conclusions regarding the role of the liver as a potential storage organ for Pi during the intestinal Pi uptake process.

2.0. Assessment of regional differences in intestinal Pi transport using *in vitro* and *in situ* techniques

2.1. Introduction

Hyperphosphatemia is one of the major associated conditions of CKD, with recent studies suggesting that even small changes in serum Pi may be linked to a higher mortality rate in this subset of the population [194]. With declining renal function in patients with CKD, the small intestine becomes the next organ to target in regulating Pi transport. Unfortunately, the mechanisms and control of Pi transport in the intestine are incompletely understood with emerging data gathered from a wide variety of methods conflicting with data from other studies.

There are opposing views concerning the contribution of specific regions of the intestine to overall Pi transport as well as the proportion of transport attributed to sodium-dependent, active transport and passive transport (either paracellular or transcellular). These variations in opinion stem from data gained through a large spectrum of methods currently being employed to investigate Pi transport. Species-specific differences in the intestinal region where the highest Pi transport takes place (e.g. mouse ileum vs. rat jejunum) also produce results which may seem paradoxical.

Intestinal Pi transport can be categorized as active or passive, the former involving the activity of sodium phosphate cotransporters and the latter occurring paracellularly or

through an unknown and/or unregulated transcellular pathway. Early studies which focused on intestinal brush border membrane vesicle (BBMV) experiments revealed a Pi transport system that was highly sodium dependent, with a K_t of around 0.1mM [195, 196]. Following this discovery, the type II sodium phosphate cotransporter, intestinal NaPi-IIb, was cloned and characterized by expression in oocytes. Upon further analysis, this transporter was eventually found to be responsible for the majority of intestinal Pi transport [197]. The type III sodium phosphate cotransporter, PiT1, is expressed ubiquitously throughout the small intestine [62]. However, its role in overall Pi homeostasis is thought to be minimal due to a lack of acute, Pi-dependent regulation of its expression at the BBM [62], the site of the rate-limiting step for transcellular Pi transport.

In contrast, *in vivo* studies using the entire rat GI tract show that relatively little Pi transport is sodium-dependent [198]. Indeed this finding, which utilized a novel method of radiolabeled Pi tracking, confirmed early observations of a sodium-independent component of intestinal Pi transport [136]; it is still unclear whether this "passive" mode of transport occurs via a transcellular or paracellular route. Therefore in contrast to renal Pi transport, where it has been established that sodium-independent Pi transport is insignificant, diffusive pathway contributes a large amount to overall intestinal Pi transport in both rats [44, 198] and man [133].

The role of tight junction proteins such as claudins and occludins, which provide a regulated and selective route for paracellular ion transport, is still unknown in the

context of epithelial Pi transport. It has been shown that intestinal claudin proteins undergo subtle changes in expression as a response to renal damage and CKD [137]. Studies also show that there are alterations in protein levels of claudin-1 and occludin, key constituents of tight junctions in intestinal epithelia, in patients with CKD [138]. Since tight junctions may regulate the still uncharacterized sodium-independent Pi transport pathway, these changes need to be investigated further to elucidate their consequences for intestinal Pi transport in CKD.

Detailed, regional specific, Pi transport patterns under both sodium and sodium-free conditions however, is still not well established in the intestine. Directly comparing methods of measuring Pi transport is important to determine the benefits and pitfalls of each technique and eventually to establish which method is a more adequate model for Pi transport.

2.1.1. Aims of this study

1. To compare the rates of sodium-dependent Pi transport utilizing two distinct *in vitro* and *in situ* techniques in five regions of the intestinal tract – the duodenum, jejunum, ileum, proximal colon, and distal colon. Three different concentrations of Pi in the uptake buffer will be used to represent low, normal, and post-prandial Pi levels in the intestinal lumen (0.1, 1, and 10mM respectively).

2. To compare the rate of Pi transport (in the presence of sodium) across the five aforementioned intestinal regions using *in vitro* and *in situ* techniques.

3. To determine changes in epithelial structure using electron microscopy to examine whether observed changes in Pi uptake reflect altered morphology or tissue damage as a consequence of the experimental protocols used.

2.2. Materials and methods

Ethics Approval: all experiments were performed under a UK Home Office Project license and protocols were approved by the Ethics Committee at University College London. All surgeries were performed under general anesthesia (details are given under relevant sections, below).

2.2.1. Animal model

Male Sprague Dawley (SD) rats of body weight 250g-300g were obtained from Charles River laboratories (UK) and used in accordance with the Animals (Scientific Procedures) Act 1986. Rats were allowed *ad libitum* access to standard rodent chow containing 0.52% phosphate (Diet RM1, SDS Ltd, Witham, Essex UK) and water.

2.2.2. *In vitro* everted intestinal sleeve technique

The everted sleeve technique exposes the mucosal surface of the intestinal segment to the uptake buffer containing radiolabelled phosphate – this allows direct measurements of Pi uptake across the intestinal BBM **(Figure 2.1).** The validation of this preparation and optimal conditions for measurements has been determined [199]. Sprague-Dawley rats were anesthetized using an intraperitoneal (IP) injection of pentobarbitone sodium (Pentoject, Animalcare York, UK), 45mg/kg body weight, and specific regions of intestine (duodenum, jejunum, ileum, proximal colon, distal colon, each about 2-4cm long) were removed from the animal. The duodenum was taken ~1cm from the stomach pylorus, jejunum was taken ~1cm distal to the ligament of Treitz, ileum began

about 3cm proximal to the cecum, proximal colon began 3 cm distal to the cecum, and the distal colon about 2 cm from the anal sphincter. A maximum of two tissue samples per specific segment were used in each animal. The order of tissue removal was randomized, and a maximum of four segments were taken from any one rat so as to not compromise tissue viability.

The segment lumen was first flushed through with room temperature, isotonic saline to remove any debris in the lumen. The intestine was then everted on a glass rod and secured with ligatures at the top and bottom edges. The tissue was subsequently incubated for 5 min in a buffer containing 16mM Na-HEPES, 3.5 mM KCl, 10mM $MgSO_4$, 1mM $CaCl_2$, and 125mM NaCl, which was oxygenated with 100% O_2 and stirred constantly with a magnetic stirrer. The tissue was then incubated for 2 min in 2 mL of Pi buffer containing 10mM glucose, 0.1, 1, or 10mM KH_2PO_4 and 0.74 MBq ^{32}P (Perkin Elmer, Bucks, UK) adjusted to a pH of 7.4 **(Figure 2.1).** A 2 min uptake period chosen for Pi uptake since this was in the middle of the linear period of Pi uptake[200], and was also brief enough to allow minimal ^{32}P to reach the serosal side of the tissue. Choline chloride (ChCl) was used as an iso-osmotic substitute for sodium chloride to allow determination of sodium-free Pi transport.

Following the 2 min incubation period, the tissue was removed from the incubation buffer and the tissue was washed for 10 min at room temperature with saline or ChCl containing Pi at a 10x greater concentration than the one used in the uptake solution, This was to displace radiolabelled Pi bound to the tissue surface. A final wash was then

carried out using phosphate buffered saline (PBS) for 5 min. These two washes were sufficient to displace most of the bound ^{32}P while retaining ^{32}P already transported into enterocytes. The tissue was then removed from the glass rod and a small segment from the middle of the tissue (~100 mg) was taken, weighed and digested overnight in Solvable (Perkin Elmer, MA, USA).

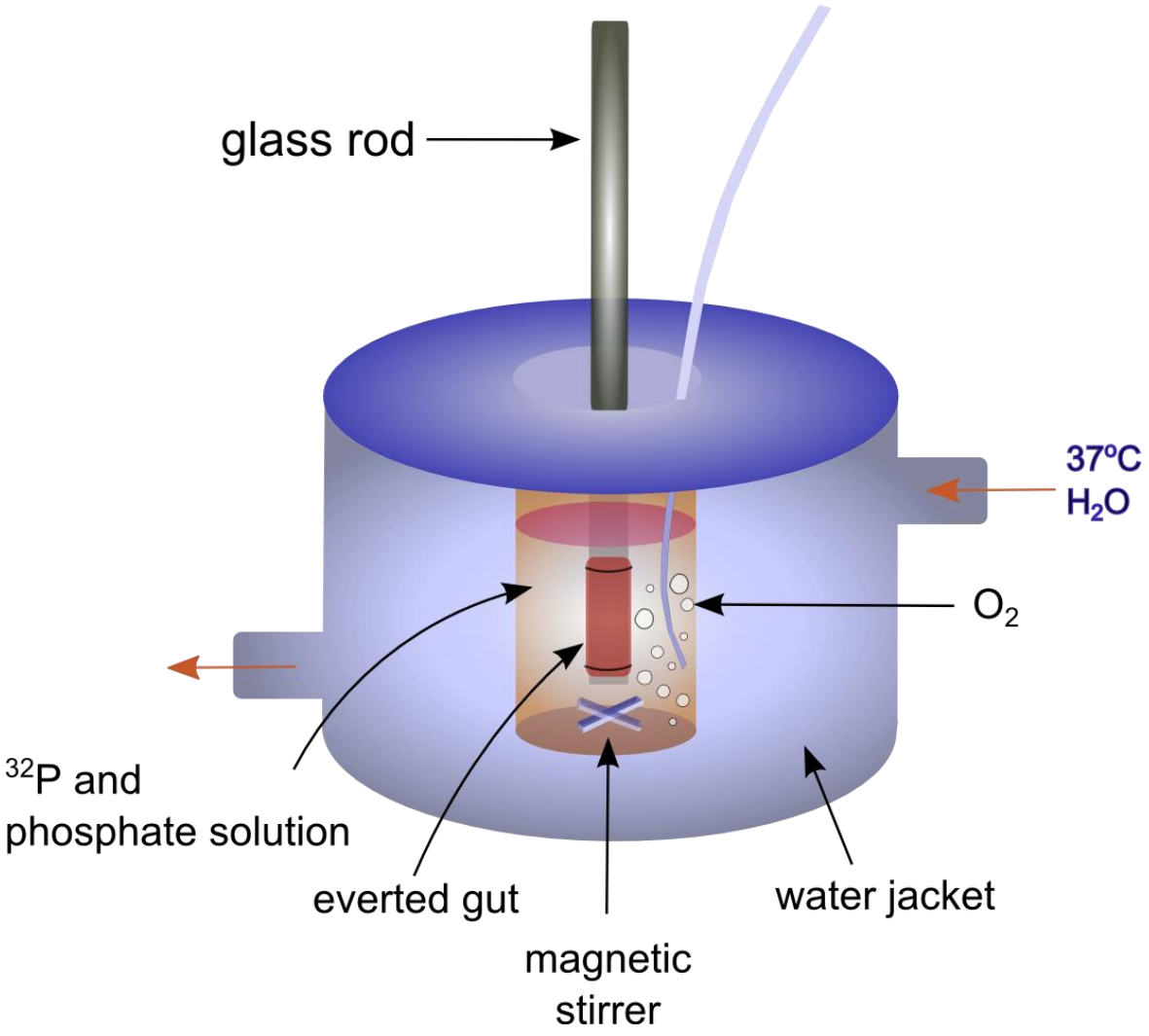


Figure 2.1. Diagram of *in vitro* everted sleeve technique method used for measuring Pi uptake

2.2.3. *In situ* intestinal closed-loop technique

The jejunum was utilized for the closed loop technique due the higher NaPi-IIb protein expression in this region of rat small intestine [12]. The closed loop method provides maximum exposure to the Pi containing buffer in the segment with highest expression of phosphate transporter.

The jejunum was identified (starting at the ligament of Treitz) and a 5cm segment was securely tied off with ligatures. An opening was made at either end of the section; a cannula-sheathed 19-gauge needle was inserted at the top and secured with another ligature **(Figure 2.2**). A 37^{o}C warmed solution (0.9% saline or 154mM choline chloride [ChCl]) was flushed through this section to remove any food debris. Following this, air was flushed through to remove as much solution as possible, then 500μL of ^{32}P radio-labelled (0.37Mbq) HEPES phosphate solution (16mM Na-HEPES, 140mM NaCl, 3.5mM KCL, 1/5/10/15mM KH_2PO_4, pH 7.4) was instilled via the cannula-sheathed needle. When the solution was visible about halfway down the section, the bottom loose ligature was tied off, and after the complete 500μL was instilled the needle was carefully removed and the ligature was tightened distal to the cannula entry **(Figure 2.2)**. For experiments involving sodium-free buffer, saline was replaced with ChCl with all other concentrations of solutes remaining the same. Osmolarity of the HEPES buffer was assessed using a freezing point depression micro-osmometer (Model 3M, Advanced Instruments Inc, Massachusetts, US) and all solutions were within isotonic range (290-310 mOsm/L). Blood samples from the femoral artery were obtained at 10, 20, and 30 min following Pi instillation and collected in tubes containing

ethylenediaminetetraacetic acid (EDTA), an anticoagulate. Plasma was obtained after spinning down blood samples at 6000rpm for 10 minute at 4°C and the data presented shows plasma Pi values at 30 min.

Calculations of Pi transport were performed by taking the ^{32}P counts, converting this to nmole Pi uptake, and normalizing to 100mg intestinal tissue (*in vitro* experiments) or 5cm of intestinal tissue per 1mL plasma collected (*in situ* experiments). ^{32}P counts were measured with a Packard tri-carb 2900tr scintillation counter using 4mL Ultima Gold (Perkin Elmer) scintillant solution to which was added 50uL plasma or 100uL digested intestinal tissue in triplicates. The average from these triplicates was taken and converted to absolute Pi uptake in nmole units using values for initial counts of undiluted ^{32}P instillate.

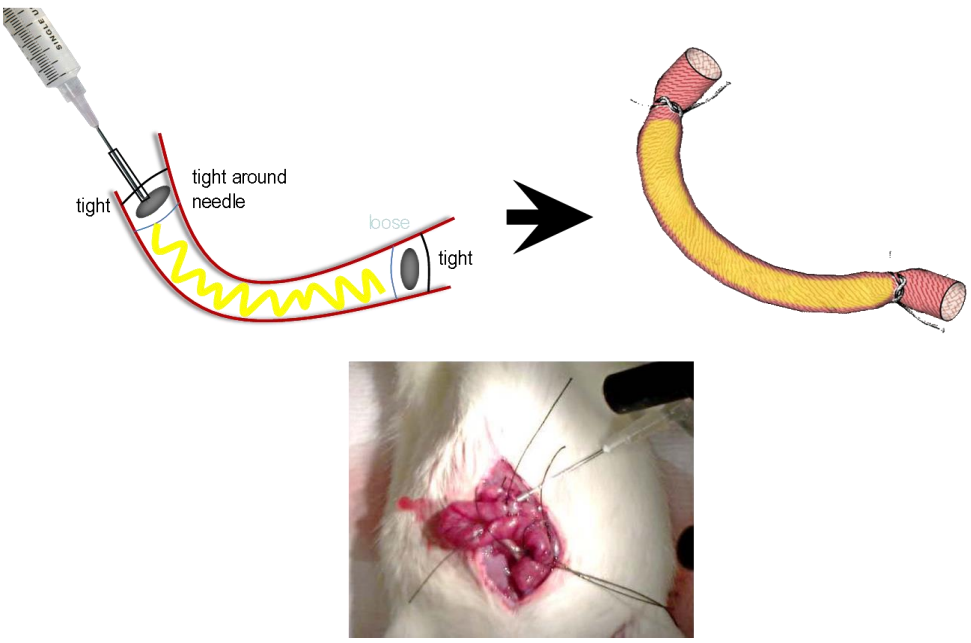


Figure 2.2. Diagram of the *in situ* intestinal closed-loop technique used to measure Pi absorption
Loose ligature in blue is tightened mid-way through Pi instillation. After full Pi instillation and cannula removal, the top blue ligature was tightened to contain Pi buffer in the specific intestinal segment.

2.2.4. Statistical analysis of Pi uptake data

Data is presented as mean ± SEM. A student's paired *t*-test or an ANOVA, as appropriate, was used and group differences were stated as statistically significant if $P < 0.05$; n represents the sample size of each experiment.

2.2.5. Electron microscopy (EM) of intestinal tissue from *in vitro* and *in situ* techniques

Segments of intestine were fixed following "mock" uptake experiments conducted in the exact manner as described previously. The aim was to assess possible differences in morphology following the two respective methodologies. A 1cm segment of intestinal tissue was then removed and fixed in ice-cold glutaraldeyhyde for 24 hours. The tissue was then sent to the EM core facility at the Royal Free Hospital for embedding, slicing, and imaging. The following protocol was used for processing of the tissue:

- washing and secondary fix in osmium tetroxide
- dehydration (ethanol gradients up to 100%)
- resin (Lemix, TAAB labs, UK)/ethanol 1:1 overnight
- 100% resin for 24 hours
- embedding at 65^{o}C overnight

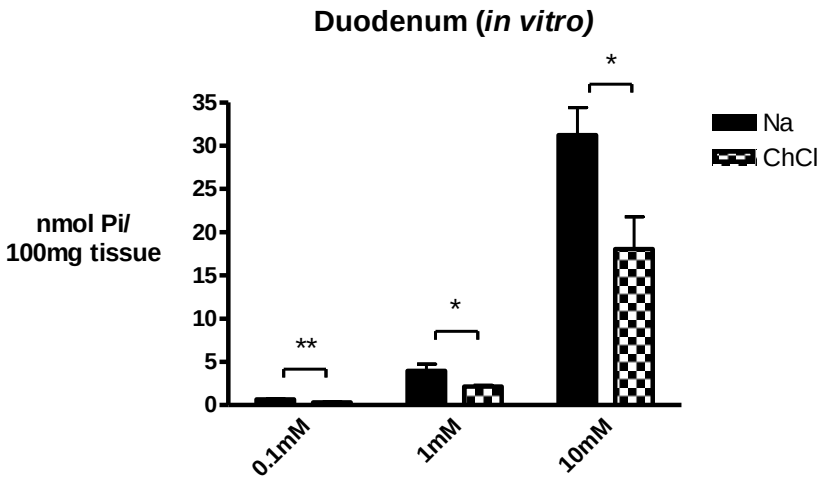
EM Images were obtained within 1-2 weeks of the above procedure using a Japanese Electron Optics Laboratories electron microscope (model JEOL 1200EX).

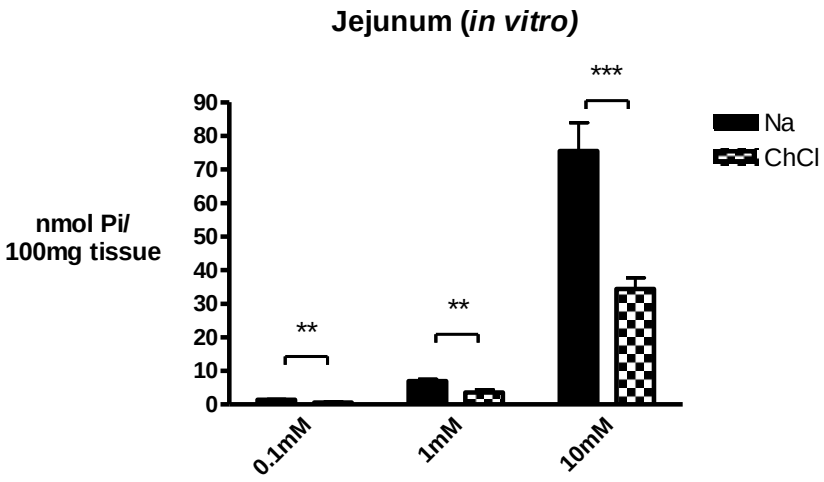
2.3. Results

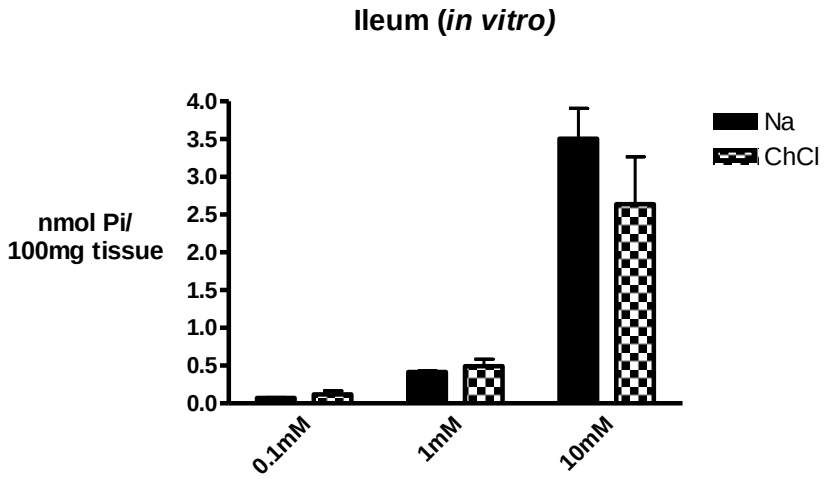
2.3.1. Sodium-dependent Pi transport: *in vitro* everted sleeve

Sodium-dependent Pi transport was measured using sections from duodenum, jejunum, ileum, proximal colon, and distal colon utilizing the *in vitro* everted sleeve technique. Uptake buffer containing 0.1mM, 1mM, and 10mM Pi was used and "sodium-free" experiments were achieved by using ChCl as a replacement for NaCl. Results are shown in **Figure 2.3.**

In both the duodenum and the jejunum, sodium dependency was seen at all three Pi concentrations used. In the ileum and proximal colon, similar rates of Pi were observed in the presence or absence of sodium. In the distal colon there was an apparent sodium dependent Pi uptake at 10mM Pi. The distal colon also transported Pi at a similar rate to the jejunum and the duodenum, which has not been previously reported. P values (Na vs ChCl): Duodenum 0.1mM = 0.00481, 1mM = 0.0341, 10mM = 0.0228; Jejunum 0.1mM = 0.005, 1mM = 0.00698, 10mM = 0.00108; Ileum 0.1mM = 0.338, 1mM = 0.411, 10mM = 0.269; Proximal colon 0.1mM = 0.678, 1mM = 0.653, 10mM = 0.837; Distal colon 0.1mM = 0.0449, 1mM = 0.374, 10mM = 0.00101 **(Figure 2.3).**


Duodenum (*in vitro*)
nmol Pi/
100mg tissue
35
30
25
20
15
10
5
0
**
*
*
0.1mM
1mM
10mM
Na
ChCl


Jejunum (*in vitro*)
nmol Pi/
100mg tissue
90
80
70
60
50
40
30
20
10
0
**
**
***
0.1mM
1mM
10mM
Na
ChCl


Ileum (*in vitro*)
nmol Pi/
100mg tissue
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.1mM
1mM
10mM
Na
ChCl

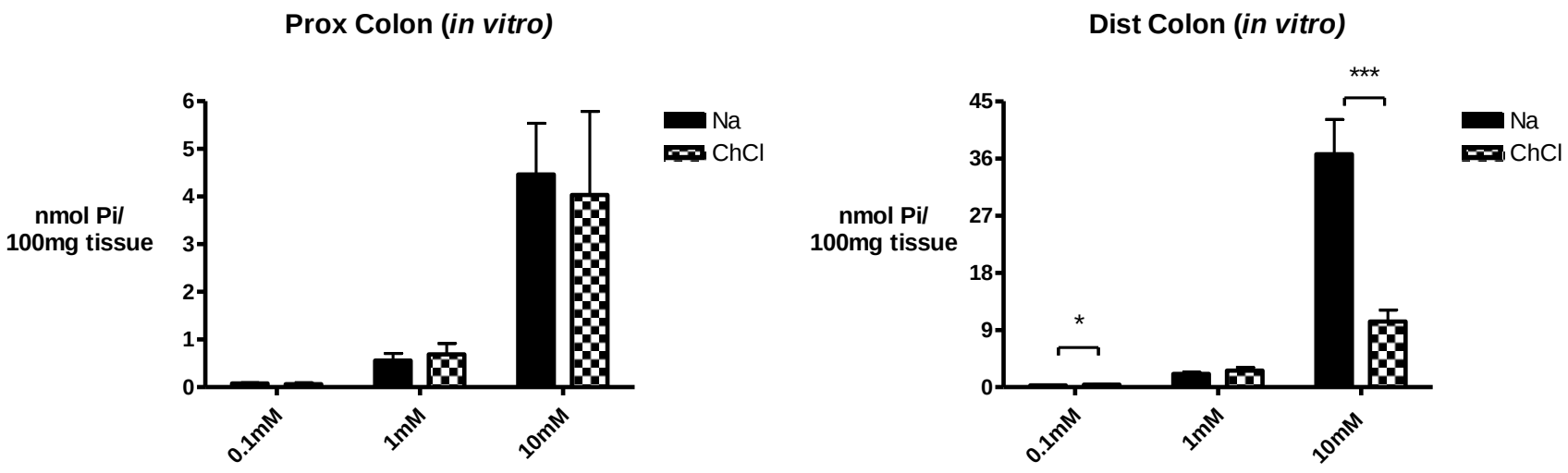
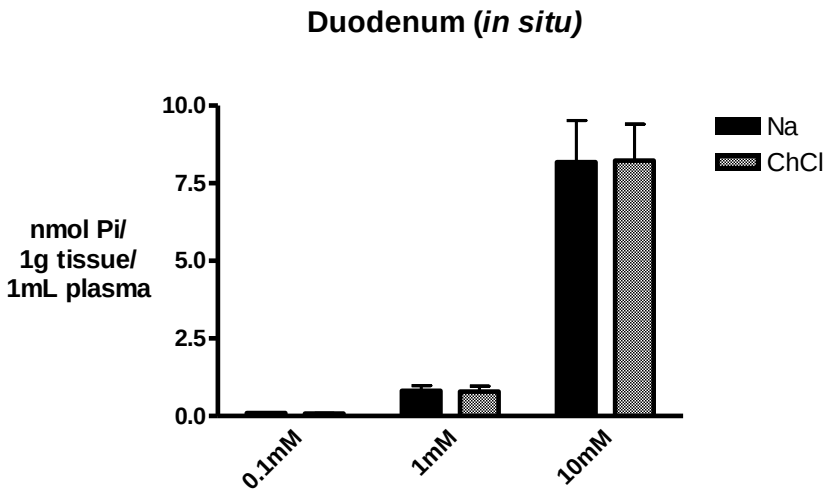


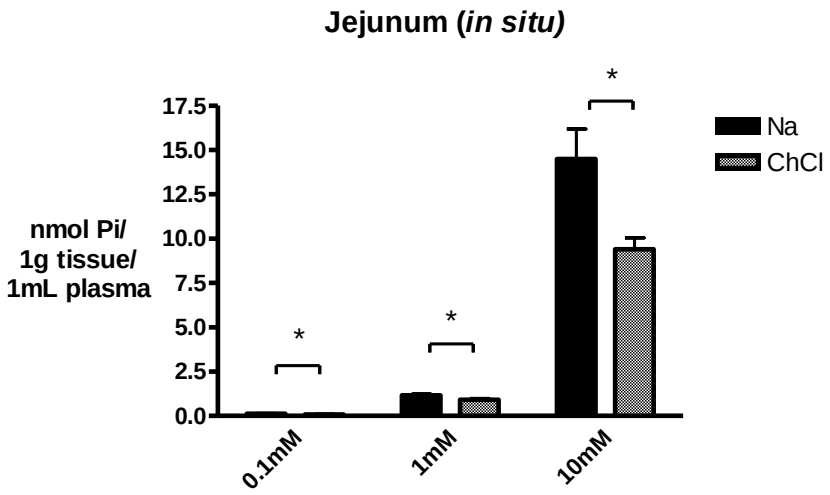
Figure 2.3. ***In vitro*** **everted sleeve. Sodium-dependent and sodium-independent Pi transport in five different intestinal regions**
Shaded bars represent Pi uptake in the presence of sodium while checkered bars represent Pi uptake with a sodium-free buffer (ChCl). Data is presented as mean ± SEM with n = 5-7 across all intestinal regions and Pi concentrations utilized. * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.005$.

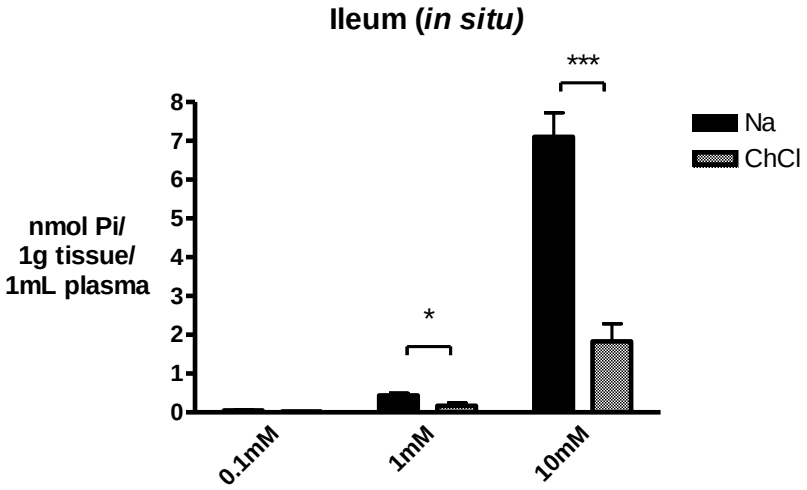
2.3.2. Sodium-dependent Pi transport: *in situ* intestinal closed-loop technique

Sodium-dependent Pi transport was measured *in situ* using the closed-loop technique and using the same three concentrations of Pi as used for the *in vitro* technique. Results are shown in **Figure 2.4.**

The jejunum exhibited the greatest Pi transport of all intestinal regions used, followed by the duodenum and the ileum. Sodium dependency was only seen in the jejunum and at two concentrations in the ileum (1mM and 10mM Pi). P values (Na vs ChCl): Duodenum 0.1mM = 0.339, 1mM = 0.907, 10mM = 0.978; Jejunum 0.1mM = 0.0292, 1mM = 0.0406, 10mM = 0.0156; Ileum 0.1mM = 0.176, 1mM = 0.019, 10mM = 4.6×10^{-5}; Proximal colon 0.1mM = 0.711, 1mM = 0.295, 10mM = 0.196; Distal colon 0.1mM = 0.946, 1mM = 0.904, 10mM = 0.753 **(Figure 2.4).**


Duodenum (*in situ*)
nmol Pi/
1g tissue/
1mL plasma
10.0
7.5
5.0
2.5
0.0
0.1mM
1mM
10mM
Na
ChCl


Jejunum (*in situ*)
nmol Pi/
1g tissue/
1mL plasma
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
*
*
*
0.1mM
1mM
10mM
Na
ChCl


Ileum (*in situ*)
nmol Pi/
1g tissue/
1mL plasma
8
7
6
5
4
3
2
1
0
*
***
0.1mM
1mM
10mM
Na
ChCl

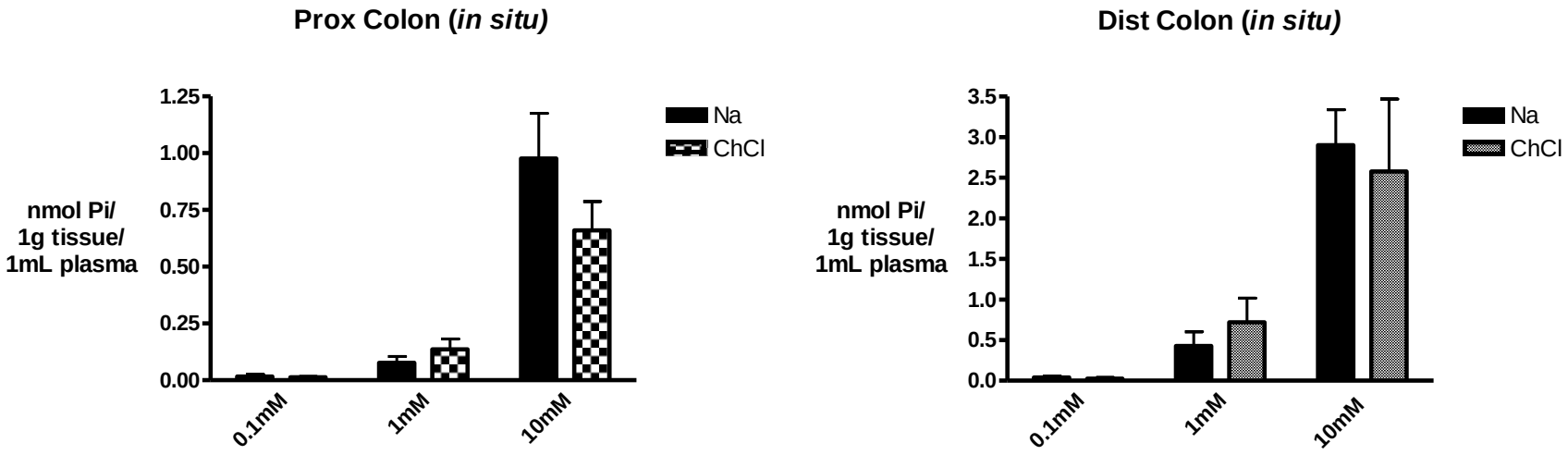


Figure 2.4. *In situ* intestinal closed-loop. Sodium-dependent and sodium-independent Pi transport in five different intestinal regions
Shaded bars represent Pi uptake in the presence of sodium while checked bars represent Pi uptake with a sodium-free buffer (ChCl). Data is presented as mean ± SEM with n = 5-7 across all intestinal regions and Pi concentrations utilized. * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.005$.

2.3.3. Regional differences in transport using *in vitro* and *in situ* techniques

Differences in the rate of Pi transport between the five intestinal regions were compared using three concentrations of Pi (0.1, 1, 10mM) in the presence of sodium. Results are shown in **Figure 2.5** using data from Figures 2.3 and 2.4.

The *in situ* method always resulted in lower measured Pi transported overall. The jejunum was the site of highest Pi transport in all experiments, with the duodenum ranked second for the majority of Pi concentrations used. For the rest of the segments however, variability was seen. The most noticeable differences occurred in the ileum and the proximal colon, where data from *in situ* experiments showed that the ileum had measurable Pi transport whereas the *in vitro* data suggests that ileal Pi transport was negligible. Interestingly, the distal colon transported Pi at a similar rate to the duodenum using 1mM Pi *in situ*, and using 10mM Pi *in vitro* which has not been previously reported **(Figure 2.5).**

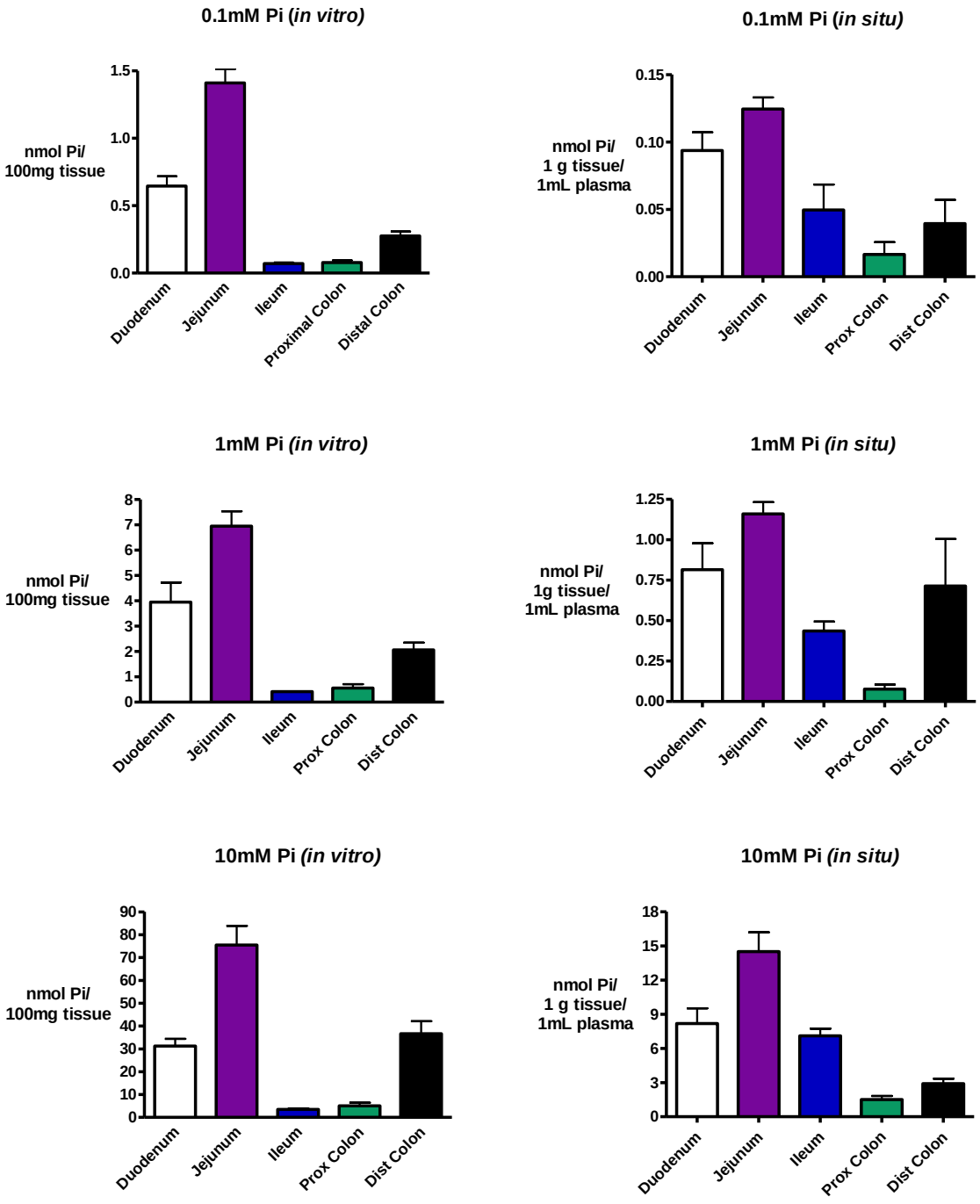


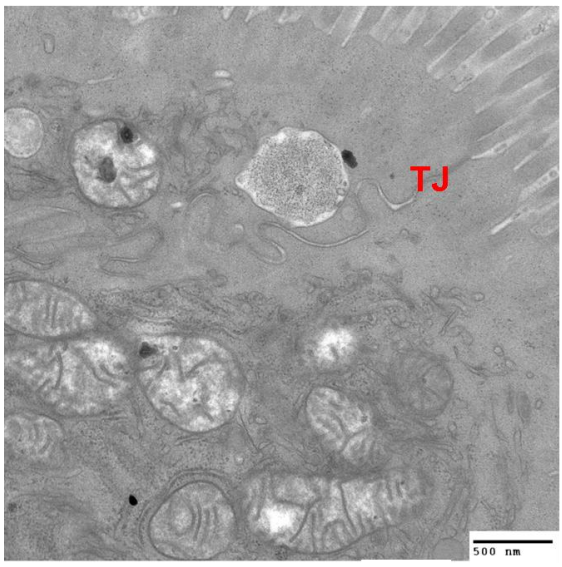
Figure 2.5. Regional intestinal Pi transport. Differences between *in vitro* and *in situ* techniques
Data is presented as mean ± SEM with n = 5-7 across all intestinal regions and Pi concentrations utilized.

2.3.4. Electron microscopy of intestinal tissue

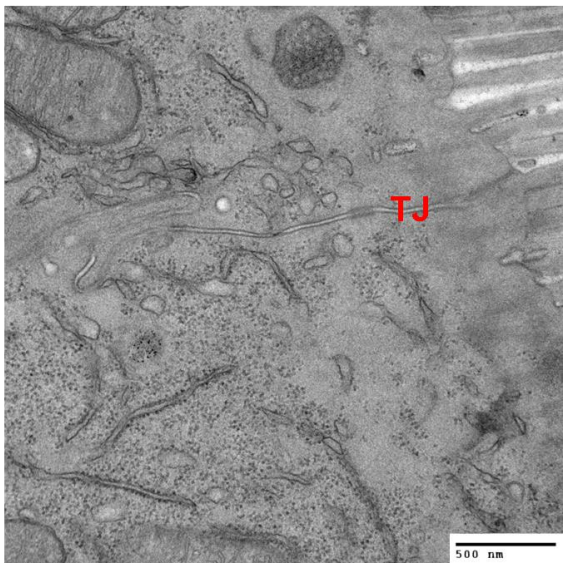
Images were obtained with the help of an on-site EM core facility at the Royal Free Hospital to visualize intestinal tissue morphology following the two different types of experiment procedures explored above. Each segment was treated in the same manner as described in the methods section and immediately fixed in cold fixative (glutaraldehyde) and processed. Intestinal tissue appeared to be intact based on tight junction observation in high magnification (10-50,000x magnification, JEOL 1200EX electron microscope) **(Figure 2.6).**

Duodenum

In vitro

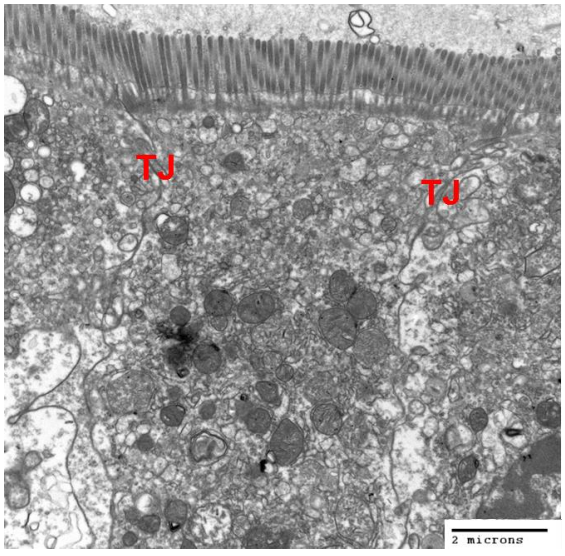


In situ

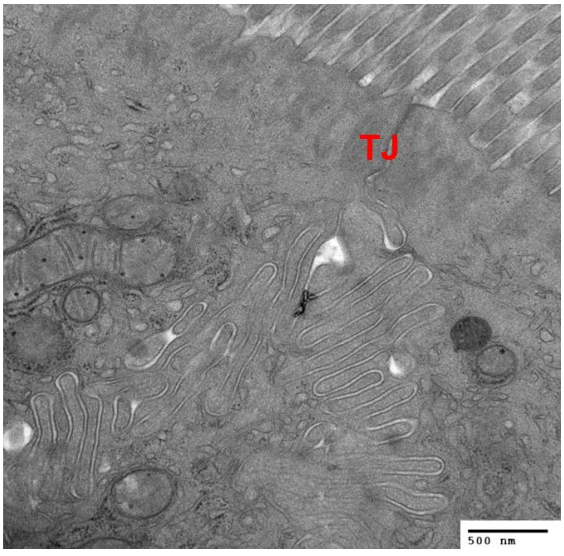


Jejunum

In vitro

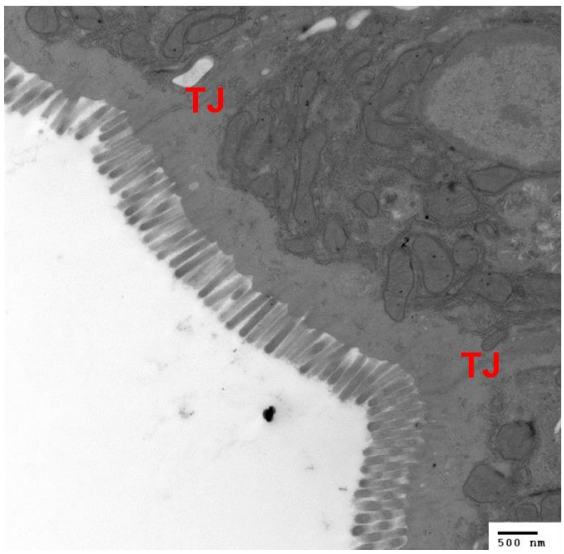


In situ

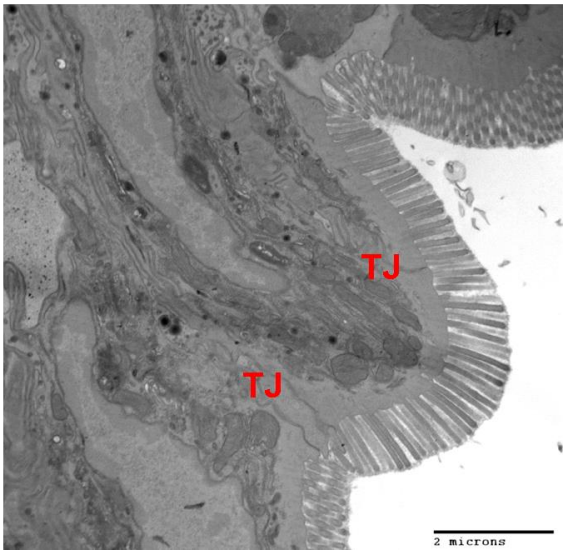


Ileum

In vitro

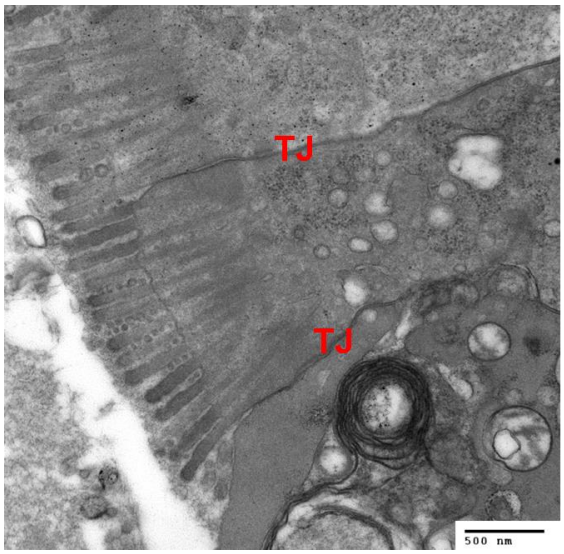


In situ

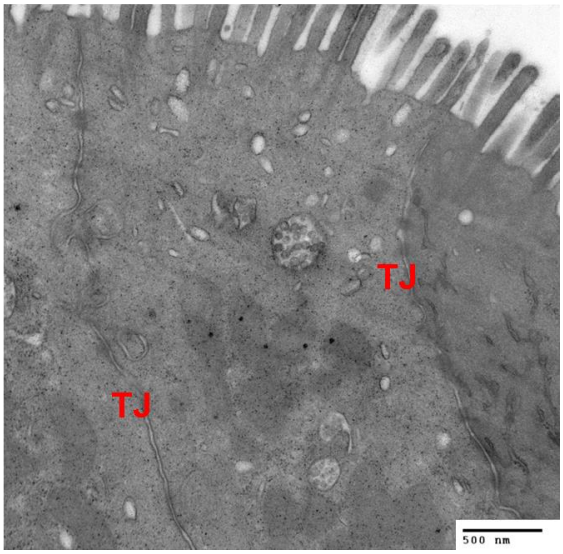


Proximal colon

In vitro



In situ



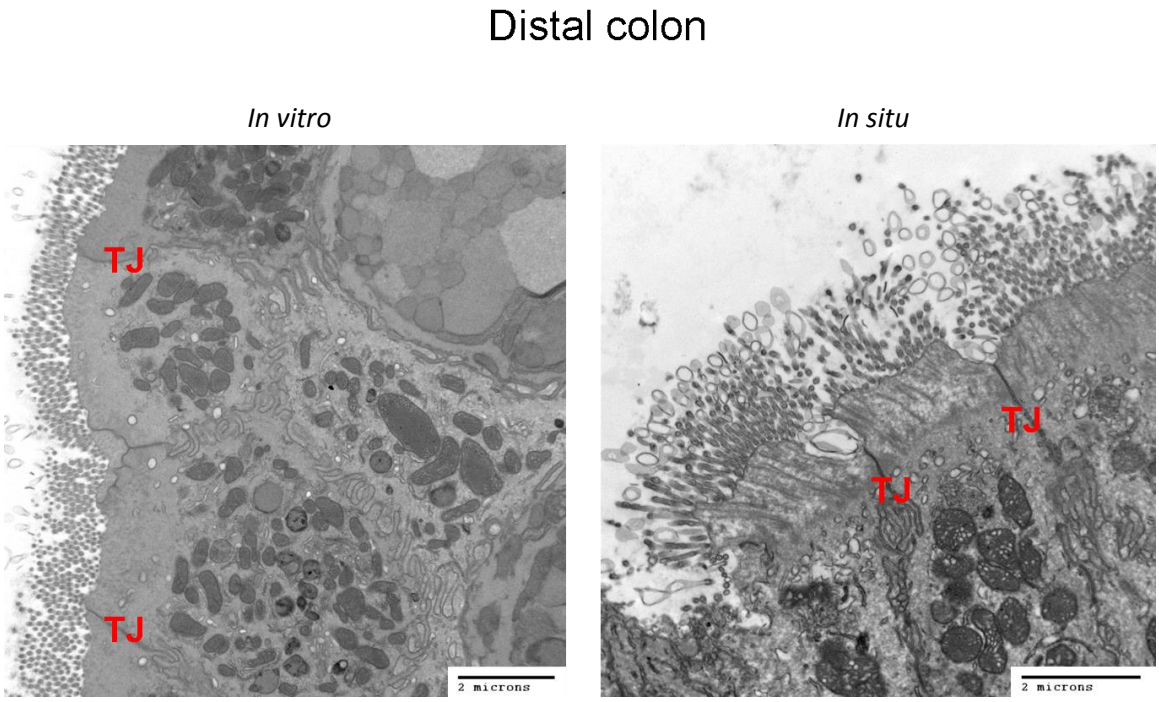


Figure 2.6. EM images of intestinal tissue obtained following *in vitro* and *in situ* methods of measuring Pi uptake
Tight junctions (TJ) are shown in red text with appropriate scale bars in the bottom right corner.

2.4. Discussion

A large range of methods have been used to study solute transport across the intestinal epithelium but comprehensive comparisons between these techniques have not been previously conducted. The aim of experiments discussed in this chapter was to elucidate the differences between Pi transport data obtained by two widely used Pi uptake methods, the *in vitro* everted sleeve technique and the *in situ* intestinal closed-loop technique.

McHardy et al established that intestinal luminal Pi concentrations were in the millimolar range [201] however, the K_t of intestinal NaPi-IIb has been measured to be around 0.1mM. Thus, most studies to date have also utilized a range of low Pi concentrations in order to assess the contribution of NaPi-IIb activity specifically. Present experiments utilized three concentrations of Pi (0.1, 1, and 10mM) to study Pi transport in five intestinal regions. It has been shown that Pi uptake observed using the everted sleeve remained linear after a 1 min exposure of the everted tissue segment to uptake buffer [200]; for that reason a 2 min uptake time was chosen as an estimated midpoint of linear Pi uptake. The *in situ* closed-loop technique has also been validated in the context of Pi transport [12]. Results of the present study indicated that Pi concentrations up to 10mM caused a corresponding rise in the rate of Pi absorption both *in vitro and in situ* **(Figure 2.3 and 2.4)**. The jejunum is the region with the highest rate of Pi measured using both techniques, which corresponds appropriately with previously published data of mRNA and protein expression [17, 62]

In terms of overall Pi transport ability, the *in situ* results using the ileum stand out from previously published data. Although the ileum does not transport Pi as effectively as the proximal small intestine (duodenum and jejunum), it exhibits significant transport ability compared to the colon. In contrast, the ileum transports the least amount of Pi *in vitro* **(Figure 2.7).** These differences seen between the *in situ* and *in vitro* data may be explained by referring to the time that intestinal contents spend in the different regions. Chyme remains in the ileum for the longest period of time compared to other segments of the GI tract (~2-3 hours) [202]. In this instance, the *in situ* method may more accurately represent the total accumulated amount of Pi transported, as plasma Pi measured in these experiments were taken after a 30 min period instead of 2 min used for uptakes using the everted sleeve.

There has been an increase in frequency of observations of hyperphosphatemia following phosphate-containing enemas, first published in 1977 [203]. When presented with a constant Pi source in flux (*in vitro)*, the distal colon surprisingly transports almost as much Pi as the small intestine. Compared with the *in situ* distal colon data presented however, this mechanism of Pi transport has no sodium-dependency at any Pi concentration. Therefore, the colon epithelium may simply be more permeable to solutes (i.e. “leaky”) as prior studies show that there is relatively little unbound Pi at this point of the rat GI tract. [200]. If the amount of unbound Pi in the large intestine is negligible, the Pi transport mechanisms revealed in present studies may not actually occur under physiological conditions. There may, however, be a mechanistic importance of Pi transport in the distal colon as short chain fatty acids, which are in

abundance in the colon, may displace small amounts of Pi from anion exchanger sites in distal colon [204] - this occurrence makes Pi available for absorption. Ultimately, whether Pi transport in this region has any significance to overall Pi homeostasis remains unclear.

Discrepancies were seen in sodium-dependent Pi transport data between the two techniques **(Figure 2.7)**. Apart from the jejunum and proximal colon, which exhibit sodium-dependency and no sodium-dependency respectively across all concentrations utilized, all other intestinal segments had varying results. Data from *in vitro* sodium-dependent Pi transport in the duodenum may be due to the pH of buffer, favoring more NaPi-IIb transport activity than PiT1. pH affects the proportion of two different Pi anions in solution (refer to section 1.2.1.3), with NaPi-IIb transporting the dilvalent form which is in abundance at a more neutral to basic pH; buffers used in present experiments were adjusted to this physiological 7.4 pH. [61]. Both NaPi-IIb and PiT1 proteins however, are not highly expressed in the duodenum [17], thus the overall Pi contributions of either may be negligible. Intestinal pH variations may also explain the data differences between these two techniques in the other intestinal regions that were investigated in these studies. The proximal small intestine starts off relatively acidic at the duodenum (pH of 5.5-6) and increases to an almost neutral state in the distal ileum since microflora become more abundant in these latter regions [205]. All solutions used in uptake experiments were adjusted to a pH of 7.4, thus PiT transporters which may function at a higher capacity in an acidic pH environment may not have been operating at full physiological capacity [61].

Interestingly, data from *in situ* experiments show a lack of sodium-dependency for duodenal Pi transport. This distinct contrast could reflect widely varying intestinal luminal transit times in difference regions of the intestine, with ingested food not remaining in the duodenum for as long as in the jejunum/ileum [202]. In this regard, the *in vitro* everted sleeve technique may be a representative model for duodenal Pi transport since transit time for chyme in rat duodenum is approximately 2 min.

	In vitro	***In situ***
Duodenum	Sodium dependent (all concentrations) 2nd highest Pi transport (0.1mM and 1mM Pi), 3rd highest Pi transport (10mM Pi)	No sodium dependence (all concentrations) 2nd highest Pi transport (all concentrations)
Jejunum	Sodium dependent (all concentrations) Highest Pi transport (all concentrations	Sodium dependent (all concentrations) Highest Pi transport (all concentrations)
Ileum	No sodium dependence Least Pi transport (all concentrations	Sodium dependent (1mM and 10mM Pi) 3rd highest Pi transport (0.1mM and 10mM Pi), 4th highest Pi transport (1mM Pi)
Proximal colon	No sodium dependence 4th highest Pi transport (all concentrations	No sodium dependence Least Pi transport (all concentrations)
Distal colon	Sodium dependent (0.1 and 10mM Pi) 2nd highest Pi transport (10mM Pi), 3rd highest Pi transport (0.1mM and 1mM Pi)	No sodium dependence 4th highest Pi transport (0.1mM and 10mM Pi), 3rd highest Pi transport (1mM Pi)

Figure 2.7. Summary of the main differences in Pi uptake seen using *in vitro* and *in situ* techniques

Black text refers to sodium-dependency of the region, lighter text compares magnitude of Pi transport between regions examined using the same method.

The Pi transport data obtained using the everted sleeve technique accurately reflects the rate of Pi taken up by the intestinal epithelium (i.e. BBM transport), however it does not represent the Pi transfer across the epithelium. In this respect, the *in situ* loop method gives a more complete picture since it reflects Pi transfer from lumen to blood. The differences in the rate of Pi transport using the two techniques (higher amount of Pi transported utilizing *in vitro* technique) correlates with this difference in the way Pi uptake is measured; the everted intestinal tissue is constantly exposured to a moving (stirred) Pi sample, while the *in situ* loop technique's Pi transport is measured as Pi transported to the blood circulation. The Pi buffer utilized in the *in situ* method is less well-mixed compared with that in intestinal closed loops, and thus less Pi may be continuously exposed to the epithelium since Pi transport depletes the Pi at the epithelial surface.

The amount of total Pi transported in both *in vitro* and *in situ* techniques is minimal (less than 0.5% of the Pi in initial Pi buffer taken up by all segments across both methods), thus differences between methods and segments may not have significant impact on overall Pi homeostasis under normal conditions. However, it is important to note that in the context of kidney disease and reduced renal function, these minute differences in intestinal Pi handling can have greater effect on blood Pi levels.

It is known that claudins control tight junctions and their selective permeability to solutes, however their role in regulating Pi transport across the small intestine is

unclear. Claudin-1, 3, 4, 5, and 8 form paracellular barriers and contribute to "tight" epithelia while other claudins such as claudin-2 , 7 and 12 form paracellular cation channels (pore-forming claudins) and mediate paracellular ion transport [206-209]. The rat jejunum has been shown to express claudin-5 and 12 which permit ion permeability, whereas the duodenum expresses the claudins associated with "tightening" of the epithelia, claudin-1, 3, 4, and 8 [210, 211]. This difference in claudin expression between the duodenum and the jejunum may explain the reduced Pi transport in the duodenum compared with the jejunum. The expression of claudin 5 and 12 in the jejunum would theoretically increase paracellular ion permeabitliy and potential paracellular Pi transport. However, it is clear from the data in this chapter that paracellular Pi transport in the jejunum is not the dominant form of Pi transport; NaPi-IIb is highly expressed in this region and sodium-dependent Pi uptake is seen across all concentrations of Pi used to study Pi uptake in this region **(Figures 2.3 and 2.4).** The colon has been shown to exhibit high expression of the "tightening" claudins, claudin-1, 3, 4, 5, 8 [211]. Data show that the distal colon transports a notable amount of Pi, which has previously never been thought to be a site of significant Pi transport **(Figure 2.5)**, thus expression of NaPi-IIb, PiT1, and potentially a novel Pi transporter should be studied in the future. Interestingly, claudin-2 and claudin-12 act as paracellular Ca^{2+} channels in the small intestine and their expression has been recently shown to be upregulated by a known Pi regulator, vitamin D [212]; no anion-selective claudins have been analyzed to date to see if they are capable of transporting the Pi anion. It would be interesting to attribute sodium-independent Pi transport to changes in claudin expression following Pi exposure.

It is essential to link changes in Pi transport to protein levels of tissue-specific Pi transporters. Several commercial antibodies are available for detection of NaPi-IIb protein in mice, however no antibodies thus far have been shown to recognize the rat isoform. Colleagues have tested a number of rat-specific NaPi-IIb antibodies on positive control rat tissues (e.g. lung brush border membrane [14, 213]) with none of the antibodies detecting consistent NaPi-IIb protein. Therefore, it has not been possible to directly correlate Pi transport data in the different regions of the rat intestine examined in this chapter with altered levels of NaPi-IIb protein.

Importantly, EM analysis indicates that intestinal ultrastructure is not compromised in either of the two techniques, and differences in Pi transport therefore did not represent diffusion across a damaged epithelium. In EM images, the tight junctions and intestinal villi remain unaffected by manipulation of tissue using both methods and across all segments of intestine utilized. Differences in the magnitude of Pi transport and sodium-dependency are thus attributed to other mechanisms that have not been explored at present.

2.4.1. Summary and conclusion

Data using *in vitro* and *in situ* techniques for measuring Pi transport indicate that the two methods can produce considerably different results. Direct comparisons of techniques have not been previously explored, and the importance of knowing what each specific technique accurately represents, and act as an appropriate model for, is emphasized in these studies. Data obtained confirm the jejunum to be the region with the highest Pi

transport capacity. Novel data was observed using the distal colon, since this region was shown to display significant Pi transport ability. It can be concluded that for some aspects of Pi transport in specific regions (e.g. duodenum) the everted sleeve method may be a more accurate representative of physiological acivity, whereas studying intestinal regions where luminal contents may remain for a longer period of time (distal small intestine and large intestine) may warrant the use of other methods such as the *in situ* closed-loop technique. These results may provide insight into how different regions of the GI tract can be targeted to regulate Pi transport in instances of altered body Pi balance, for example the hyperphosphatemia in chronic renal failure.

3.0. Measuring Pi transport across the rat intestinal epithelium using an *ex vivo* intestinal preparation

3.1. Introduction

As shown in the previous chapter, many discrepancies are seen between Pi transport data obtained from *in vitro* and *in vivo* methods. Mainly, these two methods measure different pathways of Pi transport - the *in vitro* everted sleeve method detects the amount of Pi that is taken up at the intestinal brush border and retained within enterocytes while the *in vivo* intestinal closed-loop method reflects the amount of Pi that is both transported from the intestine into blood circulation and that retained within the mucosa. While both methods have contributed to the basis of our current understanding of Pi transport, they leave gaps in information.

Discovering new treatments for hyperphosphatemia in the framework of chronic kidney disease (CKD) first involves elucidating the mechanisms of Pi transport across the intestine. Numerous studies have shown that the regional profile for intestinal Pi absorption is different across species such as between rats and mice, although the mechanisms behind this are still unclear. In rats and humans, the highest rates of transport occur in the duodenum and jejunum [133], whilst in mice maximal absorption occurs in the ileum. In both rats and mice, the profile of absorption is paralleled by levels of NaPi-IIb mRNA and protein, the main sodium-dependent Pi transporter in the

intestine [12, 54, 62]. Studies using conditional tamoxifen-inducible NaPi-IIb$^{-/-}$ KO mice have shown that this protein is responsible for ~90% of sodium-dependent transport across the mouse ileum BBM. It has also been revealed that the transporter accounted for only ~50% of total intestinal transepithelial Pi absorption in response to a dietary Pi load [54]. This finding confirms the early observations of a significant sodium-independent component of intestinal Pi transport [133, 136], although it is still unclear as to whether sodium-independent transport occurs via a transcellular or paracellular route.

Paracellular Pi transport forms a component of the sodium-independent Pi transport, however the way in which this pathway is regulated, perhaps via tight junction proteins such as claudins, is unknown. Concurrently, the exit path of Pi at the basolateral membrane of enterocytes has not been characterized and a potential Pi transporter could exist in this region. Introducing an *ex vivo* method that has never been applied to investigate Pi transport would shed light on the mechanisms of Pi transport by observing the composition of contents directly transferred across the serosal side of the intestine without the complication of dilution in systemic blood.

Fisher and Gardner established a technique in 1974 in which the transport of solutes across intact epithelium could be quantified [214]. In the *ex vivo* intestinal perfusion method, a segment of the intestine is removed from the animal and perfused with bicarbonate buffer interspersed with O_2/CO_2. These alternating gas and perfusate buffer "slugs" maintain efficient oxygenation of the mucosa which contributes towards its

viability [214]. The resulting fluid that is transferred to the serosal side of the intestinal segment can be collected at intervals and analysed for its composition. The technique has been successfully employed to measure the absorption of flavonoids, olive oil polyphenols, clinical drugs, and glucose [215-219]. The advantage of this method in investigating Pi transport is that that the amount of Pi obtained from the serosal fluid can be interpreted as the amount directly passing through the tissue as a whole, whereas *in vitro* and *in vivo* techniques only measure Pi uptake at the BBM or the appearance of solute in the blood respectively. The concentration of Pi contained in the serosal fluid of the perfused *ex vivo* intestine model can therefore be directly compared with the Pi concentration contained in the perfusate buffer. This specific *ex vivo* method has not been used to investigate Pi transport across the intestinal epithelium.

3.1.1. Aims of this study

1. To compare Pi transport across the jejunum and ileum of rats utilizing the *ex vivo* intestinal perfusion technique. Investigate sodium-dependent Pi transport across the jejunum and ileum by using choline chloride (ChCl)-based to replace sodium chloride, NaCl in perfusate for a portion of the experiment.

2. Analyze serosal fluid to assess whether concentration of Pi during the transport process across the intestinal epithelium is achieved.

3.2. Materials and Methods

3.2.1. Animal Model

Male Sprague Dawley (SD) rats between 250g-300g were obtained from Charles River Laboratories (UK) and used in accordance with the Animals (Scientific Procedures) Act 1986. Rats were allowed *ad libitum* access to standard rodent chow containing 0.52% phosphate (Diet RM1, SDS Ltd, Witham, Essex UK) and water.

3.2.2. *Ex vivo* intestinal perfusion

Rats were weighed then anesthetized by an IP injection of pentobarbitone sodium (45mg/kg body weight). Body temperature was maintained at 37^{o}C using a heating pad during sequential removal of intestinal sections.

A midline incision was made and 10-15 cm of intestinal tissue was isolated (jejunal segments began at the ligament of Treitz and ileal segments ended at the cecum). These sections were tied off with ligatures and incisions were made at either end of the section. Warmed saline was flushed through the segment to remove any food debris followed by air to remove as much luminal fluid as possible. Following this, silicone tubing was secured to both ends of the intestine with additional ligatures. One tube delivered a perfusate consisting of a segmental flow of 95% O_2/5% CO_2 interspersed with a Krebs bicarbonate solution (25mM HCO_3, 143mM Na, 133.7mM Cl, 5.9mM K, 1.2mM HPO_4, 2mM Ca^{2+}, 1.2mM Mg^{2+} , pH 7.4) containing 10 or 28mM glucose added fresh before every experiment. Using a rotary pump, the rate of perfusion was adjusted

to approximately 1 mL per minute. The tube at the distal end of the intestine served to deliver the effluent into a waste container. In experiments studying sodium-independent Pi transport, NaCl was substituted with ChCl and $NaHCO_3$ was substituted with choline carbonate ($ChHCO_3$). Once the effluent appeared, the intestinal segment was then removed from the animal by stripping it from the mesentry and then suspending it in 37^oC liquid paraffin (specific gravity 0.83-0.86) **(Figure 3.1**). The animal was then sacrificed following the removal of the last intestinal segment. Segmental flow was maintained throughout the procedure with a 40 minute equilibration period to flush blood from the vasculature and for transport to reach a steady state. Serosal fluid collections were made at 20 and 40 min post equilibration. Studies examining the effect of sodium-free buffer included a further 40 minutes of perfusion with the sodium-free buffer as well as another 40 min switch back to the sodium-containing buffer. Serosal fluid collections were centrifuged at 6000 rpm for 10 min at 4^oC and serosal fluid was removed with a 14g needle from under the paraffin layer collected with serosal fluid.

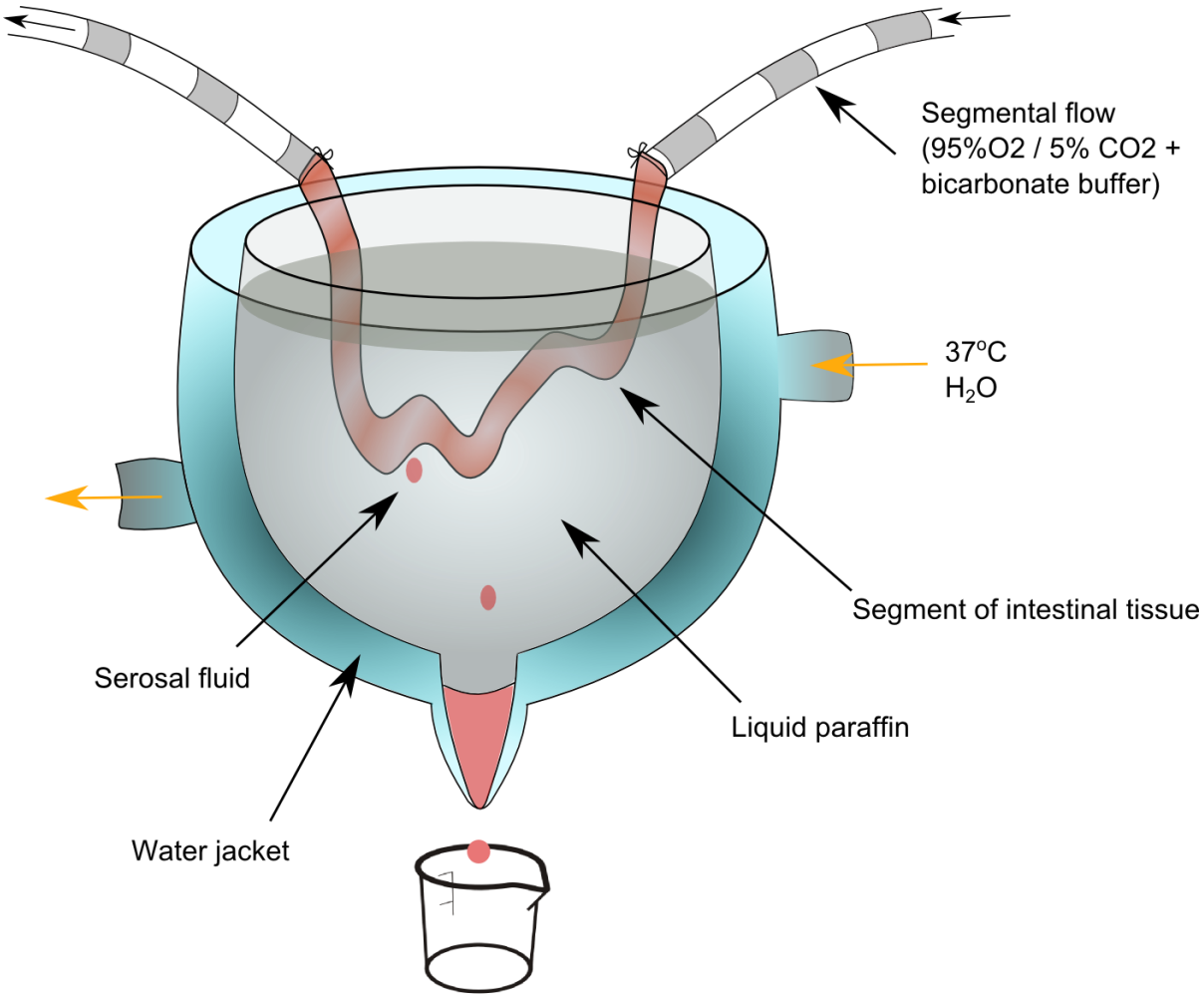


Figure 3.1. Diagram of the *ex vivo* intestinal perfusion method

3.2.3. Determining Pi and glucose concentrations

Pi and glucose concentrations were measured in the serosal fluid using a commercially available Pi assay kit (Quantichrome DIPI-500, USA) and a colorimetric glucose assay. The latter works on the principle that glucose contained in the serosal fluid is oxidised by a glucose oxidase reagent (GOR) containing glucose oxidase, peroxidise, and dianisine. Oxidation of glucose produces hydrogen peroxide which, in the presence of peroxidase, oxidises dianisidine in the reagent to produce a colorimetric response

(oxidised o-dianisidine) which can be measured using a spectrophotometer at a 450nm wavelength. The GOR was prepared with 44 mg glucose oxidase, 1.76 mL peroxidase (from a 0.53 mg/mL stock) and 2.8 mL dianisidine (from a 2.5 mg/mL stock) diluted in 176 mL dH_2O. Glucose standards were then prepared for 5, 10 and 15 mM glucose added to 500 µL dH2O and 4 ml GOR. Samples were also added to 500 µL dH_2O with 4 mL GOR and incubated for 1 hour at room temperature. Duplicates of samples and standards were measured at 450 nm with a Beckman Du650 spectrophotometer (Beckman-Coulter Research, Buckinghamshire, UK).

3.2.4. Statistical analysis

Data is presented as mean ± SEM. A student's paired *t*-test or a one-way ANOVA, as appropriate, was used and group differences were stated as statistically significant if $P < 0.05$; n represents the sample size of each experiment. Glucose and Pi concentrations were first plotted relative to perfusate concentrations, followed by an absolute (nmole) quantity transported per cm of intestinal tissue per min. Nanomole values of glucose and Pi were obtained by taking into account the overall serosal fluid volume collected at each 20 min time point.

3.3. Results

3.3.1. Pi transport across the rat intestinal epithelium

Serosal fluid was collected 20 min and 40 min post equilibration period. The fluid was then utilized to determine Pi and glucose concentration. The concentration of glucose and Pi in the serosal fluid was analyzed using a Student's *t*-test relative to the perfusate. A significant increase was seen in glucose concentration using jejunum at both 20 and 40 min post equilibration, but not using ileum **(Figure 3.2)**. Pi concentrations in the serosal fluid collected from jejunal and ileal segments were both significantly higher than the 1.2mM present in the perfusate at both time points **(Figure 3.2).**

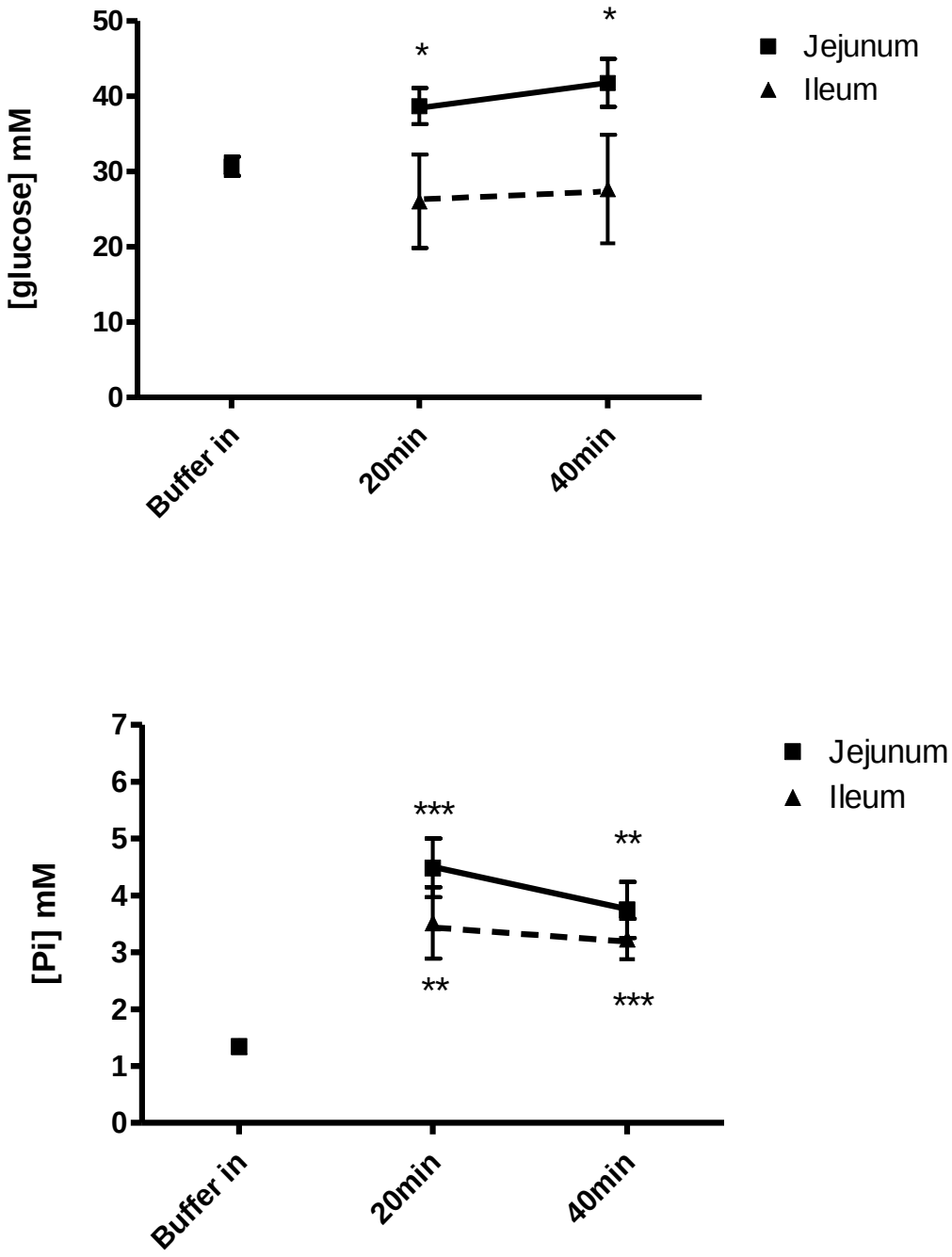


Figure 3.2. Glucose and Pi concentrations in serosal fluid
Values were analyzed for statistical significance using a Student's *t*-test (compared to the concentrations found in perfusate as indicated by "buffer in") * denotes P ≤ 0.05, ** = P≤ 0.01, *** = P ≤ 0.001 with n = 5.

3.3.2. Pi transport across the intestinal epithelium during sodium switching

Sodium-dependent Pi transport was analyzed using a perfusate switch protocol. In these experiments, normal perfusate was used and two collections of serosal fluid were made at 20 and 40 minutes post equilibration as described previously. Following this, a "sodium-free" buffer in which NaCl was substituted with ChCl and $NaHCO_3$ was substituted with $ChHCO_3$ was perfused through the segment and two collections of serosal fluid were made 20 and 40 min following the switch (represented by Ch-20min and Ch-40min). Normal, sodium-containing bicarbonate buffer was then reintroduced for 2 further serosal fluid collections at 20 and 40 min (Na-20min2 and Na-40min2).

Data for glucose concentration shows a significant increase following reintroduction of sodium-containing buffer in the last 40 minutes of the experiment in jejunal segments **(Figure 3.3a).** Interestingly, there was a significant decrease in glucose concentration at all time points using the ileum **(Figure 3.3a).** Data for Pi concentration shows that there is concentrated Pi in serosal fluid at all time points for both the jejunum and the ileum **(Figure 3.3a).**

When glucose and Pi are expressed as nmole transported relative to intestinal segment length and time, a significant decrease is seen in glucose transport across the jejunum between 20 and 40 min after sodium-free buffer (Ch) is introduced **(Figure 3.3b).** There is also a significant reduction in Pi transported between 20 and 40 min after sodium-free buffer is introduced in both jejunum and ileum **(Figure 3.3b).** There is a significant increase in Pi transported between 20 and 40 minutes after reintroduction of

normal, sodium-containing buffer in both segments of intestine **(Figure 3.3b).** A similar pattern is seen when considering the volume of serosal fluid absorbed across the epithelium (**Figure 3.3c).**

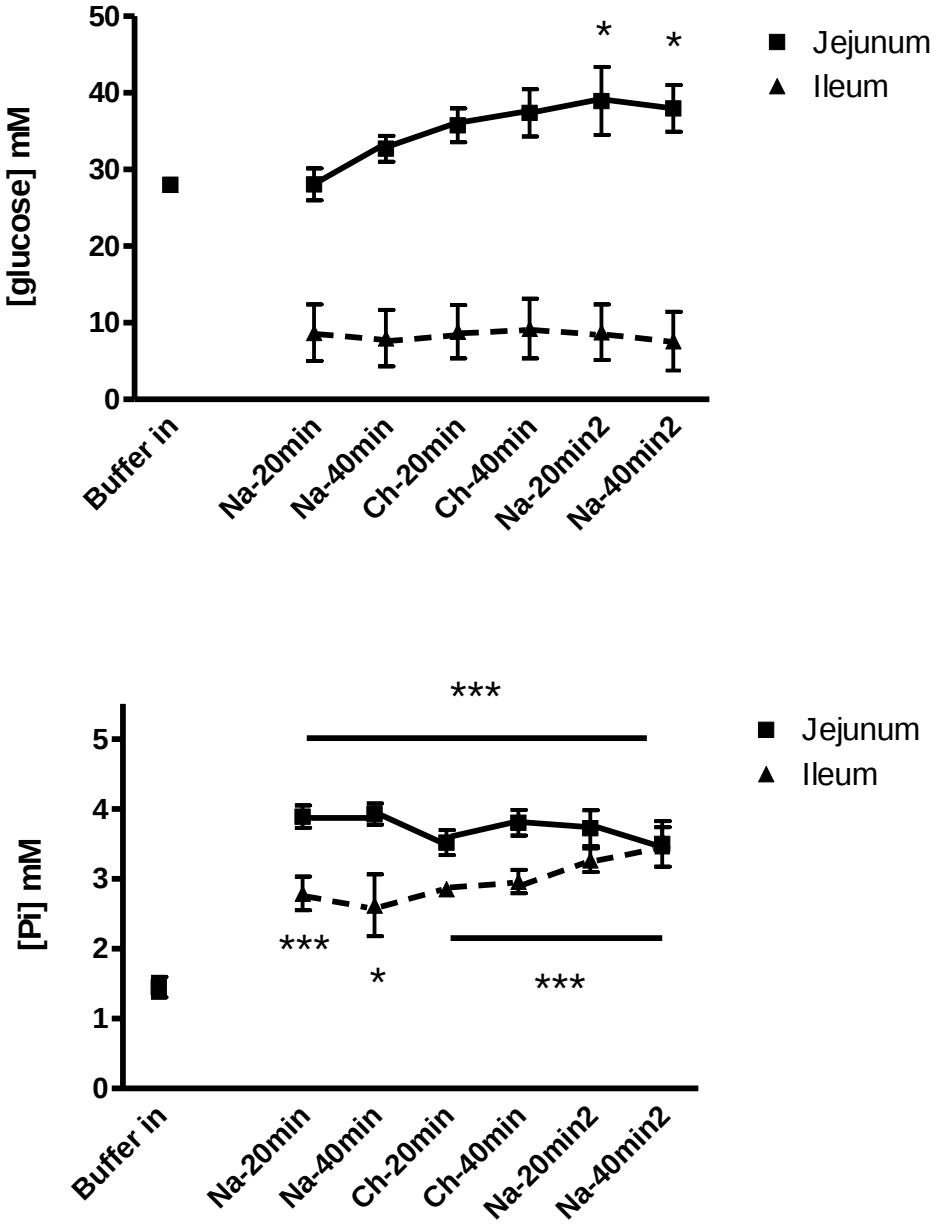


Figure 3.3a. Glucose and Pi concentrations in serosal fluid after switching between sodium containing and sodium-free perfusate buffers containing 28mM glucose
There is an approximate 2-3x increase in Pi concentration, with a variable glucose concentration seen. The only significant increase in glucose concentration appeared in the last

20 minutes of experiments utilizing jejunal segments. * denotes P ≤ 0.05, *** = P ≤ 0.001. n = 7

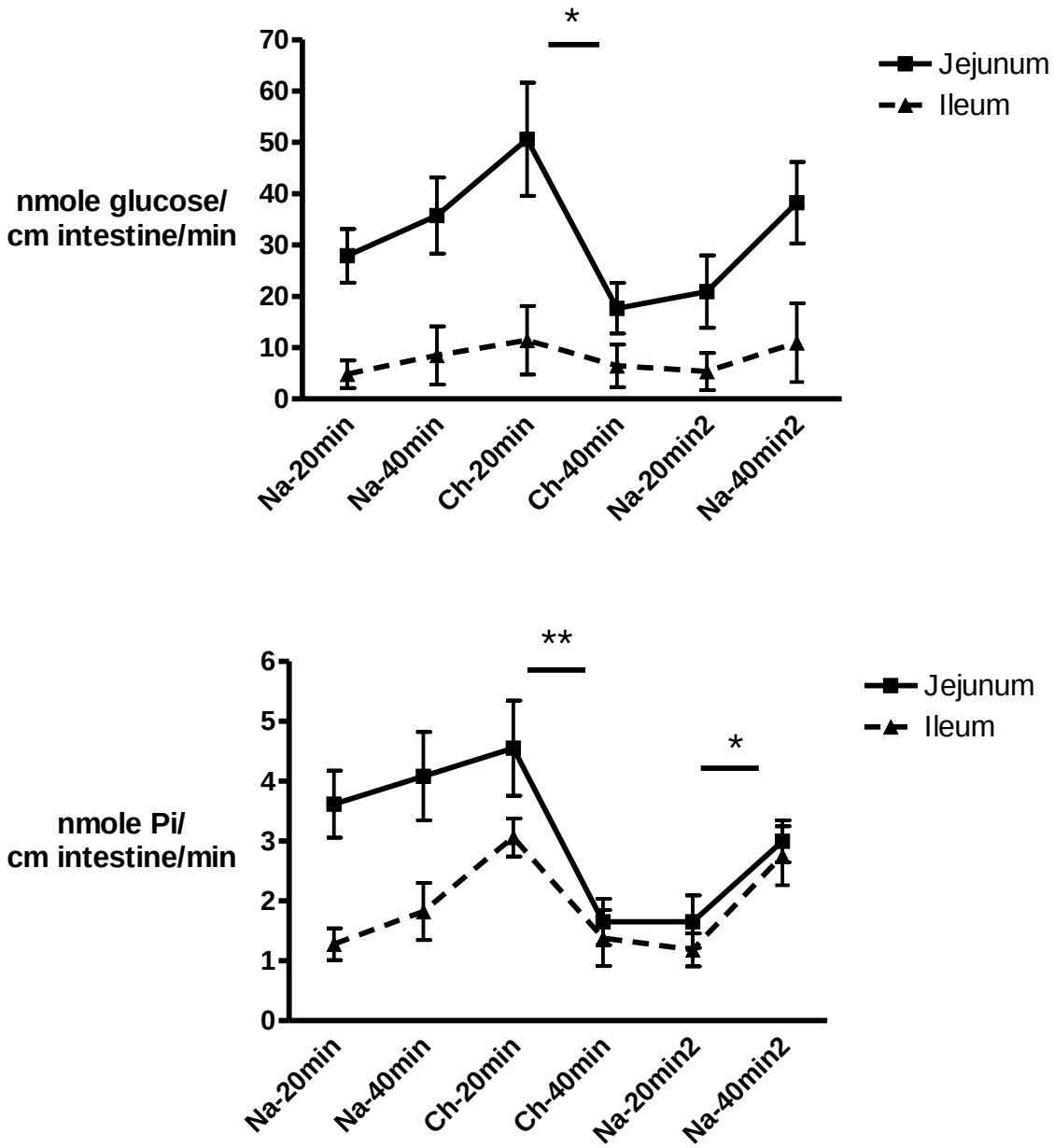


Figure 3.3b. Glucose and Pi transport expressed per cm of intestine per minute using 28mM glucose in perfusate

A significant drop in glucose transport is seen after switching to a sodium-free buffer between 20-40min in the jejunum only. A significant decrease in Pi occurred after switching to sodium-free buffer between 20 and 40 minutes as well as a significant increase following reintroduction of sodium containing buffer. * denotes P ≤ 0.05, ** = P≤ 0.01. n = 7

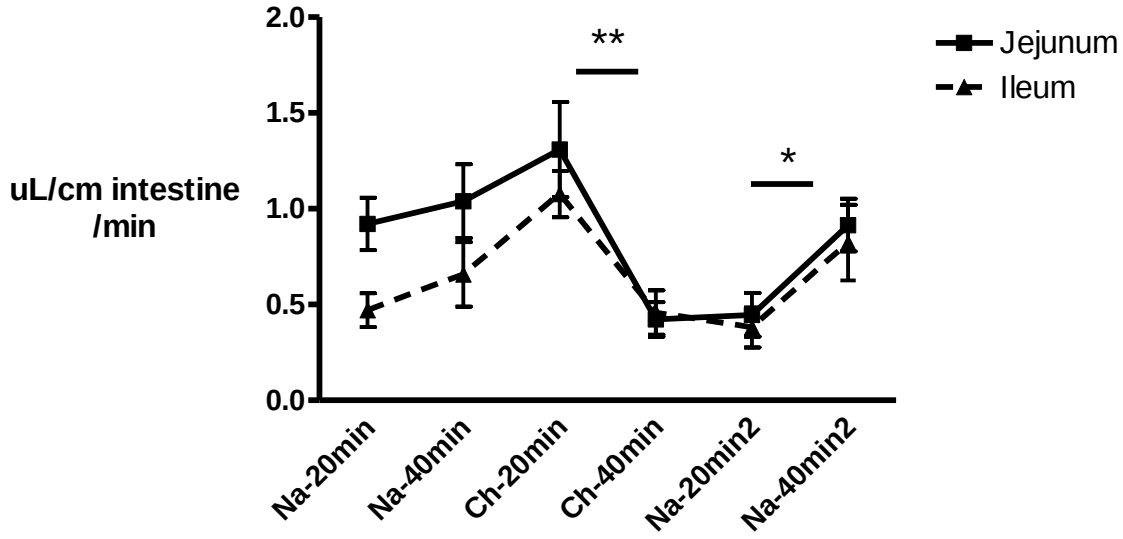


Figure 3.3c. Volume of serosal fluid collected per cm of intestine per minute using 28mM glucose in perfusate
There is a significant change in fluid transport 20-40 min after the switch to a sodium-free perfusate in both the jejunal and ileal segments. There is also a significant change between 20-40 min after the switch back to a sodium containing buffer. * denotes P $\leq$ 0.05, ** = P$\leq$ 0.01. n = 7

3.3.3. Pi transport across intestinal epithelium during sodium switching (using 10mM glucose)

10mM glucose was utilized in these experiments as opposed to 28mM glucose in order to observe changes in total serosal volume and whether this change affected the increase in Pi concentration seen in previous experiments **(Figures 3.2 and 3.3a)**. The protocol was identical to that described in the section above.

Data for glucose concentration in the serosal fluid shows a significant decrease compared to the perfusate concentration of 10mM in both jejunal and ileal segments **(Figure 3.4a).** Data for Pi concentration shows that there is significantly higher concentration Pi collected at the serosal side at all time points for both jejunal and ileal segments **(Figure 3.4a).**

When glucose and Pi data is expressed as quantity transported relative to intestinal segment length and time, a significant drop was seen in glucose transported in the ileum between 20 and 40 min after introduction of sodium-free buffer (Ch) **(Figure 3.4b).** There is a significant increase in glucose transported across the jejunum 20-40 min after sodium-containing buffer is reintroduced. There is a decrease in Pi transported between 20 and 40 min after sodium-free buffer is introduced and also an increase in Pi transported when sodium-containing buffer is reintroduced in both the jejunum and ileum **(Figure 3.4b).** In addition to this pattern, there is a significant increase in Pi transported between 20 and 40 min in the ileum at the start of this experiment. This increase in Pi transport at the beginning could be attributed to an obstruction in the luminal space of the intestine (either by a twisting of the intestine, or by luminal contents which were not completely removed during initial preparation of the segment). This might have delayed transport equilibrium. A decrease in serosal fluid volume following removal of sodium in the buffer and subsequent increase of fluid volume following reintroduction of sodium is also seen in these experiments **(Figure 3.4c).**

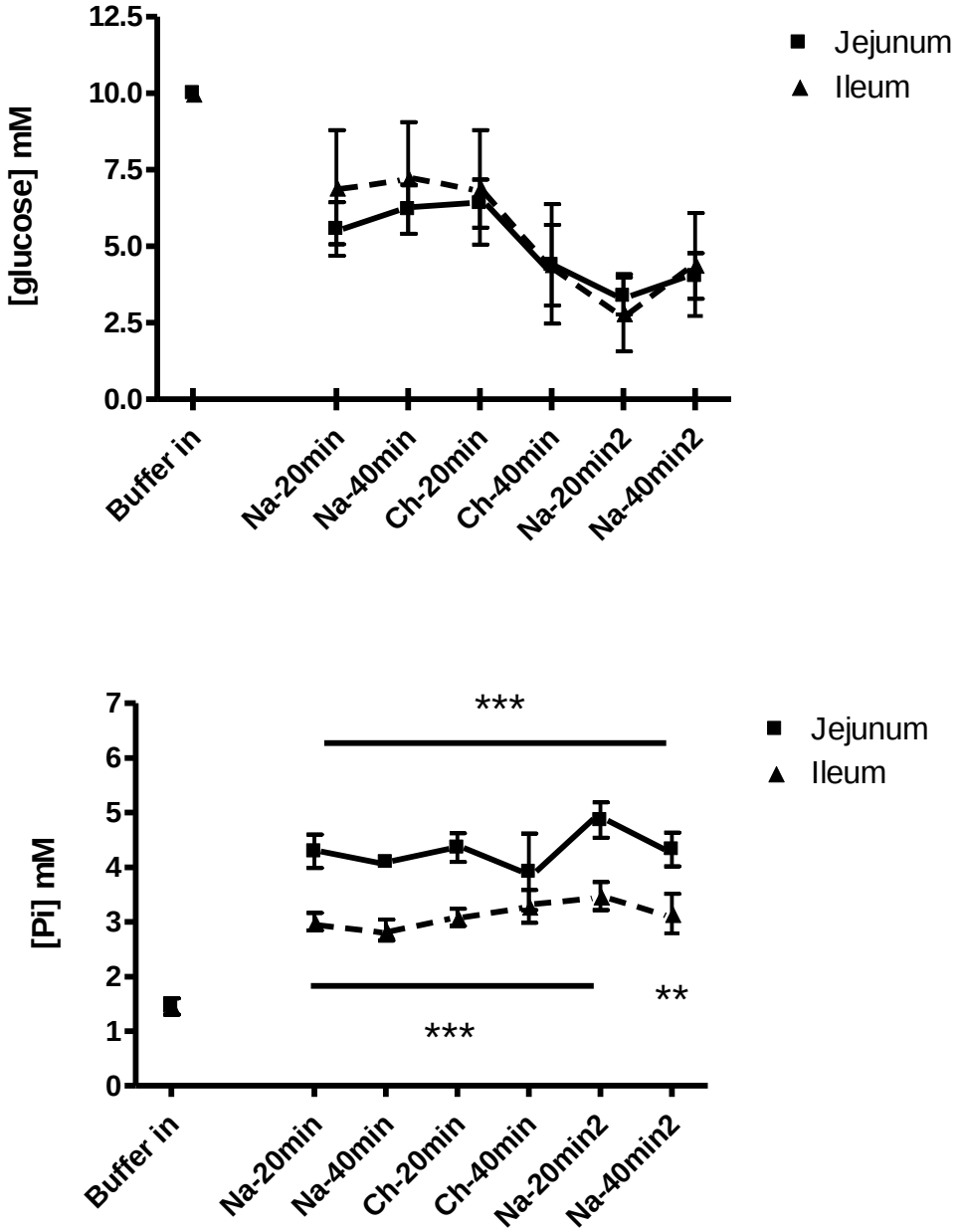


Figure 3.4a. Glucose and Pi concentrations in serosal fluid after switching between sodium-containing and sodium-free perfusate containing 10mM glucose
Both jejunal and ileal segments show no increase in glucose concentration. Pi concentrations were all significantly higher compared to the perfusate. ** denotes = P≤ 0.01, *** = P ≤ 0.001. n = 7

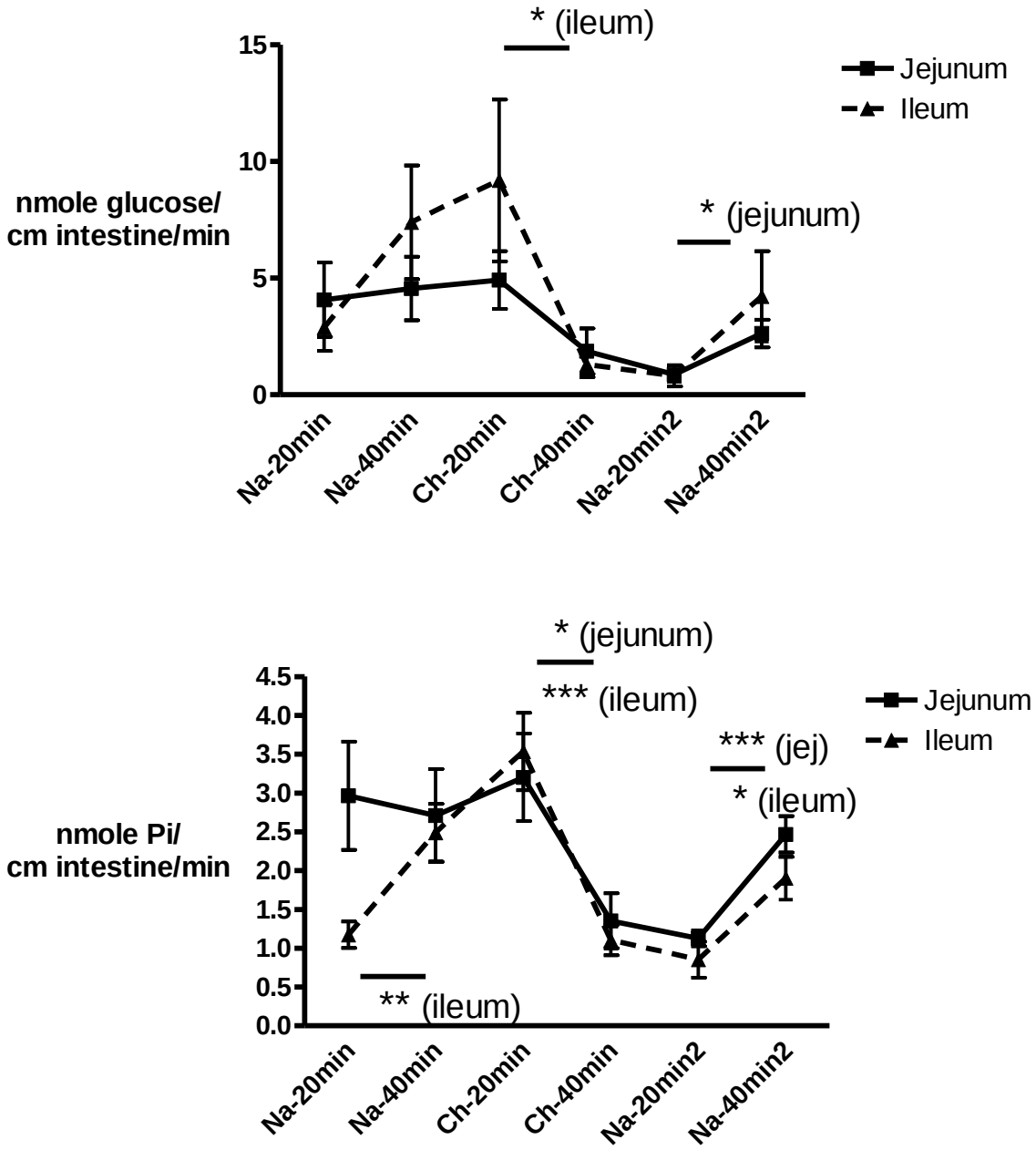


Figure 3.4b. Glucose and Pi transported per cm of intestine per minute using 10mM glucose in perfusate

A significant drop in glucose and Pi are seen after switching to sodium-free buffer between 20 and 40 minutes. Similar changes were seen with Pi, with a significant drop and rise occurring 20-40min following switch to sodium-free buffer and switch back to normal buffer respectively. * denotes $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$. n = 7

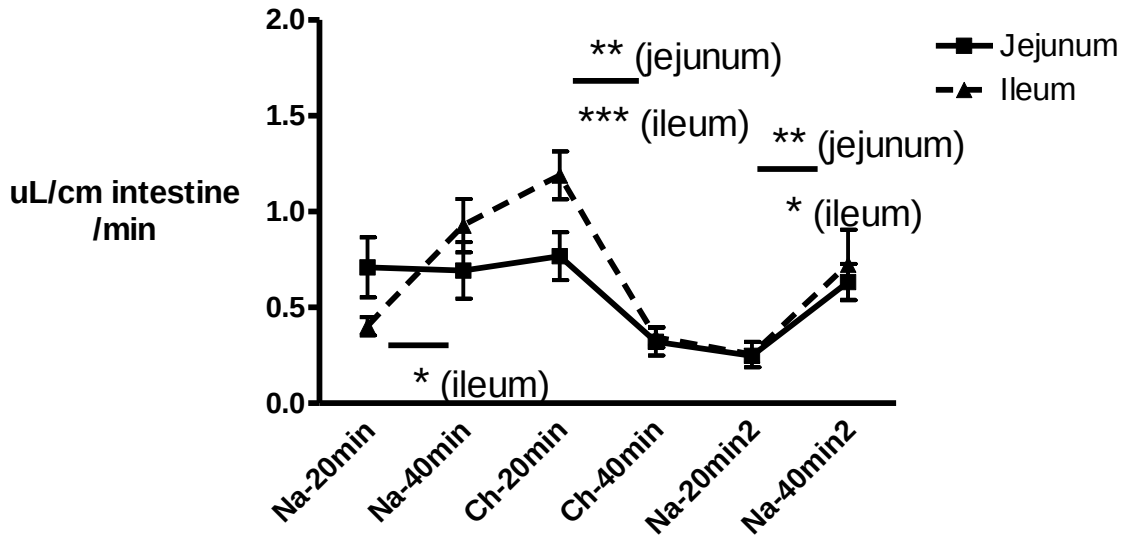


Figure 3.4c. Serosal fluid volume per cm of intestine per minute using 10mM glucose in perfusate buffer

There is a significant decrease in fluid passing through the epithelium 20-40 minutes after the switch to a sodium-free perfusate buffer in both the jejunal and ileal segments. There is also a significant increase between 20-40 min following the switch back to a sodium containing buffer. * denotes $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$. n = 7

3.4. Discussion

Results from this chapter demonstrate a novel application of the *ex vivo* intestinal perfusion technique to measure transport of Pi across the intestinal epithelium. Previous *in vivo and in vitro* methods have interpreted the amount of Pi transported by the intestine as the increase in concentration of Pi appearance in the blood or the amount of Pi taken up by everted intestinal tissue. While both of these methods have helped to form our current understanding of intestinal Pi transport, their pitfalls stem from the fact that the amount of Pi directly transported across the epithelium is not represented. Here, it is shown for the first time that there is a concentration of Pi during its transport across the intestinal epithelium, and thus a proof of principle that active transport of Pi is taking place.

The initial experiments which utilized a standard Kreb's buffer (containing 1.2mM Pi) show concentrated Pi in the serosal fluid (approx 2-3x higher compared to perfusate Pi concentration). To further show that there is a significant, active, mostly NaPi-IIb regulated Pi transport process in the intestine, additional experiments were conducted using a period of sodium-free conditions in the perfusate (ChCl and $ChHCO_3$ replacing NaCl and $NaHCO_3$ respectively). When utilizing both 28mM and 10mM glucose in the perfusate, significant changes in Pi transport are seen following the switch to sodium-free buffer (between 20-40min) as well as following the switch back to a sodium-containing buffer (also between 20-40min after the switch) **(Figures 3.3b and 3.4b)**. It is speculated that the lag-time in response to switching between the perfusates (approximately 20 min) seen in the present experiments represents transport reaching

equilibrium. Unlike the 40 minute equilibration period prior to collection of the first sample of serosal fluid, no equilibration period is allowed following the switch to sodium-free buffer and the switch back to sodium-containing buffer. Thus the serosal fluid collected during the 20 minutes following this switch may partially represent the Pi transported in the presence of sodium buffer. Additionally, the concentration of Pi measured in serosal fluid could not be attributed to contamination with liquid paraffin since a liquid paraffin control was assayed for Pi to determine if it contained substantial Pi concentration that may have affected the results. From this data, we can conclude that sodium-driven transport of Pi is indeed taking place in both the jejunum and the ileum.

Glucose is included in the perfusate buffer as it acts as a metabolic substrate for intestinal tissue and contributes to tissue viability [220]. It drives water transport across the intestinal epithelium in order to produce sufficient serosal fluid for analysis. Water transport across the epithelium occurs mainly via aquaporin channels (e.g. aquaporin 5 which is specifically expressed in the stomach and duodenum [221]), however the intestinal glucose transporters, SGLT1, GLUT1, and GLUT2 also function as a gateway for transepithelial water transport [222-224]. When comparing experiments using 28mM and 10mM of glucose in the perfusate buffer however, it is evident that serosal fluid volumes are relatively similar. This may suggest that SGLT1 and the aquaporins expressed in the jejunal and ileal segments used in these experiments function at the same water transport capacity in the presence either 10mM or 28mM glucose. Future

studies could investigate a glucose concentration that would affect transepithelial water transport by using lower amounts of glucose in the perfusate buffer.

Previous studies show an *ex vivo* intestinal tissue viability timeline of upwards of 120 min following removal from the blood supply [214, 225]. Viability was mainly determined by observing a constant, active transport component of glucose. It is of note that in the present experiments, there is inconsistent glucose transport - specifically, there is an apparent lack of active glucose transport across the ileum using 28mM glucose in the perfusate as well as in both jejunum and ileum at 10mM glucose. Accumulative glucose transport has always been a hallmark of the *ex vivo* perfusion technique, and this raises concerns about tissue viability in the present experiments. One explanation for lower than expected glucose concentration in the serosal fluid revolves around possible distension of the intestine during the perfusion period, which could stretch and damage the intestine and potentially impair solute transport mechanisms. Another factor that may contribute to inconsistent glucose transport in the intestine is buffer perfusion rate. The rate implemented using this technique varies widely, with published methods anywhere in between 0.5 - 7mL/min [225-227]. A flow rate of 1 mL/min was used in the current experiments since this was found by previous work to be the best choice to minimize perfusate build-up in the loop of intestinal segment, which could lead to distension and damage to intestinal tissue [228]. The present data shows that following the switch from sodium-free perfusate back to sodium-containing perfusate there was a trend for an increase in glucose transport **(Figures 3.3b and 3.4b)**. This pattern of increased glucose transport upon

reintroduction of sodium-containing perfusate towards the end of the experiment, along with the sustained Pi transport observed, support the fact that the intestinal tissue is intact and viable.

Intriguingly, there is an absence of sodium-dependence of glucose transport in the ileum while utilizing perfusate containing 28mM glucose **(Figure 3.3b).** The reason for a lack of sodium dependency could involve the use of 28mM glucose, as the sodium-dependent transporter SGLT1 has been shown to have a K_m of around 0.2-0.5mM [229, 230], thus SGLT1 and sodium-dependent glucose transport would function maximally in the presence of lower luminal glucose concentrations. The intestinal facilitative glucose transporter, GLUT2, which does not require sodium for glucose transport, has a high K_m of 17mM for glucose [231] and is shuttled to the apical membrane of the intestine in response to high luminal glucose concentrations [232]. Indeed when utilizing 10mM glucose **(Figure 3.4b)**, sodium-dependent glucose transport is seen in both the jejunum and the ileum following switches between the two different perfusate buffers. The serosal fluid volume for experiments using 28mM glucose show significant changes in response to sodium-free buffer switching and reintroduction **(Figure 3.3c)**, thus the lack of sodium dependency in glucose is not inhibiting water transport.

Serosal fluid collected from perfusion experiments conducted by colleagues utilizing mouse proximal small intestine show a concentration of glucose in the serosal fluid (personal communication). Glucose transporter expression and adaptation in response to changes in luminal glucose in the intestine may thus differ between species. SGLT1

is known to be expressed mainly in mature enterocytes across most species; however, mouse and rat glucose transporters are shown to have different characteristics. Phlorizin, a competitive inhibitor of SGLT1 has different inhibition constants between species (rat and rabbit) [229, 233]. GLUT2, although responsible for apical glucose transport only at high luminal concentrations, has been shown to have approximately 81% homology between human, rat, and mouse [234] and its expression level is different between humans and mouse in pancreatic ß-cells [235]. This potential difference in expression between species, in addition to the lower perfusion rate utilized in the present experiments as compared with those described in the original publication of the method [214, 220], may explain why concentrated glucose was not seen in the serosal fluid of perfused rat intestine in the present experiments.

It has recently been shown that there are morphological changes in the intestine following induction of CKD in rats - these changes occur along the tight junctions between enterocytes of the intestinal epithelium and mimic changes that occur following damage to the epithelium [137]. Specifically, these studies show that there are alterations in protein levels of claudin-1 and occludin, key constituents of tight junctions in intestinal epithelia in rat models of CKD [138]. Since tight junctions may serve important regulatory functions in the still uncharacterized sodium-independent Pi transport pathway, these anomalies in expression of tight junction proteins need to be investigated further to elucidate consequences for intestinal Pi transport in the context of CKD. Disturbances in the architecture of tight junctions may, in turn, passively release more solutes into the bloodstream perhaps in an unregulated manner.

Interestingly, mutations in genes encoding for claudin-16 produce phenotypes of hypomagnesemia with hypercalciruria [236-238], changes that may be related to the mechanisms of hyperphosphatemia seen in CKD. Future experiments could involve utilizing the *ex vivo* perfusion method using intestinal tissue from CKD-induced rats and comparing results between control and CKD animals.

Importantly, data shown in this present work illustrate that the *ex vivo* intestinal perfusion technique could provide important information regarding the transport of Pi. Serosal fluid could be analyzed in the future to see if this contains factors that are dependent on the level of Pi in luminal fluid. The composition of the serosal fluid would shed light on if and how the intestine signals to other organs, such as the kidney, in response to an acute dietary Pi load. Revealing these intestinal-derived Pi signalling mechanisms will contribute towards discovering new targets for control of body Pi balance in the context of CKD.

3.4.1. Summary and conclusion

The *ex vivo* intestinal perfusion technique reported by Fisher and Gardner in 1974 has not been utilized to date in furthering our understanding of Pi transport across the rat intestine. In the present experiments, this perfusion method has been used to show that there is a significant increase in the concentration of Pi in fluid absorbed across the rat intestinal epithelium. The significant reduction in Pi transport following a switch to sodium-free perfusate also points to an active transport mechanism most likely attributed to NaPi-IIb at the BBM, although the mechanism of Pi transport at the

basolateral membrane has not been characterized. These results further our overall knowledge of Pi transport across the rat intestine and validate the *ex vivo* perfusion method for future studies involving Pi transport.

4.0. The effect of a duodenal Pi load on urinary Pi excretion and renal phosphate transporter expression

4.1. Introduction

Communication between the small intestine and the kidney has been established in regulating such solutes as sodium, calcium and potassium (comprehensive review in [239]). For example, body sodium homeostasis has been theorized to depend, at least in part, on crosstalk between these two organs. The intestine is known to produce circulating peptides called guanylin and uroguanylin which are important for natruiresis [240, 241]. Calcium-sensing receptors are also found in both the epithelium of the intestine and the kidney as these receptors play a large role in calcium, Pi and amino acid homeostasis [242-244].

With the recent emphasis on the correlation of high serum Pi with cardiovascular events in CKD, it is now becoming apparent that new methods of detecting and treating hyperphosphatemia are needed. A present concern involves high Pi preservatives in food possibly lending to overall Pi toxicity. While only 40-60% of naturally occurring dietary Pi is thought to be absorbed, inorganic Pi from preservatives has a higher bioavailability [120]. Their presence can also increase the Pi content in food by as much as 70% [245], thus preservatives have the potential to significantly impact postprandial Pi load. A recent study showed that following ingestion of Pi salts

commonly found in additives, serum Pi levels increased for a period of up to 20 hours [246].

In 2007, Berndt et al demonstrated that instillation of a high concentration of sodium Pi (1.3M) into the proximal small intestine increased urinary Pi excretion in less than 30 min [139, 140]. This acute effect did not occur following a duodenal instillation of saline nor was an increase in urinary Pi excretion seen following a Pi load directly into the stomach. This observation pointed to a specific Pi signalling process occurring in the upper small intestine which was independent of known regulators of Pi such as PTH, FGF-23, sFRP-4, changes in GFR, or the involvement of a kidney neural reflex as demonstrated by experiments on rats with denervated kidneys. Additionally, Berndt showed that IV infusion of an extract prepared from the duodenal mucosa also had an overall phosphaturic effect. Importantly, no changes in plasma Pi levels were seen in any of these procedures suggesting that, if there was indeed some type of intestinal Pi sensing factor, it acted independently of changes in plasma Pi levels.

One main area of concern in these studies by Berndt et al involved the concentration of Pi utilized in renal clearance experiments – an erratum which was subsequently published stated that 1.3M Pi instead of 1.3mM Pi was utilized in the studies [140]. Pi concentrations in the molar range do not represent physiological concentrations and the results may have been due to changes in the structure of the intestine in response to the high osmolality of the infused buffer.

The consequences of a potential intestinal signalling pathway for Pi are potentially far reaching, especially for CKD patients. Due to declining renal function in CKD, targeting the kidney to attenuate hyperphosphatemia becomes difficult. The small intestine on the other hand, may be a suitable target for treatment since intestinal Pi absorption does not seem to be significantly altered in animal models of CKD [117]. It is therefore vital to elucidate the mechanisms of Pi transport following an acute Pi load into the intestine since the intestine could be a target for treatment in clinical hyperphosphatemia.

4.1.1. Aims of this study

1. To replicate Berndt et al (2007) *in vivo* experiments with a physiological duodenal load of 10mM Pi as well as the 1.3M concentration that the group utilized [139, 140].

2. To examine protein expression of renal NaPi-IIa and NaPi-IIc of animals utilized in these experiments.

3. Place rats on a chronic low Pi diet and repeat experiments described in aim 1 to see if low Pi diet priming is needed to illicit a (greater) sodium-cotransporter protein expression response to an acute duodenal Pi load [62].

4.2. Materials and methods

4.2.1. Animal model

Experiments were performed on male Sprague Dawley rats 12–14 weeks old (250-300g). Animals were given free access to water and standard commercial rat chow (Diet RM1, 0.52% Pi, Special Diet Services, UK) or low Pi diet, containing 0.1% phosphorous by weight (Special Diet Services, UK) and were maintained under controlled conditions of temperature and light.

4.2.2. Renal function in anesthetized rats

Sprague Dawley rats were either fed a normal diet as mentioned above, or primed with a diet of low Pi (0.1% Pi SDS diet #120426.16) over a full 7-day period before experiments took place. Rats were then anaesthetized by IP injection of thiobutabarbital (Inactin, 120 mg/kg; Sigma Aldrich UK). Unlike other anesthetics such as pentobarbital, thiobutabarbital has been shown to provide a more stable, long term analgesic effect in rats measured by stable GFR [247, 248]. A series of surgical procedures then took place: tracheotomy to maintain a clear airway, femoral artery cannulation for blood collections and mean arterial blood pressure (MAP) monitoring utilizing a blood pressure monitor (Adinstruments MLT0380/D, Powerlab 500 software, UK), bladder cannulation for urine collections, and a jugular vein cannulation for reagent infusions (1mL FITC-inulin/100g animal body weight in isotonic saline for glomerular filtration rate (GFR) determination). A cannula-sheathed 19g needle for instillation of Pi or saline solutions was placed into the duodenum (2-3cm from the stomach pylorus)

and secured with a ligature (*in situ* intestinal open loop technique). Once surgery was completed, a bolus of 0.05% fluorescein isothiocyanate (FITC) tagged inulin, diluted in isotonic saline, was given as a primer via the jugular cannula and replaced the isotonic saline for infusion. The animal was then maintained for 40 minutes for FITC-inulin equilibration and recovery from surgery **(Figure 4.1).**

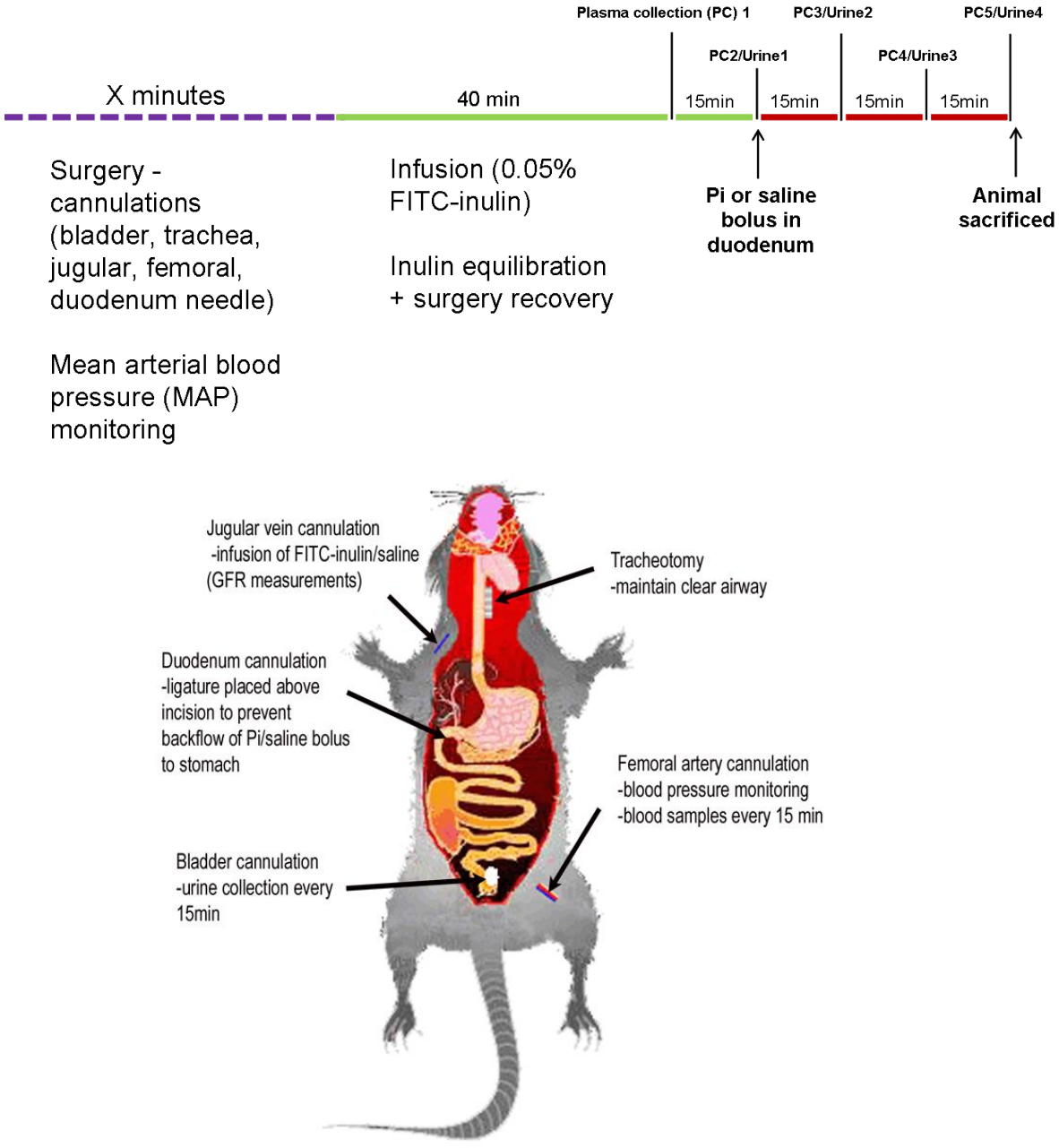


Figure 4.1. Schematic of renal clearance surgery procedures and timeline

After the equilibration period, rats underwent the following protocol: four consecutive collections of urine were made, each of 15 min. A small sample (500uL) of arterial blood was drawn at the start and then after each urine collection period for Pi and FITC-inulin measurements.

After the first control (CTRL) urine collection, 1mL of phosphate solution containing either 10mM or 1.3M diluted in HEPES buffer (16mM Na-Hepes, 140mM NaCl, 3.5mM KCl, 10mM/1.3M KH_2PO_4, pH 5) and matching saline controls (154mM and 1.35M saline respectively) was instilled into the duodenum. Osmolarity of buffers was assessed using a freezing point depression micro-osmometer (Model 3M, Advanced Instruments Inc, Massachusetts, US). All instilled solutions were within the isotonic range (290-310 mOsm/L), except the 1.3M Pi and 1.35M saline (chosen to match the osmolarity of Pi solution), which presented an extremely high osmolarity. The duodenal needle was then removed after instillation, and the intestine was tied off to prevent backflow of Pi buffer into the stomach. After the last blood collection, the rat was killed by cardiac exsanguination and the kidneys and sections of intestinal mucosa were collected for brush border membrane vesicle preparation **(Figure 4.1).** Both kidneys were decapsulated, excised and transferred to a beaker containing ice cold isotonic saline. The cortex of both kidneys containing the proximal tubules were removed by slicing off the outer layer with a scalpel on an ice-cold glass plate and snap frozen for subsequent brush border membrane vesicle preparation

4.2.3. GFR and Pi measurements/calculations

Blood was collected in tubes containing anticoagulant (EDTA) and kept on ice until processing after surgeries. After the last blood collection, all tubes were centrifuged at 6000rpm for 15 min at 4^{o}C and the plasma was separated and frozen for future use in Pi and FITC assays. Urine was immediately frozen for FITC assays.

Plasma and urine were treated with 10% trichloroacetic acid (TCA) prior to Pi assays to remove bound protein that may affect Pi concentration. Pi assays were performed according to the manufacturer's instructions (Quantichrome DIPI-500, USA). FITC-inulin assays were performed in a 96-well plate and measured using a plate reader with a filter for 480nm. FITC readings from blood collected at the beginning and end of the 15 min urine collection period were averaged to represent FITC concentrations during the whole urine collection period.

GFR was then calculated using the following equation:

[FITC value for urine x flow rate (uL/min)] / average FITC reading from first and last plasma collection for the specific time period. Absolute Pi values were obtained using this equation:

Urine Pi concentration x urine flow rate

Fractional excretion of Pi (FEPi, %) was calculated using the following equation:

[([Pi] urine / [Pi] plasma) x (FITC plasma / FITC urine)] x 100

4.2.4. Plasma PTH measurements

PTH in plasma taken from the last blood collection (45 min after Pi/saline bolus) was measured using an enzyme-linked immunosorbent assay (ELISA) targeting rat bioactive PTH (Immunotopics, USA). The ELISA was performed according to manufacturer's protocol.

4.2.5. Kidney brush border membrane vesicle preparation

Cortex from both kidneys, collected in the manner mentioned above, was suspended in 30mL resuspension buffer 1 (300mM mannitol, 5mM EGTA, 12mM Tris-HCl, protease inhibitors, pH 7.4) and homogenised using an Ultra Turrax homogenizer for 2 min. The homogenizer was then rinsed with 42mL cold dH_2O and collected with the homogenate. This solution was then stirred on ice with the addition of 720 µL 1.2M $MgCl_2$ (final concentration of 12mM $MgCl_2$) for 15 min and then centrifuged at 4500rpm for 15 min at 4^oC. Supernatant was obtained after centrifugation and further centrifuged at 16000rpm, for 30 min to obtain a BBM containing pellet. The pellet was resuspended in 20mL of resuspension buffer 2 (150mM mannitol, 2.5mM EGTA, 6mM Tris-HCL, pH 7.4) and hand homogenized using a Teflon hand homogeniser. The homogenate was gently stirred on ice and a further addition of 200uL of 1.2M $MgCl_2$ (final concentration of 12mM $MgCl_2$) for 15 min and spun at low and high rpm as previously described. The resulting pellet was resuspended with 20mL resuspension buffer 3 (300mM mannitol, 12mM Tris-HCl, 2.5mM EGTA, pH 7.4) and hand homogenized in the same manner was above. The homogenate was spun one final time at 16000 rpm for 30 min and the

resulting pellet was resuspended with 1mL of resuspension buffer 3 using a 21 gauge needle and syringe. All steps were performed at 4°C.

An alkaline phosphatase assay was utilized to determine enzymatic activity of the initial BBMV homogenate and the final protein concentration was obtained using a Bradford assay. Taken together, these assays allowed overall purity of the BBM preparation to be determined [249, 250].

4.2.6. Western blotting

BBMVs (40 µg protein) were combined with 2x Laemmli Buffer (Bio-Rad) at a 1:1 ratio and further denatured at 90°C for 2 min. Samples and a molecular weight marker (Bio-Rad) were then run on a 10% SDS-PAGE gel and transferred onto a PVDF membrane using a semi-dry transfer system (Bio-Rad) for 1.5 hours at 120mA for 2 gels and 75mA for 1.5 hours for 1 gel. The membranes were then blocked with 5% powdered milk diluted in 1x PBS and 0.1% Tween (PBST) for 45 min and incubated with a primary antibody overnight on a shaker 4°C. Blots were washed 3 x 10 min with PBST and incubated with a horseradish peroxidase conjugated secondary antibody for 1 hour at room temperature on a shaker. Membranes were washed again as described previously and proteins were detected using a homemade electrochemiluminscence reagent (1.25mM 3-aminophthalic acid hydrazide aka luminol, 0.2mM p-coumeric acid, 500mM tris-HCl, 100mM NaCl, 21.5mM H_2O_2) . Antibodies used: primary: rabbit anti NaPi-IIa, rabbit anti NaPi-IIc, (generous gifts from Biber J, Zurich Switzerland), loading

control ß-actin (Abcam); secondary – HRP conjugated donkey anti rabbit/mouse (Sigma).

4.2.6.1. Protein quantification and statistical analysis

Protein bands were detected and quantified using densitometry using the Fluor-S MultiImager system (BioRad, Hertfordshire, UK). Values are expressed in arbitrary units (a.u.) as a ratio of primary antibody protein intensity to ß-actin. Data is presented as mean ± SEM and statistical significance is shown after analysis with a Student's paired *t*-test with * denoting a P value of ≤ 0.05 and ** denoting a P value of ≤ 0.01. P values were obtained comparing protein expression relative to the saline control.

4.3. Results

4.3.1. Renal function and blood pressure of anesthatized rats maintained on a regular diet or low Pi diet over the course of the surgical procedure

No significant changes in GFR and MAP were seen during surgeries for all groups of animals. There was a trend in decrease of MAP as surgeries progressed - this is well-documented effect of anesthesia [251] **(Figures 4.2a and 4.2b).** There was one time point (45 min post-bolus) in which animals receiving a 1.35M saline load into the duodenum load showed a significant decrease in MAP compared to the control period ($P = 0.016$).

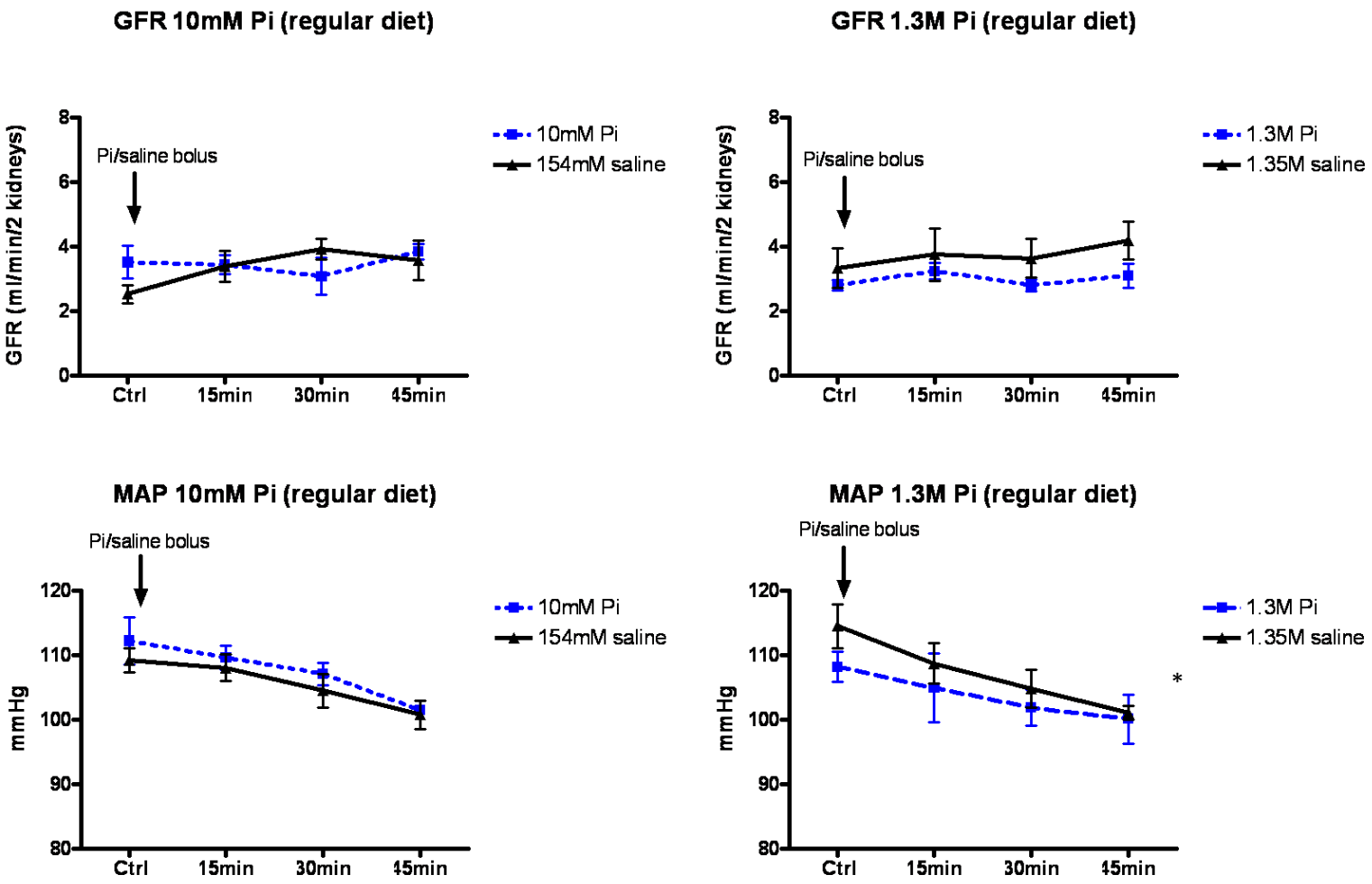


Figure 4.2a. GFR and MAP data from clearance surgeries (regular diet)

GFR values and MAP for clearance surgeries on animals maintained on a regular diet. n = 4 (154mM saline), 5 (10mM Pi), 5 (1.35M saline), 7 (1.3M Pi). Data is presented as mean ± SEM, * denotes $P \leq 0.05$.

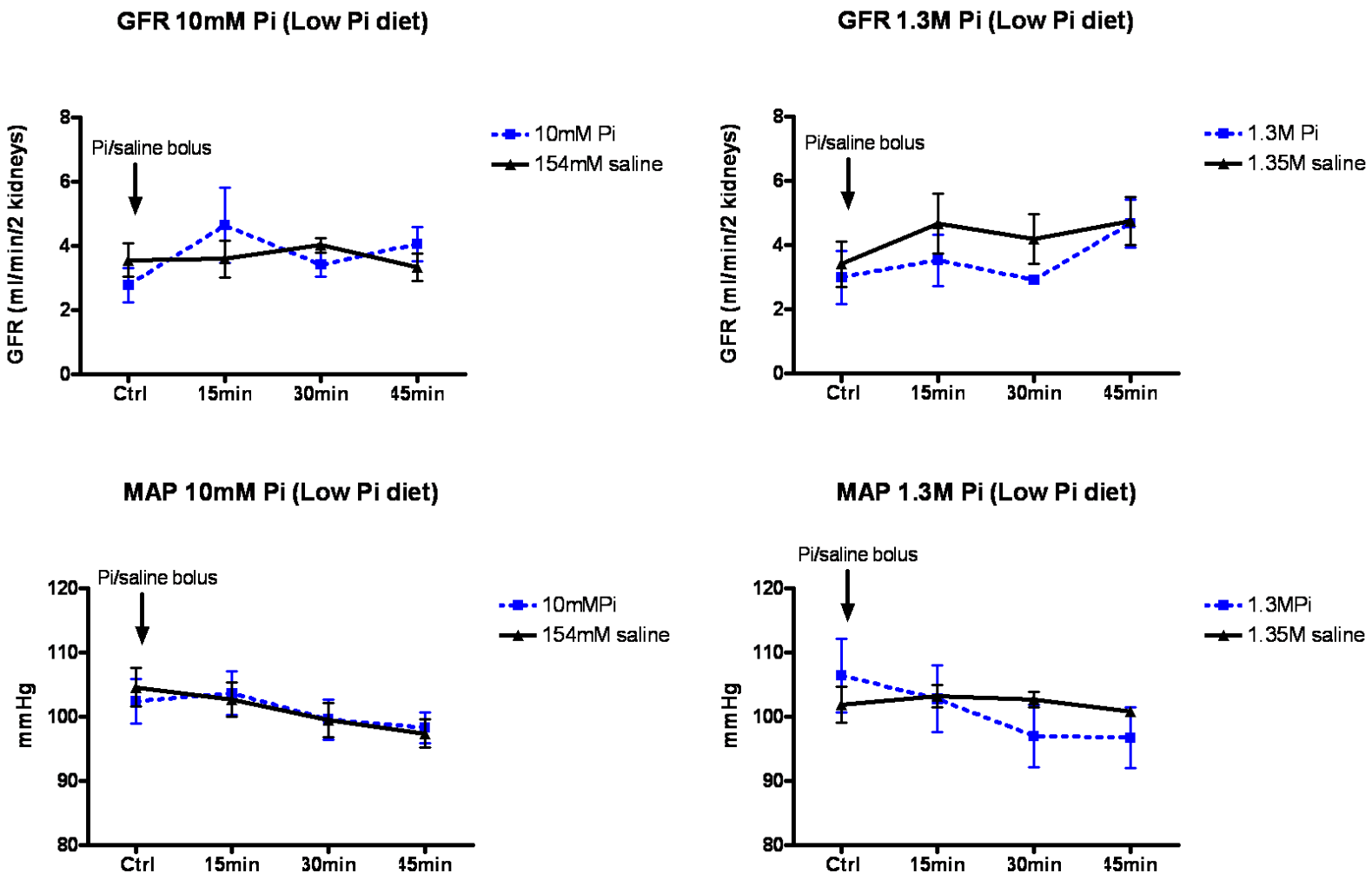


Figure 4.2b. GFR and MAP data from clearance surgeries (low Pi diet)

GFR values and MAP for clearance surgeries on animals maintained on a low Pi diet. n = 5 for all parameters. Data is presented as mean ± SEM.

4.3.2. Effect of a 10mM Pi duodenal instillation on plasma Pi levels and urinary Pi excretion (regular diet)

A 10mM Pi concentration was used to represent a physiological, post-prandial Pi concentration in the intestinal lumen. No significant changes were seen in plasma levels, FEPi, or absolute urinary Pi excretion following a 10mM Pi duodenal challenge **(Figure 4.3a).**

4.3.3. Effect of a 1.3M Pi duodenal instillation on plasma Pi levels, and urinary Pi excretion (regular diet)

A 1.3M Pi concentration was used to directly replicate experiments conducted by Berndt et al (2007) [139, 140]. A significant increase in plasma Pi concentration was seen following Pi instillation into the duodenum (P values to control period: 15 min = 0.0018, 30 min = 1.8 x 10-5, 45 min = 0.00039). There was a significant increase in absolute urinary Pi excretion 45 min post 1.3M Pi instillation into the duodenum. (P = 0.0435) and a trend in increase of FEPi in this group. A similar trend in increase in FEPi and absolute urinary excretion of Pi was seen in animals that received a 1.35M saline bolus, however changes were not significant. Data for absolutely excretion of Pi for 1.3M Pi and 1.35M saline duodenal instillation were expressed on separate graphs due to large differences in Pi excretion values **(Figure 4.3b).**

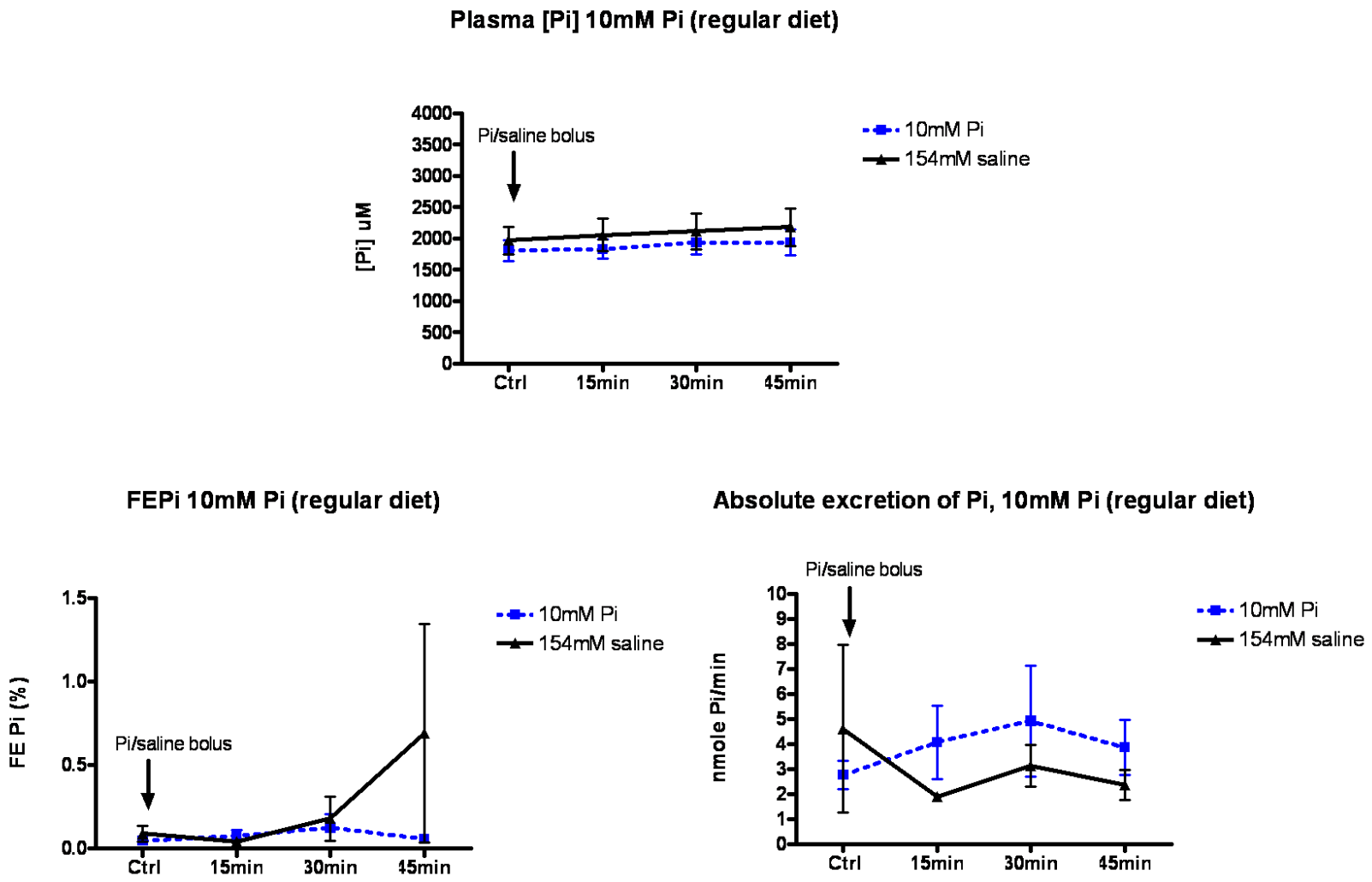


Figure 4.3a. Plasma Pi concentration and urinary Pi excretion data for animals that received a 10mM Pi duodenal instillation, maintained on a regular diet

n = 4 (154mM saline), 5 (10mM Pi). Data is presented as mean ± SEM.

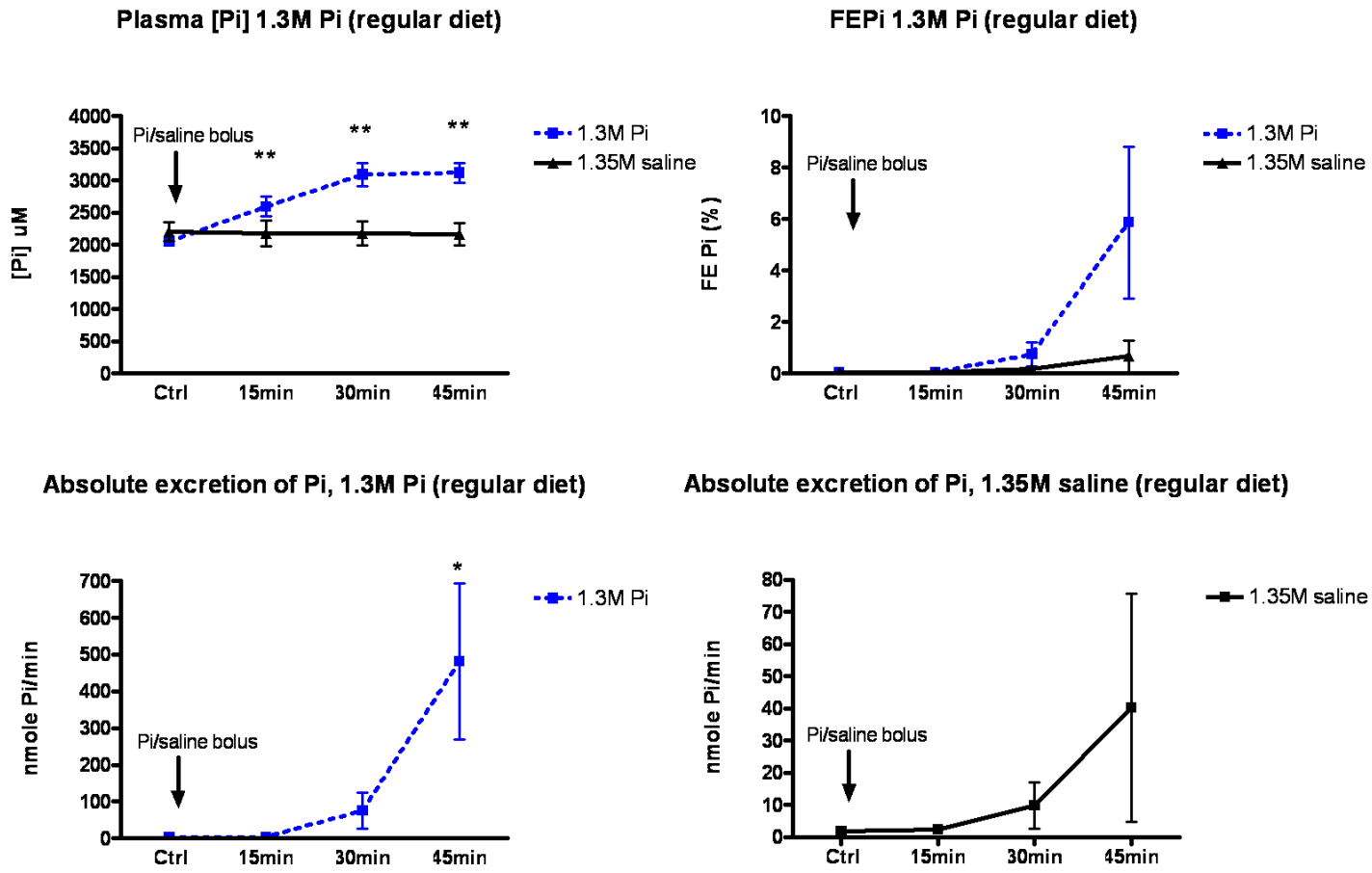


Figure 4.3b. Plasma Pi concentration and urinary Pi excretion data for animals that received a 1.3M Pi duodenal instillation and were maintained on a regular diet.

n = 5 (1.35M saline), 7 (1.3M Pi). Data is presented as mean ± SEM, * denotes $P \leq 0.05$.

4.3.4. Effect of 10mM and 1.3M Pi instillation in the duodenum on expression of NaPi-IIa and NaPi-IIc protein at the renal BBM (regular diet)

Western blots were performed utilizing kidney BBMVs prepared from corresponding animals post-mortem. No changes was seen in renal protein expression of NaPi-IIa or NaPi-IIc following duodenal Pi instillation **(Figure 4.4a and 4.4b)**

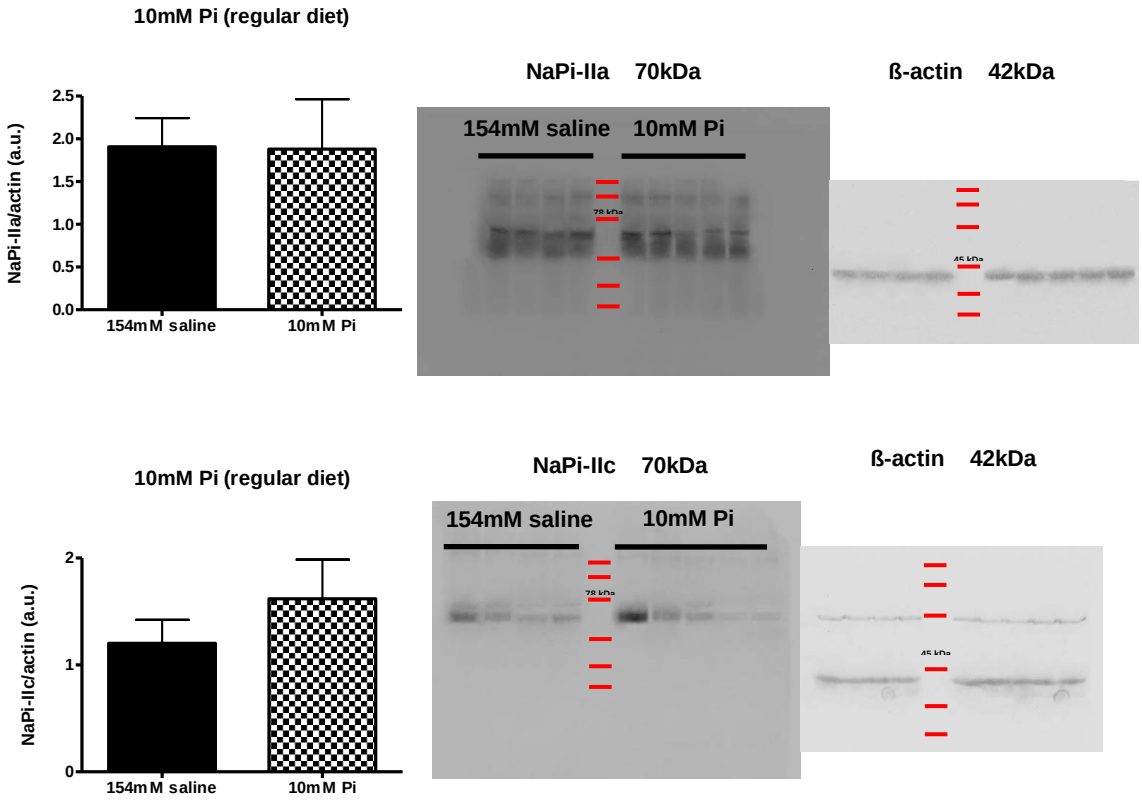


Figure 4.4a. NaPi-IIa and NaPi-IIc expression following a 10mM Pi duodenal instillation in animals maintained on a regular diet

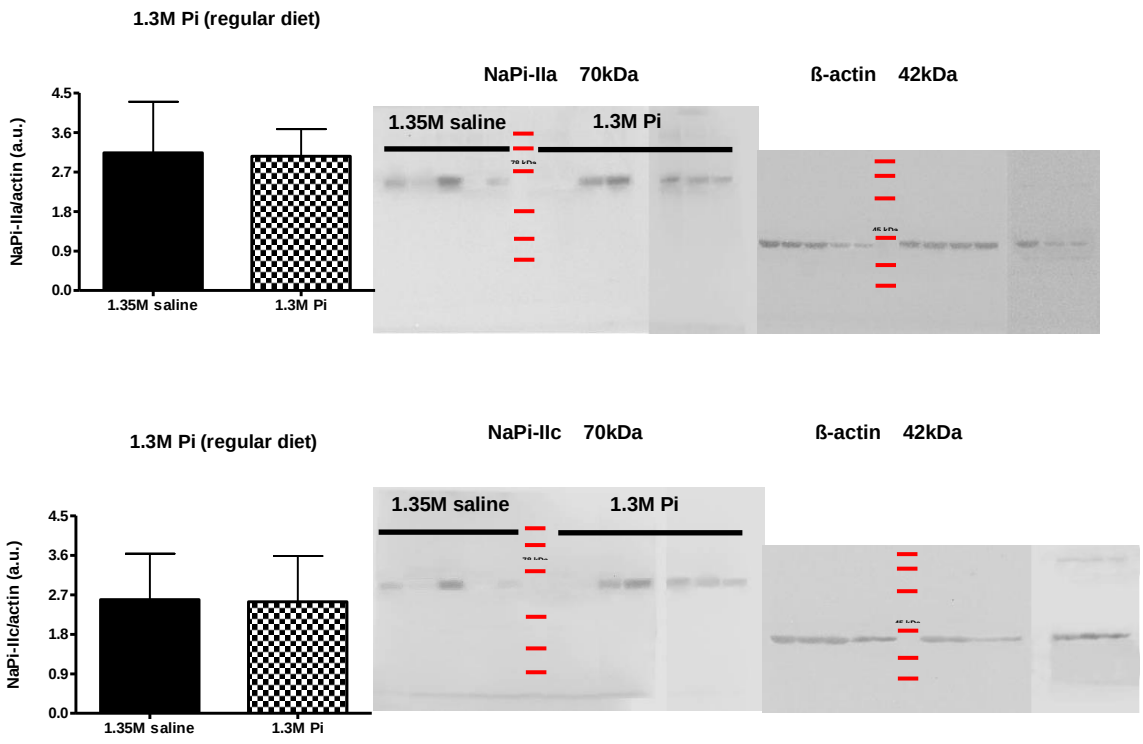


Figure 4.4b. NaPi-IIa and NaPi-IIc expression following a 1.3M Pi duodenal instillation in animals maintained on a regular diet

4.3.5. Effect of 10mM Pi instillation in the duodenum on plasma Pi levels and urinary Pi excretion (low Pi diet)

No significant changes were seen in plasma Pi levels, FEPi, or absolute urinary Pi excretion following a 10mM Pi duodenal challenge in animals maintained on a chronic low Pi diet **(Figure 4.5a).**

4.3.6. Effect of 1.3M Pi instillation in the duodenum on plasma Pi levels and urinary Pi excretion (low Pi diet)

No significant changes were seen in FEPi, or absolute urinary Pi excretion following a 1.3M Pi load into the duodenum in animals maintained on a low Pi diet. At all time points following duodenal Pi instillation, there was a significant increase in plasma Pi concentration. P values: 15min = 2.3×10^{-4}, 30min = 7.9×10^{-6}, 45min = 5.8×10^{-5}) **(Figure 4.5b).**

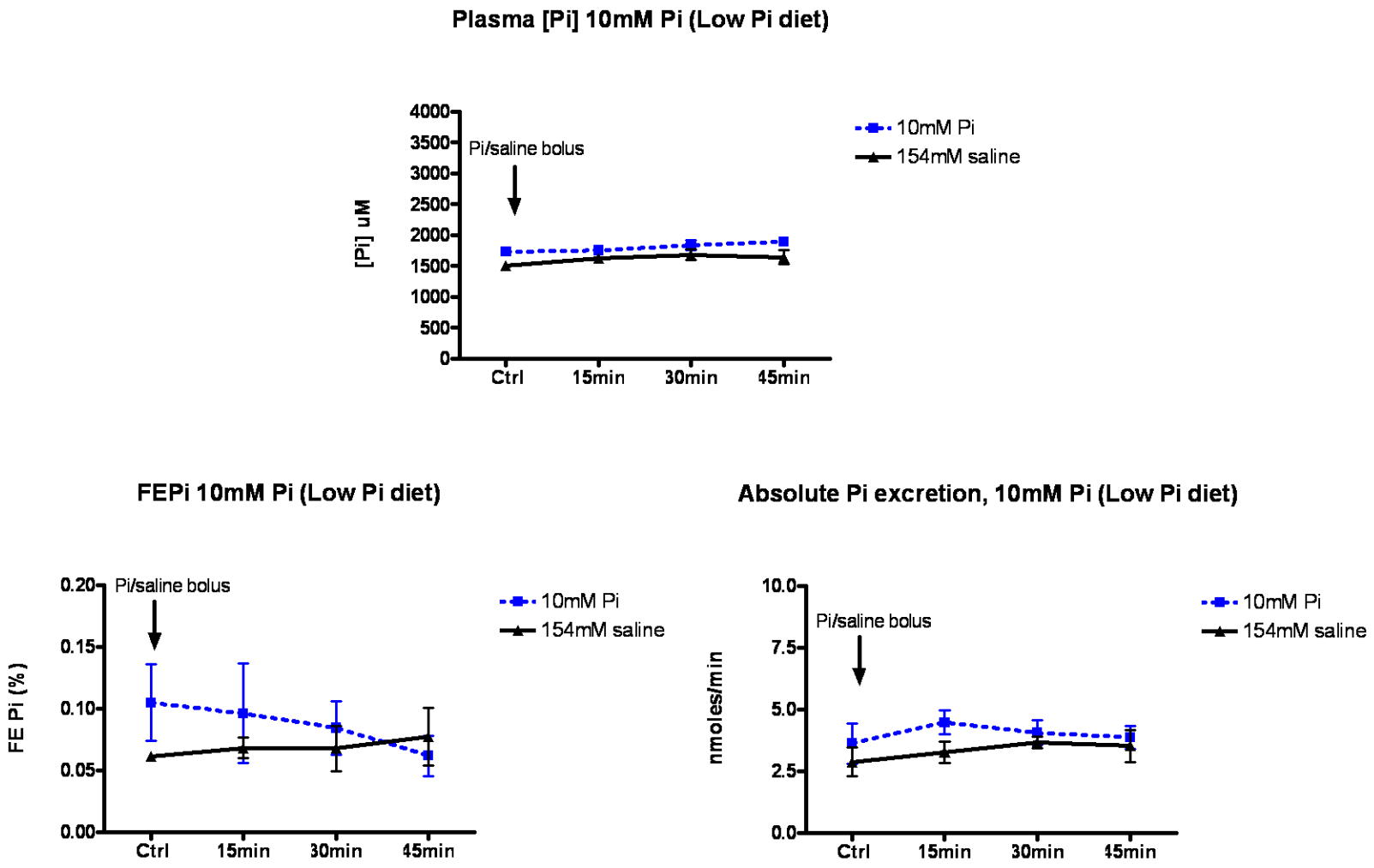


Figure 4.5a. Pi concentration and urinary Pi excretion data for animals that received a 10mM Pi duodenal instillation, maintained on a chronic low Pi diet

n = 4 (154mM saline), 5 (10mM Pi). Data is presented as mean ± SEM.

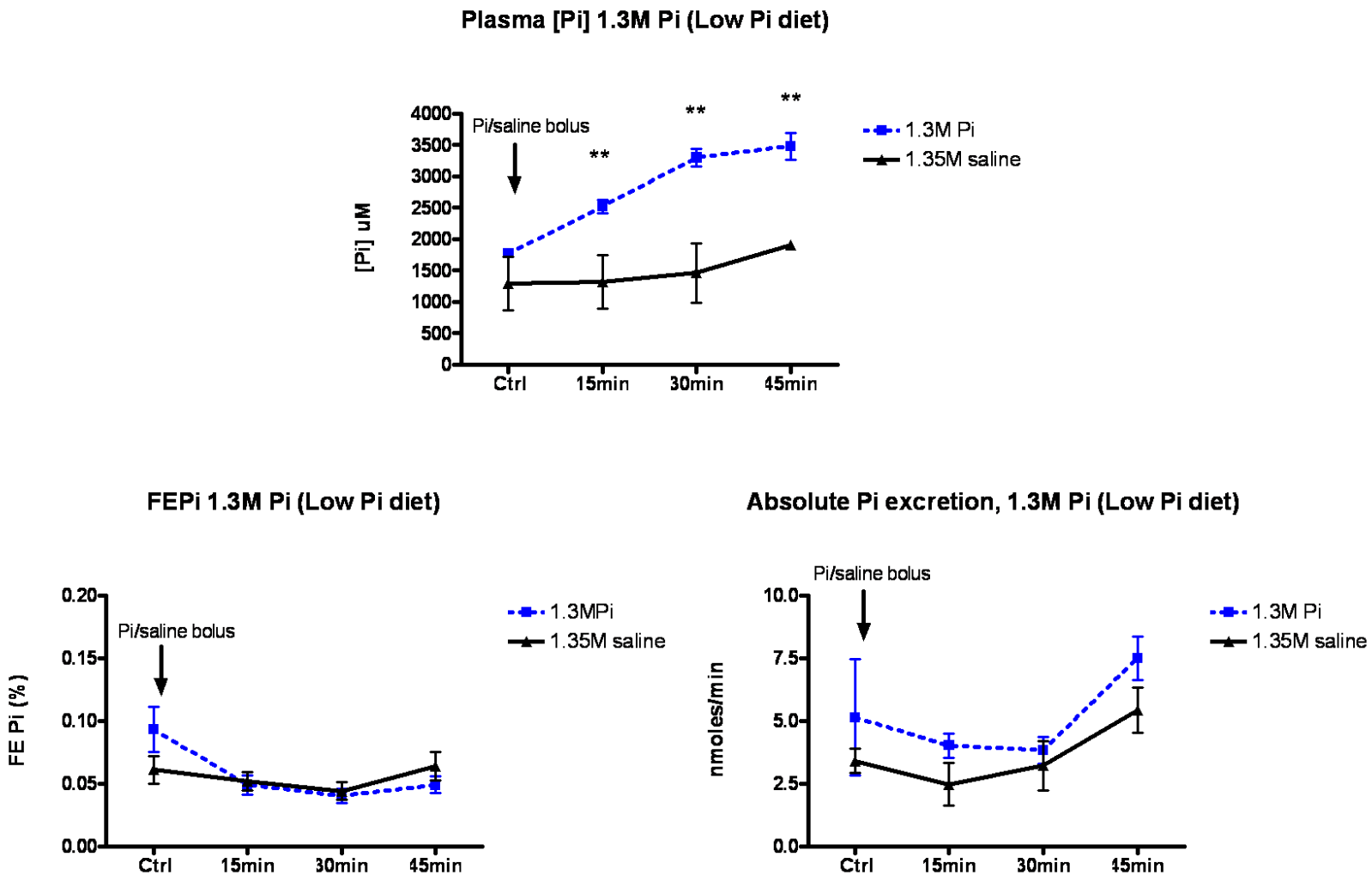


Figure 4.5b. Pi concentration and urinary Pi excretion data for animals that received a 1.3 M Pi duodenal instillation, maintained on a chronic low Pi diet

n = 4 (154mM saline), 5 (10mM Pi). Data is presented as mean ± SEM, ** denotes P $\leq$ 0.01.

4.3.7. Effect of 10mM and 1.3M Pi duodenal instillation on NaPi-IIa and NaPi-IIc protein expression at the renal BBM (low diet)

No changes was seen in protein expression of NaPi-IIa or NaPi-IIc following duodenal Pi instillation in animals maintained on a low Pi diet **(Figure 4.6a and 4.6b).**

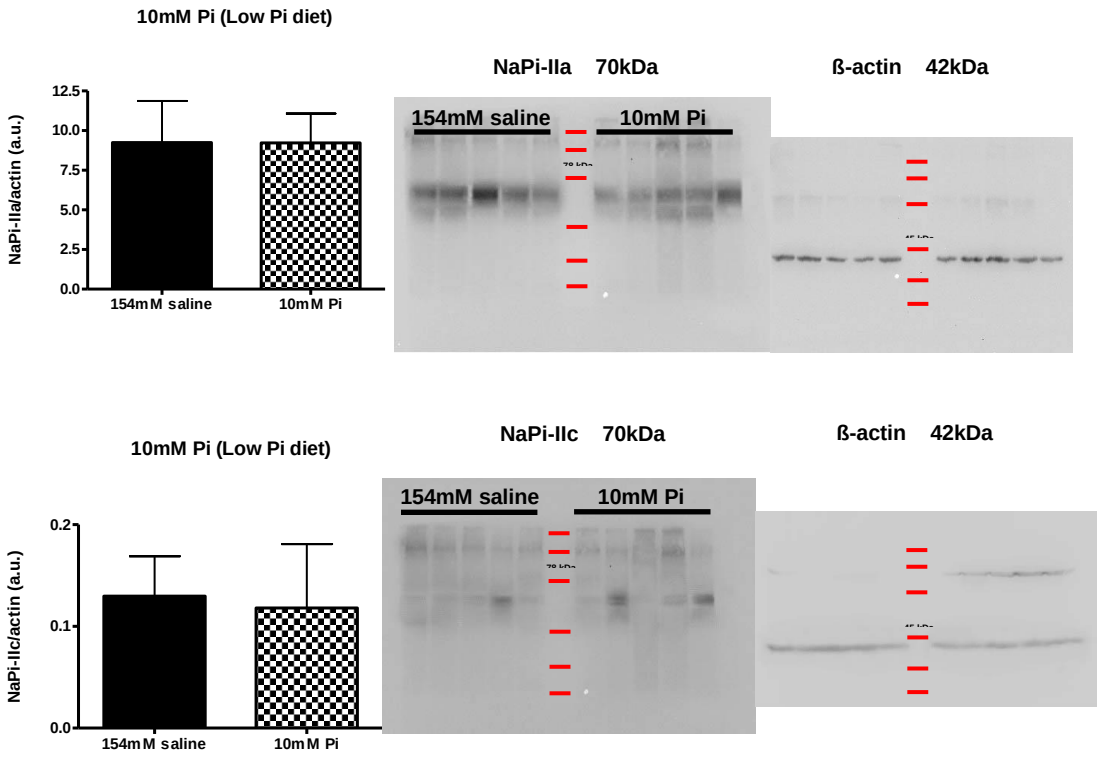


Figure 4.6a. NaPi-IIa and NaPi-IIc expression following a 10mM Pi duodenal instillation in animals maintained on a chronic low Pi diet

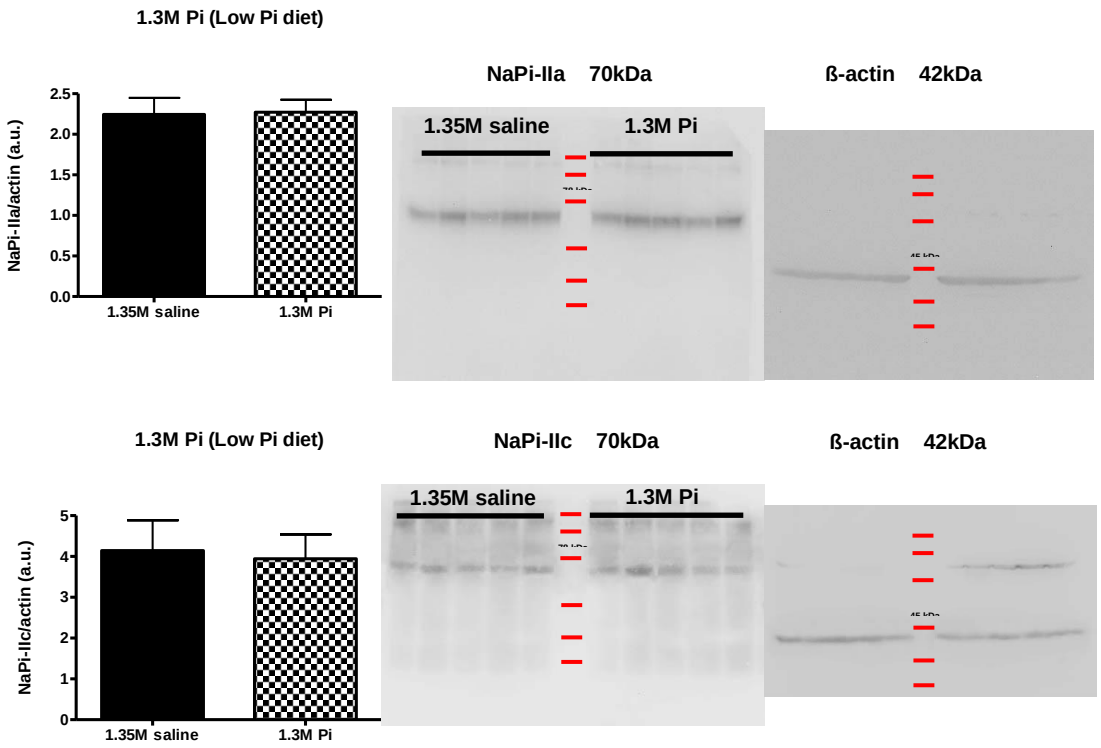


Figure 4.6b. NaPi-IIa and NaPi-IIc expression following a 1.3M Pi duodenal instillation in animals maintained on a chronic low Pi diet

4.3.8. Effect of duodenal Pi load on plasma PTH levels

There was no significant difference in plasma PTH levels between animals receiving a saline or 10mM Pi duodenal bolus in animals maintained on regular or a low Pi diet. Animals which received a 1.3M Pi duodenal load, whether on both regular and low Pi diets, showed significant increases in levels of PTH. The increase in plasma Pi and PTH concentrations may explain the phosphaturia that was observed in these animals **(Figure 4.7).**

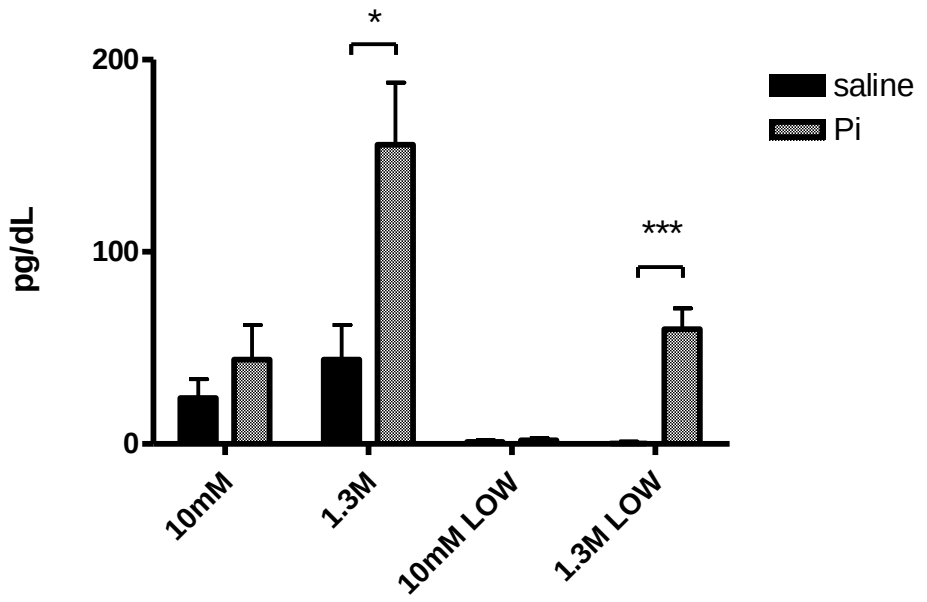


Figure 4.7. Plasma PTH levels in animals 45 min after a Pi or saline bolus into the duodenum PTH assays were performed using the last plasma collection in renal clearance surgeries as described. "LOW" denotes animals maintained for 7 days on a low Pi diet prior to clearance surgeries.

4.4. Discussion

Our knowledge of Pi homeostasis remains vague with regards to the initial events that occur after the intake of food and the subsequent Pi transport processes that take place in the GI tract. In 2007, Berndt and colleagues proposed a novel mechanism in which Pi was excreted rapidly (less than 20 min) following an acute duodenal Pi load. This striking phosphaturia was independent of all known regulators of Pi [139, 140]. Furthermore, when duodenal mucosa from animals that received a 1.3M Pi duodenal load was homogenized and infused into the blood circulation of other rats, phosphaturia also occurred – suggesting the secretion of an uncharacterised factor from the mucosa of upper small intestine [139, 140]. It has been theorized that this factor may be a yet undiscovered phosphatonin (see section 1.3), or one that has not been implemented in this proposed gastro-renal signalling, such as MEPE.

There has been an increasing interest in the role of phosphatonins in regulating extracellular Pi as they have the potential to act more acutely on NaPis than known Pi regulators. The rapid phosphaturic response to an intestinal Pi load shown by Berndt et al (2007) [139, 140] seemed to point to an unknown phosphatonin-like factor that induced a rapid Pi excretion without affecting the levels of known Pi regulators. Crucially, the phosphaturia occurred without a change in plasma Pi concentration. Berndt et al utilized intricate *in vivo* surgical techniques and present experiments were conducted as similar as possible in order to examine urine and plasma Pi concentration and expression of NaPi-IIa and NaPi-IIc protein following an acute Pi load. Both a Pi

solution of a physiological concentration (10mM) was used as well as the 1.3M Pi buffer utilized by Berndt et al [140].

One of the main concerns that led to the decision to reproduce these experiments involved the subsequent correction of Pi concentration utilized by Berndt et al which stated that an extremely concentrated 1.3M Pi solution was instilled into the duodenum rather than the originally stated 1.3mM Pi concentration [139, 140]. With this 1000x more concentrated solution used in their experiments, other factors such as the effects of increased osmolarity of instillate and damage to the apical membrane of the small intestine come into question and may potentially render previous results unphysiological. Indeed, an observation noted during the present experiments was that intestinal morphology seemed markedly disturbed with an increase in mucous content in both 1.35M saline and 1.3M Pi compared to experiments utilizing isotonic solutions **(Figure 4.8).** This phenotype was very drastic and immediately noticeable upon opening of the peritoneal cavity. An increase in the production of mucous is usually viewed as a protective mechanism to prevent damage to epithelium [252] and the manner in which this affects Pi transport is unknown.

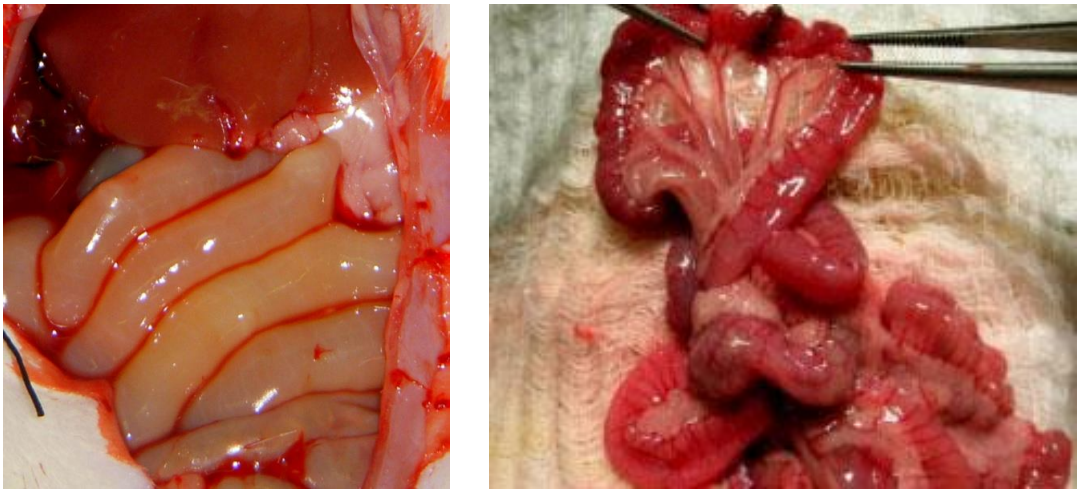


Figure 4.8. Rat GI tract following duodenal instillation of 1.3M Pi (left)
An increase in mucous production is clearly visible as compared to a segment of small intestine instilled with isotonic saline (right - image from [253])

According to the results from this study, there was only one time point in which a significant increase in urinary Pi excretion was seen and this was at 45 min after duodenal instillation of 1.3M Pi in animals maintained on a normal diet. The fact that this increase in excretion was coupled with an increase in plasma Pi concentration leads to the conclusion that other factors are driving this phosphaturia such as the correlated increase in PTH. The increase in urinary excretion also takes place at a later time point following duodenal Pi instillation than the phosphaturia observed by Berndt et al (phosphaturia occurred within 20 min) [139]. The variability of urinary Pi excretion in animals on a regular diet was also much greater than the group primed on a chronic low Pi diet - whether this has to do with the variability of existing intestinal luminal Pi concentrations immediately prior to surgery is unknown. Present data represented surgeries performed around the same time each day, thus future experiments may address this issue by fasting animals briefly (4-5 hours) to normalize intestinal food content.

One of the responses to a high plasma Pi concentration is a rapid increase in plasma PTH levels (refer to section 1.8), which is shown in the present data. There does not seem to be a significant change in plasma PTH in rats which received a physiological concentration of duodenal Pi (10mM) maintained on both low Pi and regular diets. However, 45 min after duodenal instillation of a 1.3M Pi solution, there is a marked increase in serum PTH in animals in both diet groups **(Figure 4.7).** This increase in PTH may be the cause of the few cases of phosphaturia observed in regular diet-fed animals at 45 min following Pi bolus. Moreover, this result is in direct contrast with the data from Berndt et al [139], which showed no increases in plasma Pi or PTH. Occurrence of phosphaturia in animals receiving a 1.3M Pi duodenal load can therefore be attributed to these changes in plasma Pi. The animal group receiving a 1.35M saline duodenal load showed no significant increases in plasma PTH, thus the significant increase in urinary Pi excretion seen in this group was not a result of PTH. The instance of significant increase in urinary Pi excretion 30 min following duodenal load of concentrated saline may thus be attributed to damage to intestinal structure and function.

Interestingly, baseline FEPi of all animals used in present experiments was very low, with values all under 0.15%; the range of published FEPi values in Sprague Dawley rats vary from 2 - 28% [139, 254, 255]. The low FEPi could be directly attributed to observed low urinary Pi excretion; urinary Pi concentrations were confirmed by utilizing more than one established Pi assay (Quantichrome DIPI-500 and [256]). The low FEPi values was the reason behind expressing Pi excretion data as an absolute urinary Pi

excretion value concurrently, as this method focused on absolute amounts of Pi rather than a percentage of Pi excreted. Previous publications which have studied Pi handling in rats have also used absolute urinary Pi excretion to communicate their data [90, 91]. Whether this low FEPi value is a trait of all Sprague Dawley rats from Charles River should be investigated as this may not represent physiological renal Pi handling. It is important to note that plasma Pi levels of all animals used in the present study were within normal range, thus Pi homeostasis was not dysregulated.

Utilizing BBM prepared from kidneys of Pi infused rats, it was apparent that a duodenal Pi load and the potential phosphaturic factor proposed by Berndt et al does not downregulate the expression of NaPi-IIa or NaPi-IIc at the apical membrane of the kidney. The aforementioned variability of Pi excretion, in particular a few animals which were given a 1.3M Pi duodenal load and exhibited phosphaturia, do in fact correspond to a lower expression of NaPi-IIc **(Figure 4.4b).** As mentioned in section 1.2.1.5, NaPi-IIc does not play a large role in kidney handling of Pi; however, in response to a very high Pi load the mechanisms of Pi regulation may be altered.

A recent study re-evaluating the established gastro-renal natiuretic signalling axis of guanylin, revealed that crosstalk between these two major organs may involve complex tangential pathways, other organs, and humoral factors [257]. Similarly, in the context of Pi regulation, it appears that the intestine and kidney interplay to maintain Pi homeostasis by mechanisms which are still unknown. Studies in man show that a Pi load presented to the jejunum increases related Pi regulatory factors such as PTH

without affecting other factors thought to be upstream of this sensing pathway (i.e. calcium levels) [258]. Other studies in CKD patients have shown that in contrast to the well documented association between FGF-23 and dietary Pi intake [142], acute non-dietary modulation of serum Pi levels (e.g. IV) within the normal range does not induce changes in FGF-23 [259]. Therefore, it appears that intestinal Pi load and/or sensing may have the capability of regulating FGF-23 and PTH, which in turn would directly affect Pi reabsorption by the kidney.

Patients with CKD are encouraged to reduce their dietary Pi load and there have been many subsequent reports of the impact, or lack thereof, of dietary intervention. In rats, a decrease in dietary Pi coupled with Pi binders significantly reduced aortic calcification and kidney damage [164]. On the contrary, other comprehensive patient studies provide evidence that reduction of dietary Pi load does not reduce the probably of developing cardiovascular conditions and has no effect on mortality and the future development of renal damage [260]. What is known is that dietary Pi restriction and Pi binders both have poor patient compliance and more effective methods of Pi control are needed to manage hyperphosphatemia in CKD.

4.4.1. Summary and conclusions

The present study investigated kidney Pi clearance in rats and showed that there is no increase in urinary Pi excretion up to 45 min following duodenal instillation of a 10mM or 1.3M Pi solution. Duodenal instillation of these Pi concentrations also did not significantly affect the overall expression of NaPi-IIa or NaPi-IIc proteins at the renal

BBM, however, a correlation was seen between reduced expression of these renal Pi transporters and phosphaturia presented in a few, specific animals given a supraphysiological (1.3M) Pi duodenal bolus. These decreases in NaPi protein expression could be, in part, due to increases in plasma Pi and PTH concentrations seen in animals which received a 1.3M duodenal Pi load.

5.0. Pi uptake by the liver

5.1. Introduction

Pi signalling between organs is potentially important for supplementing the classical hormonal responses in the maintenance of Pi homeostasis. Although the data presented in Chapter 4 suggests does not support the hypothesis of acute regulation of Pi between the intestine and the kidney, other organs may be involved in overall Pi balance. The notion to investigate the role of the liver role in Pi handling was prompted by observations of low serum Pi following hepatectomy and in patients with liver diseases. The initial reasoning for the occurrence of hypophosphatemia immediately following hepatectomy was the fact that the regenerating liver would require extra metabolic needs and, subsequently, more Pi from circulating blood [149]. However, the magnitude of the observed hypophosphatemia could not be accounted for solely by the demands of extra Pi being utilized for liver regeneration, thus other potential mechanisms were investigated to explain this clinical observation. Nafidi et al as well as Salem et al revealed an early post-operative increase in fractional excretion of Pi post-hepatectomy, which was independent of plasma pH changes, and levels of FGF23, FGF7, sFRP-4 and PTH (after day one) [150, 151, 261]. The mechanisms behind this phosphaturic response have still not been uncovered.

Certain liver processes are closely related to body Pi handling and regulation. The liver produces cathepsin B, a protease that cleaves a more recently characterized

phosphatonin MEPE into ASARM, another biologically-active phosphaturic factor; the basis for liver secretion of this enzyme and the method of its regulation are currently unknown [152]. Vitamin D, a main factor in Pi regulation, also has strong ties to the liver - one of the first steps of conversion of 7-dehydrocholesterol (cholecalciferol) into the active form of vitamin D occurs in the liver through the production of cholecalciferal 25-hydroxlyase in its microsomes [146].

In instances of CKD, parallels are also seen between alterations in receptor expression that happen within the liver and the kidney. It has been shown that the PTH receptor in the liver is downregulated in a similar fashion to the renal PTH receptor amongst a surgically-induced CKD rat cohort [153], the function of which is again, still unknown. Interestingly, the calcium-sensing receptor (CaSR) has been shown to be expressed in hepatocytes and, when activated by excess levels of calcium, stimulates bile flow [262]. This mechanism was thought to protect the liver against calcium accumulation that could potentially damage hepatocytes. CaSR is vital in the PTH pathway of Pi regulation, in which activated CaSR prompts release of PTH and a subsequent increase in excreted Pi and a decrease in Pi absorption across small intestine (refer to section 1.4.2.2.). These findings further the notion that the liver may be a key player in Pi regulation and may be an intermediary in the previously proposed gastro-renal axis of Pi homeostasis.

Another occurrence which points to the liver as a possible organ involved in Pi signalling and regulation involves the expression of type II and III sodium phosphate

cotransporters (NaPi-IIb and PiT1 and PiT2) in hepatocytes and cholangiocytes of the rat liver. Experiments utilizing brush border vesicles prepared from these cells revealed that the sodium-dependent transport mechanisms and transport kinetics show similarities to transporters expressed in the kidney and small intestine. The possible roles of these transporters in the liver were speculated to involve regulating biliary Pi concentration, as hepatocytes and cholangiocytes release ATP into bile which degrades to adenosine and Pi [13].

A recent finding concerning liver x receptor (LXR) brings another receptor into the proposed function of the liver in the context of Pi handing. Activation of the ubiquitously expressed LXR is known to modulate cholesterol trafficking out of various cell types in different tissue Studies now show that agonists of the β isoform of LXR, which is expressed in greatest abundance in the liver, trigger a decrease in NaPiIIa and NaPiIIc expression in the kidney and subsequently, Pi transport in both the intestine and the kidney [263]. Again, the mechanistic significance of this regulation, and the role of LXR in the greater scheme of Pi homeostasis, is still unclear.

Investigating the liver as a potential player in Pi regulation, in conjunction with the intestine and kidney, seemed to be a natural progression due to the location of the liver in the body and its function as an important metabolic organ. The liver also receives 70% of its venous blood supply from the portal vein, which consists of nutrient rich blood from the small intestine and pancreas [145]. Following this, the liver also metabolizes a substantial variety of compounds that are brought to it by the portal and systemic

circulations (refer to section 1.7), however, the direct interplay between the liver and Pi balance has not been previously investigated *in vivo*.

5.1.1. Aims of this study

1. To characterize Pi transport in the liver as compared with the established Pi transport mechanisms of the intestine; these studies will utilize 4 different concentrations of Pi in the buffer perfusing the small intestine (1, 5, 10, and 15mM) to represent a range of physiological luminal Pi concentrations.

2. To investigate the sodium dependency of Pi transport in the liver by utilizing intestinal luminal buffer with and without sodium. Sodium-free buffers contained choline chloride (ChCl) as a substitute for sodium chloride (NaCl).

5.2. Materials and Methods

5.2.1. Animal model

Male Sprague Dawley (SD) rats between 250g-300g were obtained from Charles River laboratories (UK) and used in accordance with the Animals (Scientific Procedures) Act 1986. Rats were allowed *ad libitum* access to standard rodent chow containing 0.52% phosphate (Diet RM1, SDS Ltd, Witham, Essex UK) and water.

5.2.2. Liver Pi uptake, following *in situ* perfusion of the small intestine using the closed loop technique

Anesthetic administration and femoral artery cannulation was performed as described in Chapter 2.2.3 to acquire blood samples at 10, 20, and 30 min following intestinal Pi instillation for Pi concentration analysis. Plasma was obtained after centrifuging blood samples at 6000rpm for 10 minute at 4^{o}C.

Intestinal loop preparation was performed as mentioned in Chapter 2.2.3. 500μL of ^{32}P radio-labelled (0.37Mbq) Pi solution was added to buffer containing 16mM Na-HEPES, 140mM NaCl, 3.5mM KCl, 1/5/10/15mM KH_2PO_4, (pH 7.4). For experiments involving sodium-free buffer, NaCl was replaced with ChCl with all other concentrations of solutes remaining the same. The HEPES buffer which was instilled into the jejunum was first assessed for its osmolarity using freezing point depression osmometry (micro-osmometer; Model 3M, Advanced Instruments Inc, Massachusetts, US). All solutions used for *in vivo* Pi uptake studies were within isotonic range (290-310 mOsm/L).

5.2.3. Liver removal

After 30 minutes, the last blood collection was performed and the animal was killed via cardiac exsanguination. The whole liver was removed lobe by lobe, weighed, and approximately 100g was dissolved in Solvable (Perkin Elmer, MA, USA) protease solution overnight.

5.2.4. Exsanguination

Experiments involving exsanguinations were performed immediately after blood collection at the last time point of 30 min. An incision was made in the right atrium of the heart and 200mL of an ice-cold heparin/saline solution (1000iu/100mL saline) was manually infused via the femoral artery using a 50mL syringe to thoroughly flush out the blood from systemic organs.

5.2.5. Statistical analysis of radioactive counts

Plasma was obtained after spinning down blood samples at 6000rpm for 10 min at 4^{o}C and dissolved tissue was obtained via the method mentioned in the above section. ^{32}P counts were measured using a Perkin-Elmer tri-carb 2900tr scintillation counter using 4mL of Ultima Gold (Perkin Elmer) scintillant combined with 50uL plasma or 100uL dissolved liver tissue in triplicates. The average was taken and converted to absolute Pi quantity in nmole units using values obtained by initial counts of undiluted ^{32}P instillate solution. Data is presented as means ± SEM and shows plasma Pi values at 30 min. A student's paired *t*-test or an ANOVA, as appropriate, was used and group differences

were stated as statistically significant if $P < 0.05$; n represents the sample size of each experiment.

5.2.6. Calculations of Pi transport

Calculations of Pi transport were performed by taking the ^{32}P counts as mentioned in the section above and converting this to nmole Pi quantity and normalizing this value to 1g of jejunal tissue per 1mL plasma collected.

5.3. Results

5.3.1. Proof of principle - intestinal Pi transport as measured by appearance of ^{32}P in systemic blood

For closed-loop surgeries looking at Pi transport in the liver, it was important to check that the animal was physiologically stable whilst anesthetized; this was determined by observing if there was constant Pi transport from the jejunum to systemic blood over time. Blood was taken at 10, 20, and 30 min via the femoral artery and was counted for ^{32}P. Significant increases were seen in Pi transport at 30 min between all concentrations of Pi instilled into the jejunum **(Figure 5.1a)** as well a steady amount of Pi transport over time during all experiments **(Figure 5.1b).** P values Pi transport in the jejenum: 1 vs 5mM Pi = 2.80 x 10^{-5}; 5 vs 10mM Pi = 1.65 x 10-4; 10 vs 15mM Pi = 0.010. n values for systemic blood: 1mM = 5; 5mM = 6; 10mM = 6; 15mM = 6.

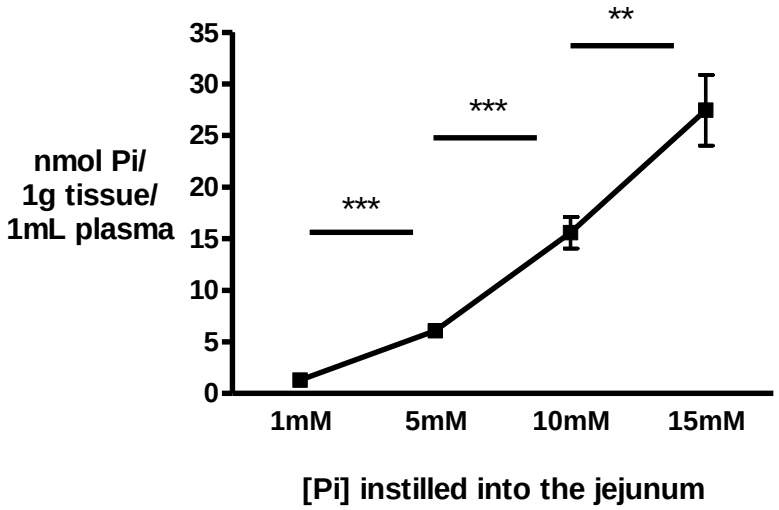


Figure 5.1a. Pi transferred by the jejunum as measured by appearance of Pi in plasma, a comparison between four Pi concentrations instilled into the jejunum
1, 5, 10, and 15mM Pi diluted in HEPES buffer was used as the uptake solution. Results represent Pi contained in plasma 30 min post-jejunal Pi load. Significant changes are seen between each increase in Pi buffer concentration. Data is presented as mean ± SEM with n=5-7.

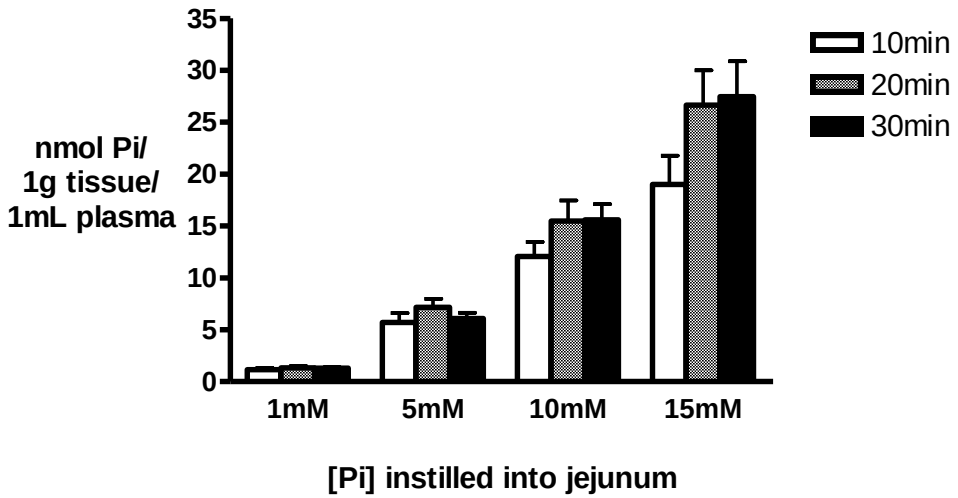


Figure 5.1b. Pi transferred by the jejunum as measured by appearance of Pi in plasma over time
Three samples of plasma were collected during the course of the experiment, at 10, 20, and 30 min post-jejunal Pi load. There is a steady and maintained level of Pi in the blood over time with no significant decreases by 30 min, which validates the animal model used.

5.3.2. Pi transferred into the liver following jejunal Pi load *in situ*

Pi uptake by the liver was investigated using an *in situ* jejunal closed-loop technique utilizing 500 μL of 1, 5, 10, and 15mM radiolabelled Pi solution containing sodium. Results showed a steady and significant increase of Pi uptake at increasing Pi concentration and an insignificant change between 10mM and the highest concentration of 15mM, perhaps signifying that the organ has reached Pi saturation **(Figure 5.3)**. P values liver: 1 vs 5mM = 7.94 x 10^{-6}; 5 vs 10mM = 0.0181; 10 vs 15mM = 0.549. n values for liver: 1mM = 5; 5mM = 4; 10mM = 5; 15mM = 7.

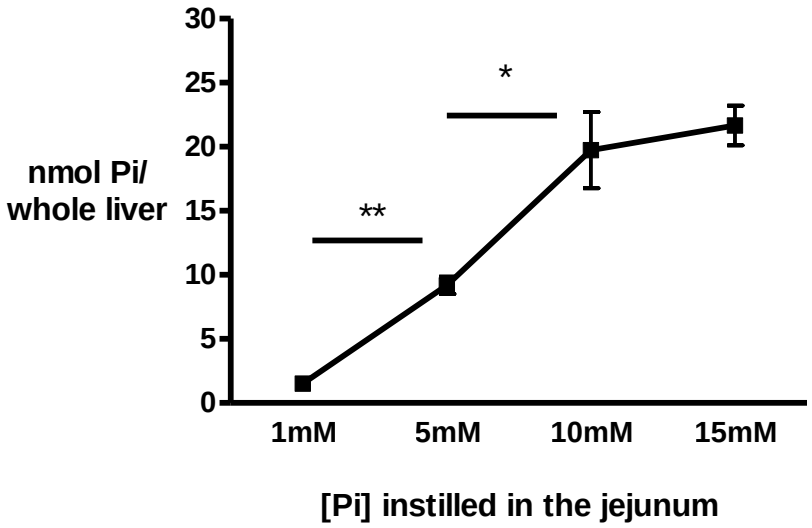


Figure 5.2. Pi transferred into the liver, a comparison between four Pi concentrations instilled into the jejunum

Significant increases in the amount of Pi taken up by the liver were seen between 1 and 5mM as well as between 5 and 10mM. There is a noticeable plateau between 10 and 15mM Pi which may signify saturation of the transport process. Data is presented as mean ± SEM with n=4-7.

5.3.3. Sodium-dependent Pi uptake into the liver following Pi load into the jejunum *in situ*

Sodium-dependent uptake by the liver was investigated by instilling radiolabelled Pi solution with or without sodium directly into the jejunum utilizing the *in situ* closed-loop technique described previously in Chapter 2.2.3. Results indicate significant sodium dependent Pi transport by the jejunum (as measured by Pi appearance in blood) at all concentrations of Pi instilled into the jejunum **(Figure 5.4a).** However, sodium dependency was not seen in the liver apart from at 15mM Pi jejunal instillation **(Figure 5.4b).** Results compared animals that received Pi into the jejunum in the presence of sodium (Na) with animals that received Pi into the jejunum in a choline chloride-based buffer (ChCl). P values were: 1mM = 0.427, 5mM = 0.0175, 10mM = 0.103, 15mM = 0.028). n values for animals receiving Pi in presence of Na were same as in figure 5.1. n values for Pi uptake in the presence of ChCl: 1mM = 5, 5mM = 6, 10mM = 6, 15mM = 7. P values of Pi uptake by the liver in presence of Na vs ChCl animals at 30min were: 1mM – 0.658, 5mM – 0.838, 10mM – 0.759, 15mM – 0.0156). n values for Pi uptake by the liver in the presence of Na were the same as in figure 5.2. n values for Pi uptake by the liver in the presence of ChCl: 1mM = 5, 5mM = 4, 10mM = 5, 15mM = 7.

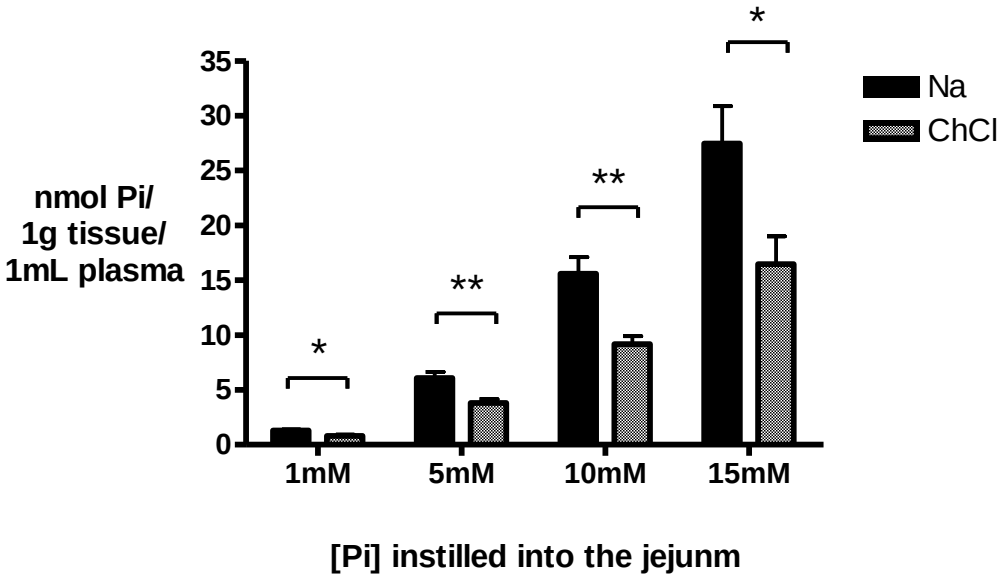


Figure 5.3a. Sodium-dependent Pi transport by the jejunum following Pi load, as measured by appearance of Pi in plasma

Shaded bars represent Pi uptake in the presence of sodium (Na) while checkered bars represent Pi uptake with a sodium-free buffer (ChCl). Data is presented as mean ± SEM with n = 5-7.

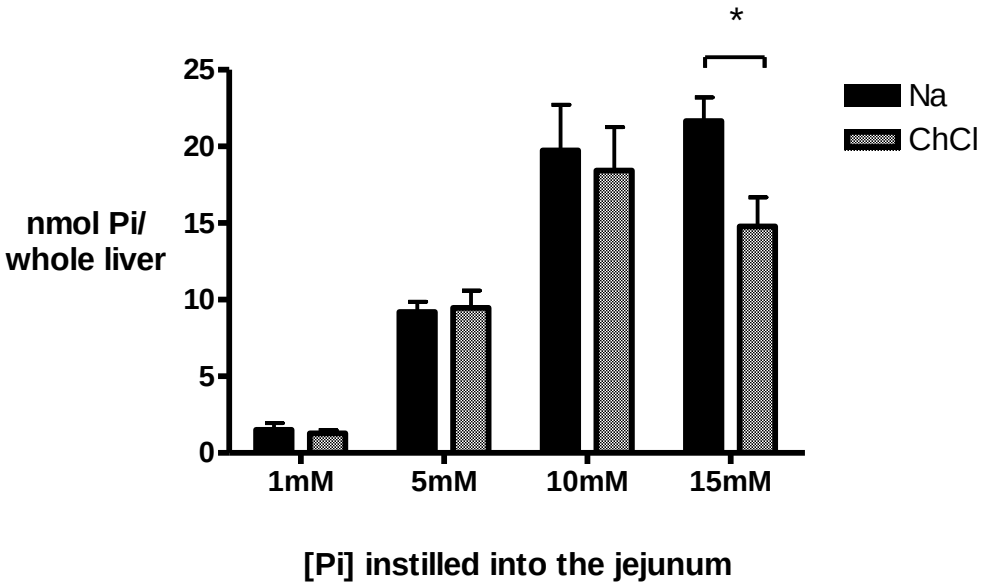


Figure 5.3b. Sodium-dependent Pi uptake by the liver following a jejunal Pi load

Shaded bars represent Pi uptake in the presence of sodium (Na) while checkered bars represent Pi uptake with a sodium-free buffer (ChCl). Data is presented as mean ± SEM with n = 5-7.

5.3.4. Pi transferred into the liver following exsanguinations

Exsanguinations were performed to confirm that measurements of Pi uptake represented the Pi contained in liver hepatocytes and not the Pi contained in the blood present in the liver. Results showed no significant differences in Pi amounts contained in liver obtained without exsanguinations and those with exsanguinations **(Figure 5.5).** n values for regular liver collection: 1mM = 5; 15mM = 7; exsanguinations: 1mM = 5; 15mM = 5.

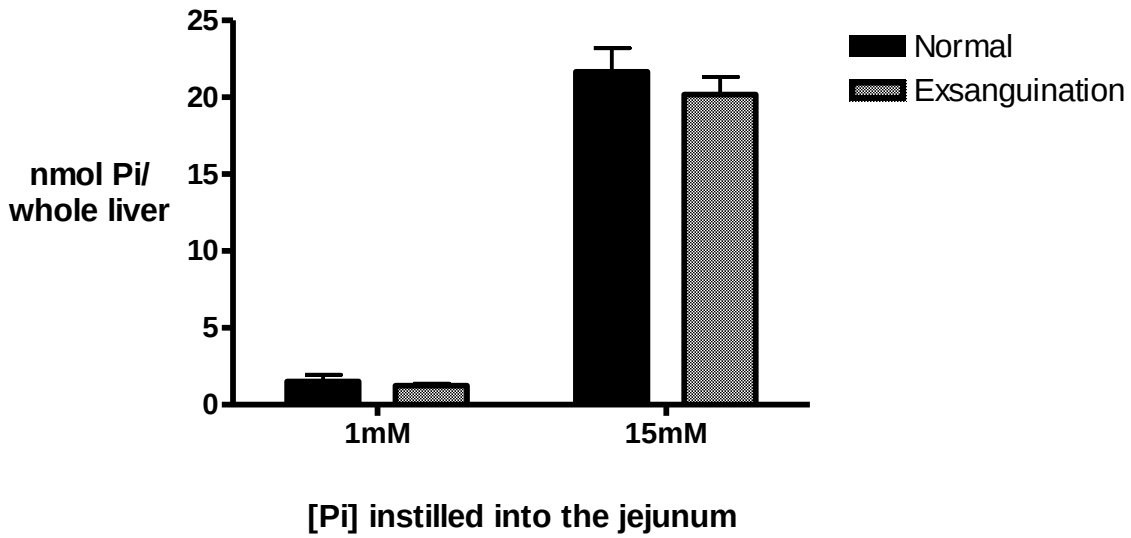


Figure 5.4. Amount of Pi taken up by the liver in animals following exsanguination compared to standard liver extraction following sacrifice
Liver taken from animals immediately after surgery are grouped as "normal". There are no significant differences in Pi amount contained in the liver between these two groups.

5.3.5. Pi in hepatic portal vein blood vs. systemic blood

Pi was measured in plasma collected from the hepatic portal vein (HPV) in animals instilled with 10mM Pi solution and compared with the Pi levels in systemic plasma to assess whether there were any significant differences between Pi concentrations in these two points of the circulatory system. Animals instilled with Pi in NaCl or ChCl buffer were also compared to examine if there were different sodium-dependent mechanisms displayed at different locations of plasma collection. Results showed that Pi contained in portal blood and systemic blood collected from the femoral artery was comparable with no significant differences **(Figure 5.6).** P values: systemic vs HPV +Na = 0.915; systemic vs HPV ChCl = 0.457; n values: systemic plasma = 6; HPV = 4.

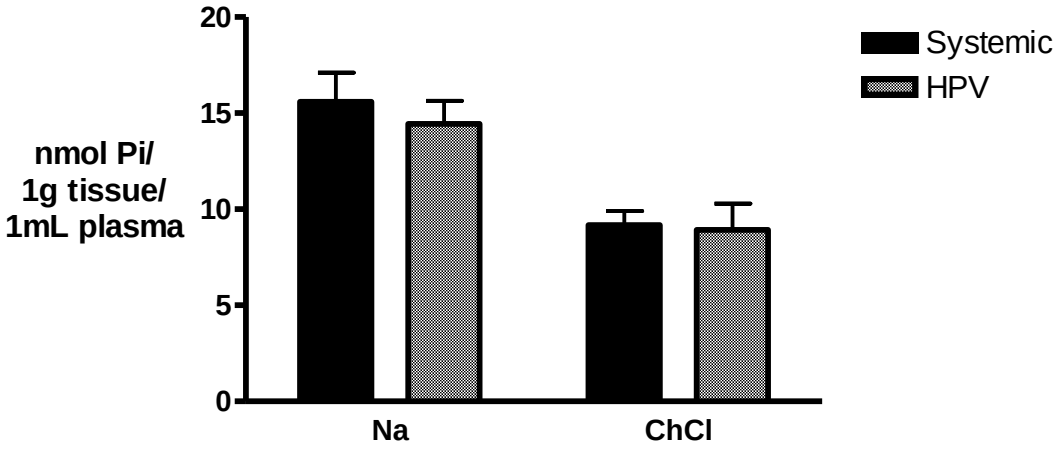


Figure 5.5. Pi content of plasma collected from the hepatic portal vein (HPV) compared to blood collected from the femoral artery following 10mM Pi instillation into the jejunum
Pi measured in systemic plasma is represented by a solid bar, and Pi in HPV plasma shown by a checkered bar. No significant differences are seen between plasma Pi from systemic and HPV collection sites in both groups of animals (Pi instilled in presence of NaCl or ChCl). Data is presented as mean ± SEM.

5.4. Discussion

The idea that the liver may potentially be involved in Pi homeostasis was speculated following observations of Pi imbalances seen after certain liver procedures. This idea related back to observations of acute hypophosphatemia in patients following hepatectomy [149]. This phenomenon was eventually attributed to increased phosphaturia which occurred independently of the major known mediators of Pi homeostasis (PTH and phosphatonins) [150]. The underlying mechanism and cause for hepatic-related phosphaturia remain unidentified.

In 2004, both type II and III sodium phosphate transporters were found to be expressed in the liver and their roles in this organ revolved around regulating bile Pi concentrations [13]. The function of the liver in secreting phosphatonin-specific proteases such as cathespin B, also suggested that the liver may have a role in Pi homeostasis. Combined, this knowledge about the function of the liver made it an appealing organ to focus on in the present Pi transport experiments.

The results from liver Pi uptake experiments utilizing the *in situ* intestinal closed-loop technique conclude that the liver accumulates more Pi when higher concentrations of Pi is instilled into the jejunum. This pattern is equivalent to Pi transport mechanisms seen in the small intestine as measured in systemic plasma. The trend for apparent saturation of Pi uptake when 15mM Pi is instilled into the jejunum is interesting, since it represents a Pi concentration saturation range for the liver. The range of intestinal

luminal Pi concentration in man has been shown to be approximately 0.7-12mM depending on the timing and composition of food [119]. Previous work in our group has shown that intestinal luminal Pi concentration in Sprague Dawley rats ranged from 7-11.5mM [200], thus 15mM may be just over this physiological spectrum. Data from these experiments suggest that there is a Pi secretory function for the liver at supraphysiological Pi concentrations which may protect the rest of the body from excess Pi by secreting it back into the bile to be recirculated into the small intestine. It would be interesting to see whether this seemingly saturable Pi uptake would maintain its pattern at Pi concentrations even higher than 15mM. However, higher concentrations of Pi were not used since these levels of Pi are not physiologically relevant.

Sodium dependent Pi uptake of the liver was also investigated by substituting ChCl for NaCl in the Pi buffer instilled into the jejunum. Results show a differing pattern in sodium dependency between the small intestine and the liver. Sodium-dependent Pi transport was evident at all concentrations of Pi instilled into the jejunum. In the liver, however, sodium dependency was only seen at 15mM Pi. Again, at this concentration, which is on the cusp of being a high luminal Pi load, different mechanisms of Pi uptake seem to be triggered. This may suggest that the sodium-phosphate cotransporters expressed in the liver may only be responsible for a small role in Pi transport at physiological levels and/or could be a part of a greater network of Pi handling that is still yet to be discovered.

The total amount of Pi taken up by the liver was similar to the amount transported by the jejunum, with less than 0.5% of Pi taken up from the starting buffer. This may signify that under normal physiological conditions, the role of the liver may not weigh heavily on maintaining overall Pi homeostasis. It is possible however, that when Pi regulation is dysregulated under the conditions of chronic kidney disease, the liver may play a more important part in Pi buffering.

One observation taken from the results of HPV compared with systemic plasma Pi concentrations was the phenomenon of a seemingly lack of concentrated Pi being actively transported by the jejunum. In theory, following an acute Pi load in the intestine, the excess Pi in the intestinal lumen would trigger Pi transport and the Pi amount in HPV plasma (plasma made up, in part, from the small intestine) would be greater than the amount in the circulation. However, at 30 min, when these samples were taken, no significant changes were seen between HPV and systemic plasma. One possible reason for this observation could be that at 30 min, Pi levels have normalized throughout the circulatory system and the initial predicted high levels of Pi concentration in the HPV would be at a level equal to Pi concentration in the rest of the circulatory system. An experiment that could perhaps address this suggested explanation would be to cannulate the hepatic portal vein and take blood samples at earlier time points to see if there is an initial increase in Pi transport, proving the principle of active transport. Furthermore, experiments involving exsanguinations also confirmed that the Pi measured was contained in hepatic cells and not in the blood of the liver. These

experiments were necessary to conclude that the liver cells themselves were taking up Pi.

In addition to the experiments presented in this chapter, future studies should include the investigation of bile composition. Bile is an intricate secretory product produced by the liver and serves two principal functions: 1) eliminate many endogenous and exogenous waste products from the body, such as bilirubin and cholesterol, and 2) promote the digestion and absorption of lipids from the intestine. The composition of bile is modified significantly as a result of the absorptive and secretory properties of epithelial cells that line the intrahepatic and extrahepatic bile ducts. Knowing that PiT-1 and PiT-2 are expressed in bile duct cholangiocytes is a starting point in investigating the involvement of bile as a vehicle of Pi excretion. Further studies could involve cannulating the bile duct and observing whether changes in bile Pi concentration occur following jejunal instillation of supraphysiological levels of Pi and whether inhibition of the process of Pi transport into bile leads to a dysregulation of body Pi homeostasis. Protein expression of PiT-1 and PiT-2 could also be investigated to reveal Pi uptake or secretion mechanisms along the bile duct.

In 1980, a potential “portal factor” responsible for regulating calcium homeostasis was suggested [264] . Using experiments similar to those mentioned in this chapter, the group measured plasma calcium levels in portacaval shunted Lewis rats following parathyroidectomy. The results showed that there was correction of hypocalcaemia in shunted mice that was independent of PTH, and the data suggested a substance

originating in the portal region that bypassed modifications in the liver and was able to correct the hypocalcemia following parathyroidectomy [264]. The portal factor regulating whole body calcium balance could be similar to the unknown factor proposed for Pi regulation, since hepatectomy could alter the modification abilities of the liver.

5.4.1. Summary and conclusions

The liver absorbs more Pi with increasing levels of Pi instilled into the jejunum (1, 5, 10, and 15mM) and the uptake process reaches a saturation point at 15mM since no significant increase is seen between the amount transported at 10mM. The liver also transports Pi in a mostly sodium-independent manner *in vivo* up to supraphysiological concentrations (15mM) where saturation occurs. The mechanisms involved in this physiological response, however, are unknown but the excess Pi may be released into the bile for secretion into the intestine. In this way, the liver may serve as a Pi sensor and act as a protective mechanism and act in concert with the phosphaturic action of PTH to minimize increases in plasma Pi concentration.

6.0. General discussion and future experiments

6.1 Brief background to intestinal, renal, and hepatic Pi handling

To date, there have been numerous studies focusing on the mechanisms of renal and intestinal Pi transport, while also elucidating the specific impact of sodium-dependent phosphate cotransporters (NaPis and PiTs) in these tissues on overall Pi homeostasis. It is slowly becoming evident that there is complex interplay between the small intestine and kidney and that the liver may also play a role in these signalling pathways. The proposed cross-talk between the intestine, kidney, and liver needs to be characterized in order to offer more targeted treatments of diseases of Pi imbalance such as hypo- and hyperphosphatemia, specifically in the context of chronic kidney disease (CKD).

It has been previously established that the kidney plays a major role in maintaining Pi homeostasis via the sodium phosphate cotransporter, NaPi-IIa, at the proximal tubules. The kidneys filter Pi in the blood and about 80% of this load is then reabsorbed mainly by NaPi-IIa at the proximal tubule **(Figure 1.1).** However, in specific subgroups of patients with CKD and subsequent hyperphosphatemia, impairment of renal function rules out the kidney as a target to treat Pi overload. Much focus has since been placed in investigating the mechanisms of intestinal Pi transport and its potential as a main option for therapeutic intervention. Studies have attempted to tease out the overall involvement of intestinal NaPi-IIb in postprandial Pi absorption and ascertain the relative

importance of paracellular (passive) and transporter-mediated (active) transport to overall Pi transport [12, 131, 132].

During the investigation of intestinal Pi transport, Berndt et al [139] discovered that introducing a high concentration of Pi into the upper small intestinal lumen produced a rapid increase in urinary Pi excretion, which was not due to known regulators of serum Pi levels such as PTH and FGF-23 [139]. It was concluded that a novel signalling factor originated from the duodenum, as mucosal extracts from Pi treated animals infused into other animals also elicited this phosphaturic response. More recently, a group in Japan found a liver-derived factor that affected renal reabsoprtion of Pi in rat models of CKD as well as in proximal tubule cell lines [154]. These latest findings point to an intricate Pi regulatory system which may involve multiple organs and different pathways.

A primary aim of the experiments reported in this thesis was to characterize intestinal Pi transport by directly comparing widely-used *in vitro and in situ* techniques in five different regions of the intestinal tract (duodenum, jejunum, ileum, proximal colon, and distal colon). These experiments utilized the *in vitro* everted sleeve and the *in situ* intestinal closed-loop techniques and served to clarify discrepancies seen in published results regarding intestinal Pi transport. Critical analysis of which specific technique would be more appropriate for investigating Pi transport in different regions of the intestine was also conducted based on how Pi uptake is measured using *in vitro* and *in situ* methods.

The next goal was to establish an *ex vivo* intestinal perfusion technique to investigate intestinal Pi transport. Data obtained from this method offers advantages in understanding how Pi is transported directly across the intestinal epithelium since serosal fluid is collected and analyzed from perfused intestine. The composition of this serosal fluid can be interpreted to directly represent the Pi passing through the intestine whereas previously discussed *in vitro* and *in situ* methods measure Pi taken up at the BBM and Pi appearance in blood circulation respectively. The *ex* vivo technique has already been used to investigate the absorption of flavonoids and hydroxycinnamates, olive oil polyphenols, clinical drugs, and glucose [215-219]. Present studies investigated the sodium-dependent component of Pi transport utilizing this technique by perfusing the intestinal segment with a sodium-free perfusate.

One of the main issues to address during this project was the hypothesized gastro-renal Pi signalling axis. The objective was to directly replicate and further the renal clearance studies by Berndt et al [139, 140] using a physiological Pi concentration instilled into the duodenum (10mM). The reported phosphaturia by Berndt et al followed a 1.3M Pi duodenal load [140] – this concentration of Pi was also utilized in the renal clearance experiments contained in Chapter 4. Renal NaPi-IIa and NaPi-IIc protein levels were also quantified by preparing BBM vesicles from the kidneys of animals receiving a Pi or saline duodenal bolus and probing with antibodies against these transporters. It has previously been shown that acute adaptations to an intestinal Pi load, in the form of altered expression of NaPi transporters, are more pronounced after priming animals on a chronic low Pi diet [62]. Thus another set of animals were also maintained on a low Pi

diet before clearance surgeries to prime renal NaPi-IIa and NaPi-IIc expression; the low Pi diet group would potentially exhibit quantifiable changes in protein expression following intestinal Pi load.

Lastly, the role of the liver in overall Pi handing and homeostasis was investigated using the aforementioned *in situ* closed-loop technique with ^{32}P labelled Pi buffer using four different Pi concentrations (1, 5, 10, and 15mM). The liver was subsequently removed and ^{32}P counts were measured; from this data, the total Pi transferred into the liver was calculated and compared with known Pi transport mechanisms of the jejunum.

6.2 Assessment of regional differences in intestinal Pi transport using *in vitro* and *in situ* techniques

A large range of methods is relevant to the study of solute transport across the intestinal epithelium; however comprehensive comparisons between these techniques have not been previously conducted. The experiments in Chapter 2 aimed to reveal the differences between Pi transport data obtained by two broadly used methods, the *in vitro* everted sleeve technique and the *in situ* intestinal closed-loop technique. It is important to emphasize that the *in vitro* everted sleeve technique produces data that represents Pi uptake at the intestinal BBM and the *in situ* intestinal closed-loop technique measures transepithelial Pi transport by appearance of Pi in the blood.

In these studies, three different concentrations of Pi (0.1, 1, and 10mM) were used to examine Pi transport in the intestine since postprandial intestinal luminal Pi concentrations are within this range [119]. Data showed that increasing the Pi concentration up to 10mM caused a corresponding rise in total Pi absorption in both *in vitro* and *in situ* data using both techniques. The jejunum is also the area with the highest magnitude of Pi transport, which corresponds appropriately with previously published data of high levels of intestinal phosphate transporter NaPi-IIb mRNA and protein expression in this region of rat intestine [17, 62].

In terms of overall Pi transport ability, *in situ* data from the ileum show higher a magnitude of Pi transport than previously published data. Although the ileum does not

transport as much Pi quantitatively as the proximal small intestine (duodenum and jejunum), it still exhibits substantial Pi transport compared to the colon. In contrast, the ileum transports the least amount of Pi of all the intestinal regions *in vitro*. These discrepancies may be related to known variations in intestinal content transport time. Chyme remains in the rat ileum for the longest period of time (~2-3 hours) [202], thus a 2 min incubation time with ^{32}P may not have accurately represented physiological Pi transport ability in this region.

Using the *in vitro* everted sleeve technique, the distal colon unexpectedly transported near equivalent amounts of Pi as the small intestine, also with significant sodium dependency. To date there have been numerous clinical observations of hyperphosphatemia following Pi-containing enemas, first described in 1977 [203], and current results may explain these clinical occurrences. On the other hand, the *in situ* distal colon data shows that this mechanism of Pi transport is sodium-independent at all Pi concentrations used, thus following *in situ* manipulation, the distal colon epithelium may simply be more permeable to solutes. In either instance, the amount of unbound Pi in the large intestine is thought to be negligible, hence the Pi transport data from both of these techniques may not actually occur under physiological circumstances. Although small amounts of Pi is available for binding at this point of the GI tract, there may be a mechanistic importance of Pi transport in the distal colon. Short chain fatty acids, which are in abundance in the colon, displace Pi from anion exchanger sites in distal colon [204], thus making small quantities of Pi available for absorption. Furthermore, the effectiveness of current treatments of hyperphosphatemia with oral Pi

binders may be affected if displaced Pi becomes available at this part of the GI tract. Although it has been reported that mRNA of both NaPi-IIb and PiT1 [265] are present in the distal colon whether Pi transport in this region has any significance to overall Pi homeostasis is still yet to be investigated. Interestingly, Capuano et al [265] detected mRNA of NaPi-IIb and PiT1 in the distal colon and both appeared to be regulated by a low Pi diet in a similar fashion to the known PiT1 expression in the small intestine [62].

Discrepancies are seen in sodium-dependent Pi transport between data obtained from *in vitro* everted sleeve and *in situ* closed-loop techniques. Apart from the jejunum and proximal colon, which exhibits sodium dependency and no sodium dependency respectively across all concentrations utilized, all other segments displayed varying results. *In vitro* data showing sodium dependent Pi transport in the duodenum might have been due to the pH of solution, representing a greater amount of NaPi-IIb transport activity than PiT1 as NaPi-IIb activity is maximized at a neutral to basic pH [31]. PiT transporters are also not solely dependent on sodium to drive Pi transport as lithium has been shown to be able to substitute for sodium and transport Pi at a lower capacity [61]. Both NaPi-IIb and PiT1 proteins, however, are not highly expressed in the duodenum [17], thus data contained in Chapter 2 may point to an unidentified transporter. Known variations in intraluminal pH along the intestinal tract might also explain the differences in data obtained from the two techniques in different regions of the intestine. The proximal small intestine starts off relatively acidic (pH of 5.5-6) and increases to an almost neutral state in the distal ileum [205]. All of the solutions used in these experiments were adjusted to a physiological pH of 7.4, thus transporters which

may function at a higher capacity in an acidic pH environment may not have been operating (e.g. PiT1) [61].

Data from *in situ* experiments show no sodium dependency of Pi transport in the duodenum. This distinct contrast to data from *in vitro* studies which showed sodium-dependency at all concentrations of Pi in the duodenum may be explained by regional differences in intestinal luminal transit times; ingested food remains in the duodenum for minutes compared to hours in the jejunum/ileum [202]. In this regard, the *in vitro* everted sleeve technique may be a representative model for duodenal Pi transport, since Pi uptake was measured for a 2 min period in this intestinal segment.

The *in vitro* everted intestinal tissue is constantly exposured to stirred Pi buffer ensuring a consistant Pi concentration close to the BBM, whilst the Pi buffer utilized in the *in situ* technique is relatively stagnant within the closed-loop. The differences in Pi transport magnitude between the two techniques (higher amount of Pi transported utilizing the *in vitro* technique) may be explained by this difference in Pi measurement. It is also important to recognize that the Pi exit pathway through the basolateral membrane is still unknown.

Importantly, EM analysis on intestinal tissue segments used in both *in vitro* and *in situ* techniques show that the intestinal ultrastructure is not compromised and differences in Pi transport does not represent increased passive permeability across a damaged epithelium. EM images revealed the tight junctions and intestinal villi to be intact

following manual handling using both methods and all segments of intestine utilized. Differences in magnitude of Pi transport and sodium dependency using both methods are thus attributed to factors other than structural explanations.

6.3. Pi transport across the rat intestinal epithelium

In Chapter 3, a novel application of the *ex vivo* intestinal perfusion technique to measure transport of Pi across the intestinal epithelium is demonstrated. Here, data obtained from the serosal fluid of these experiments show for the first time that there is an increased Pi concentration directly absorbed across the intestinal epithelium compared to that in the perfusate. These results confirm that active transport of Pi is taking place in the small intestine.

Initial experiments using buffer containing 1.2mM Pi show an approximately 2-3x concentrated level of Pi in the serosal fluid. Additional experiments were conducted using sodium-free perfusate to investigate sodium-dependent Pi transport. A significant decrease in Pi transport was seen 20-40 min following the switch to sodium-free buffer as well as an increase in Pi transport following the switch back to a sodium-containing buffer (also between 20-40 min after the switch). The lag-time in the transport response to switching between the two perfusates is speculated to represent the time taken for solute transport to reach equilibrium. Thus the serosal fluid collected during the first 20 min of exposure to sodium-free buffer may partially represent the Pi transported in the presence of sodium-containing buffer. Additionally, the concentration of Pi measured

in the serosal fluid was confirmed to not be attributed to contamination of serosal fluid with liquid paraffin. From these data, it can be concluded that sodium-driven accumulation of Pi is indeed a feature of transport across the small intestinal epithelium.

In the present perfusion experiments, glucose transport across the intestine is inconsistent; when utilizing 10mM glucose since a significant reduction in glucose concentration was detected in serosal fluid compared to perfusate. Studies by others have used glucose transport as a reflection of intestinal tissue viability [220], however, it is evident that following the switch from sodium-free perfusate back to sodium-containing perfusate, there is a trend towards an increase of glucose transport. This pattern of glucose transport upon reintroduction of sodium-containing perfusate towards the end of the experiment, along with the continuous and sustained active Pi transport observed, indicates viability of the intestinal tissue. Previous studies show that intestinal tissue remains viable upwards of 120 min following removal from the blood supply [214, 225] and perfusion experiments presented in this thesis were designed to fit within that time frame.

There are changes in protein levels of claudin-1 and occludin, key constituents of tight junctions in the intestine, following induction of CKD in rats [138]. These alterations in the tight junctions of the intestinal epithelium mimic changes that occur following damage to the epithelium [137]. Disturbances in the architecture of tight junctions may, in turn, passively release more solutes into the bloodstream perhaps in an unregulated manner. Interestingly, mutations in genes encoding for claudin 16 produce phenotypes

of hypomagnesemia with hypecalciruria [236-238], features that may perhaps be related to the mechanisms of hyperphosphatemia seen in CKD. Since tight junctions may regulate the still uncharacterized sodium-independent Pi transport pathway, these changes need to be investigated further to elucidate their relevance to intestinal Pi transport in the context of CKD. Future experiments could involve utilizing the *ex vivo* perfusion method using intestinal tissue from CKD-induced rats and comparing results between control and CKD animals.

The use of mannitol to examine paracellular intestinal transport of solutes could also be conducted utilizing this same technique. However the charge and size of mannitol are markedly different to the Pi anion (PO_4^{2-}) and thus the amount of paracellular transport occurring would only be a rough estimate of overall intestinal epithelial permeability. Although there are pitfalls, intestinal mannitol transport data can be used to correct for paracellular transport when observing Pi concentrations in serosal fluid. The serosal fluid could also contain phosphaturic factors that have been implicated in the intestinal-kidney signalling axis such as FGF-23 or MEPE. In the present studies, very few samples of serosal fluid were of sufficient volume to conduct assays for components other than glucose and Pi.

6.4. Gastro-renal Pi signalling axis

In 2007, Berndt and colleagues reported a novel mechanism by which Pi was rapidly excreted (within a 20 min period) following an acute duodenal Pi load. This

phosphaturia was independent of most known regulators of Pi excretion such as PTH, FGF-23, sFRP-4, and elevated serum Pi in general [139, 140]. Furthermore, when a homogenate of duodenal mucosa from animals receiving a duodenal Pi load was infused into the blood of a separate group of rats, phosphaturia also occurred – suggesting that there is a uncharacterized factor located in the mucosa of the proximal small intestine. It has been theorized that this Pi sensing element may be a yet undiscovered phosphatonin (see section 1.3), or one that has not been implicated in this proposed gastro-renal signalling, such as MEPE.

The *in vivo* clearance surgeries described in Chapter 4 were conducted as similar as possible to those described by Berndt et al [139, 140] in order to examine urine and plasma Pi concentration and expression of NaPi-IIa and NaPi-IIc protein following an acute intestinal Pi load. The experiments utilized both a physiological concentration of Pi (10mM) as well as the 1.3M Pi buffer utilized by Berndt et al [140]. According to the results from experiments contained in this thesis, there was only one time point in which a significant increase in urinary Pi excretion was seen and this was at 45 min after duodenal-instillation of 1.3M Pi in animals maintained on a normal diet. The fact that the increased urinary Pi excretion was coupled with an increase in plasma Pi concentration reveals that other factors may be responsible for this phosphaturia, such as the correlated increase in blood levels of PTH. The increase in urinary Pi excretion was also shown to occur at a later time point following duodenal Pi instillation than that observed by Berndt et al, who noted that phosphaturia occurred within 20 min [139, 140].

In addition to replicating the experiments of Berndt *et al*, another group of animals were also fed a low Pi diet for 7 days prior to clearance measurement in order to increase the expression of renal sodium-phosphate transporters and make it more likely to observe changes in protein expression of NaPi-IIa and NaPi-IIc following an intestinal Pi load [62]. One of the main differences between data obtained from animals on a low Pi diet and animals on a normal diet was the variability of urinary Pi excretion. Animals on a regular diet had a wider range of urinary Pi excretion than the animals fed a chronic low Pi diet - whether this has to do with the variability of existing intestinal food content and luminal Pi concentrations prior to surgeries in animals on normal diet is unknown. Future experiments could address this issue by fasting animals briefly (4-5 hours) to normalize the amount of intestinal food content.

One of the responses to a high plasma Pi concentration is a rapid increase in plasma PTH levels (refer to section 1.8), which is also shown by the data in Chapter 4. There was no significant change in plasma PTH levels of rats maintained on both low Pi and regular diets which received a physiological concentration of Pi (10mM) in the duodenum. However, 45 min after duodenal instillation of a 1.3M Pi solution, there is a significant increase in serum PTH amongst animals in both diet groups. This increase in PTH may be the cause of the few cases of phosphaturia observed in regular-diet fed animals at 45 min following a Pi bolus. Moreover, this result is in direct contrast with the data of Berndt et al, which failed to detect elevations in plasma levels of plasma Pi or PTH. The most obvious interpretation of data obtained from present experiments is that

phosphaturia in animals which received a 1.3M Pi intestinal load can be attributed to the resultant increase in plasma Pi and PTH concentration.

Utilizing BBM prepared from kidneys of rats receiving a duodenal Pi or saline bolus, it is shown that a duodenal Pi load and the potential phosphaturic factor proposed by Berndt et al does not downregulate the expression of NaPi-IIa or NaPi-IIc at the renal BBM. The aforementioned variability of Pi excretion, in particular a few animals which received a 1.3M Pi duodenal load and exhibited phosphaturia, do in fact correlate with a lower expression of NaPi-IIc. As discussed in section 1.2.1.5, NaPi-IIc does not play a dominant role in kidney handling of Pi; however in response to a very high Pi load the mechanisms of Pi regulation may be altered and NaPi-IIc may assume a more important role in renal Pi reabsorption.

Berndt et al [139] corrected their published 1.3mM Pi buffer utilized in their experiments to 1.3M Pi solution [140]. With this 1000x more concentrated Pi solution, present work revealed morphological changes in intestinal ultrastructure. Particularly noticeable was a marked increase in intestinal mucus production following both 1.35M saline and 1.3M Pi compared to experiments utilizing isotonic solutions. An increase in mucus production is usually viewed as a protective mechanism to prevent damage to epithelium [252] and the manner in which this excess mucus and/or already damaged epithelium affects Pi transport is unknown. Future studies may involve direct visualization of the epithelium following exposure to a high osmolarity buffer utilizing immunohistochemistry or electron microscopy.

Human studies have shown that a Pi load presented to the jejunum increases blood levels of Pi regulatory factors such as PTH without affecting other factors thought to be upstream of this sensing pathway (i.e. calcium levels) [258]. Other studies in CKD patients have shown that in contrast to the well documented association between FGF-23 and dietary Pi intake [142], acute non-dietary modification of serum Pi levels (i.e. via dialysis) within the normal range does not induce changes plasma levels of FGF-23 [143, 144]. Thus, it appears that intestinal Pi load and/or sensing may have the capability of regulating secretion of FGF-23 and PTH, which in turn would directly affect Pi reabsorption at the kidney. Most recently, a group investigated the time of onset of phosphaturia following IV and duodenal Pi loading in humans [266]. Results show that there was no difference in onset of phosphaturia following IV and duodenal Pi loads, and both plasma Pi and PTH were elevated before any increase in FEPi. This study furthers the evidence that the previously reported, acute gasto-renal Pi signaling does not exist. With the knowledge that dietary Pi intervention and treatment with oral Pi binders have poor patient compliancy in CKD, an understanding of Pi sensing in the intestine is likely to be useful for new potential targets of treatment for hyperphosphataemia.

6.5 Liver and Pi handling

The idea that the liver may release a factor which influences Pi homeostasis relates back to observations of clinical cases involving acute hypophosphatemia in patients following hepatectomy [149]. This phenomenon has been attributed to a marked increase in phosphaturia which occurred independently of the major known players of Pi homeostasis (PTH and phosphatonins) [150]. The underlying mechanism and cause for this phosphaturia remains unidentified.

The results from experiments shown in Chapter 5 confirm that the liver accumulates more Pi when higher concentrations of Pi are instilled into the intestine; this pattern parallels the Pi transport mechanisms observed in the intestine. One main different between Pi uptake by the liver and the intestine is that liver Pi accumulation plateaus between 10mM and 15mM Pi instillation into the intestine and may signify a saturation point for Pi transport into hepatocytes. The normal range of intestinal luminal Pi concentration is approximately 0.7-12mM depending on the timing and composition of food [119], thus 15mM may exceed this physiological maximum. The liver Pi uptake plateau revolving around this physiological maximum suggests that there is a Pi secretory function for the liver at supraphysiological Pi concentrations, potentially to protect the body from excess Pi by secreting it back into the bile to be recirculated into the small intestine in an analogous fashion to waste products, i.e. bilirubin. The utilization of higher concentrations of Pi to investigate this apparently saturable liver Pi

transport process was not furthered since these levels of Pi would not be considered physiologically relevant.

The sodium dependency of Pi accumulation by the liver was investigated by substituting ChCl for NaCl in the buffer instilled into the jejunum. Results show a differing pattern in sodium-dependent Pi uptake between the intestine and the liver. Sodium-dependent Pi transport was seen following a jejunal Pi load at all four concentrations of Pi used (1, 5, 10, and 15mM). For the liver however, there was no sodium dependency seen until 15mM Pi was instilled into the jejunum. At this concentration, which is at the level of a supraphysiological Pi load, different mechanisms of Pi uptake are apparent. This possibly suggests that the sodium phosphate cotransporters expressed in the liver may only be responsible for a small component of Pi transport at physiological levels and/or could be a part of a greater network of Pi handling that is still yet to be discovered. The fact that no sodium-dependency was seen in liver Pi uptake prior to 15mM may not also rule out the function of sodium phosphate cotransporters in the liver, as the sodium-free buffer used as a jejunal Pi load would not be completely sodium-free upon arrival to the liver via the Na-plenty circulatory system. Exsanguinations were also performed following Pi transport experiments to confirm that the Pi measured was contained in hepatic cells and not present in the blood vasculature of the liver.

Future studies focusing on the role of the liver in Pi transport should include measurements of Pi levels in the bile. Secreted bile serves to transport endogenous and exogenous waste for excretion and thus could potentially remove postprandial

excess levels of Pi that are sensed by the liver following uptake across the small intestine. The knowledge that PiT1 and PiT2 transporters are expressed in cholangiocytes of the bile duct also provides grounds for investigating the role of bile in Pi transport. Further studies could involve cannulation of the bile duct and observing whether changes in Pi concentrations occur in the bile following intestinal instillation of high concentrations of Pi (>15mM) and whether inhibition of this process of biliary Pi uptake leads to a dysregulation of body Pi homeostasis.

A reported portal factor which was shown to regulate whole body calcium [264] could be similar to the unknown factor that is playing a role in Pi regulation. Using experiments similar to the ones conducted in this thesis, this group observed plasma calcium levels in portacaval shunted Lewis rats following parathyroidectomy. The results showed that there was correction of hypocalcemia in shunted mice which was independent of PTH, and the group proposed that a substance originated in the portal region which bypassed modifications in the liver. This unaltered factor was then able to correct for the hypocalcemia following parathyroidectomy [264]. The clinically observed hypophosphatemia seen in hepatectomy could result from a factor with similar properties, since hepatectomy could temporarily disturb the liver's modification abilities.

6.6. Overall conclusions

Data obtained utilizing *in vitro* and *in situ* techniques confirm that the jejunum is the intestinal region with dominant Pi transport capacity and that there is a significant sodium-dependent Pi transport pathway in the jejunum, likely dependent on NaPi-IIb, which is highly expressed in this region. A significant Pi transport ability in the distal colon is also evident from current studies and there has been no previous evidence of considerable Pi transport in this region of the intestine. It can be concluded that for the study of Pi transport in regions of the small intestine where chyme only remains for a short period of time, the everted sleeve method may be a more accurate representative of physiological mechanisms taking place *in vivo*. Studying intestinal regions where food remains for a longer period of time (distal small intestine/large intestine) may warrant other methods such as the *in situ* closed-loop technique. Utilizing an *ex vivo* perfusion technique never previously used to investigate Pi transport, a concentration of Pi occurred in serosal fluid compared to perfusate buffer. This concentration of Pi in the serosal fluid appears to be sodium-dependent since replacing normal perfusate buffer with one which was absent of sodium resulted in a significant decrease in Pi transport. The acute, gastro-renal signalling axis of Pi regulation proposed by Berndt et al [139, 140] was also refuted following identical clearance surgeries in which a physiological Pi concentration (10mM) was instilled into the duodenum of rats. A supraphysiological Pi concentration (1.3M), as used by Berndt et al [140] was also given to rats; data from these experiments showed an increase in serum Pi and plasma PTH. These changes in plasma composition could explain the phosphaturia seen in some animals during

these experiments. Lastly, the liver was shown to posses Pi transport properties by measuring the amount of Pi taken up by the liver following administration of Pi buffer into a closed-loop of intestine. The concentration of Pi in the liver stabilised at luminal Pi levels of between 10 and 15mM, which is the range of Pi concentration present in the intestinal lumen. Thus, the liver may play a regulatory role in Pi release into the systemic circulation following absorption across the small intestine **(summary, Figure 6.1).**

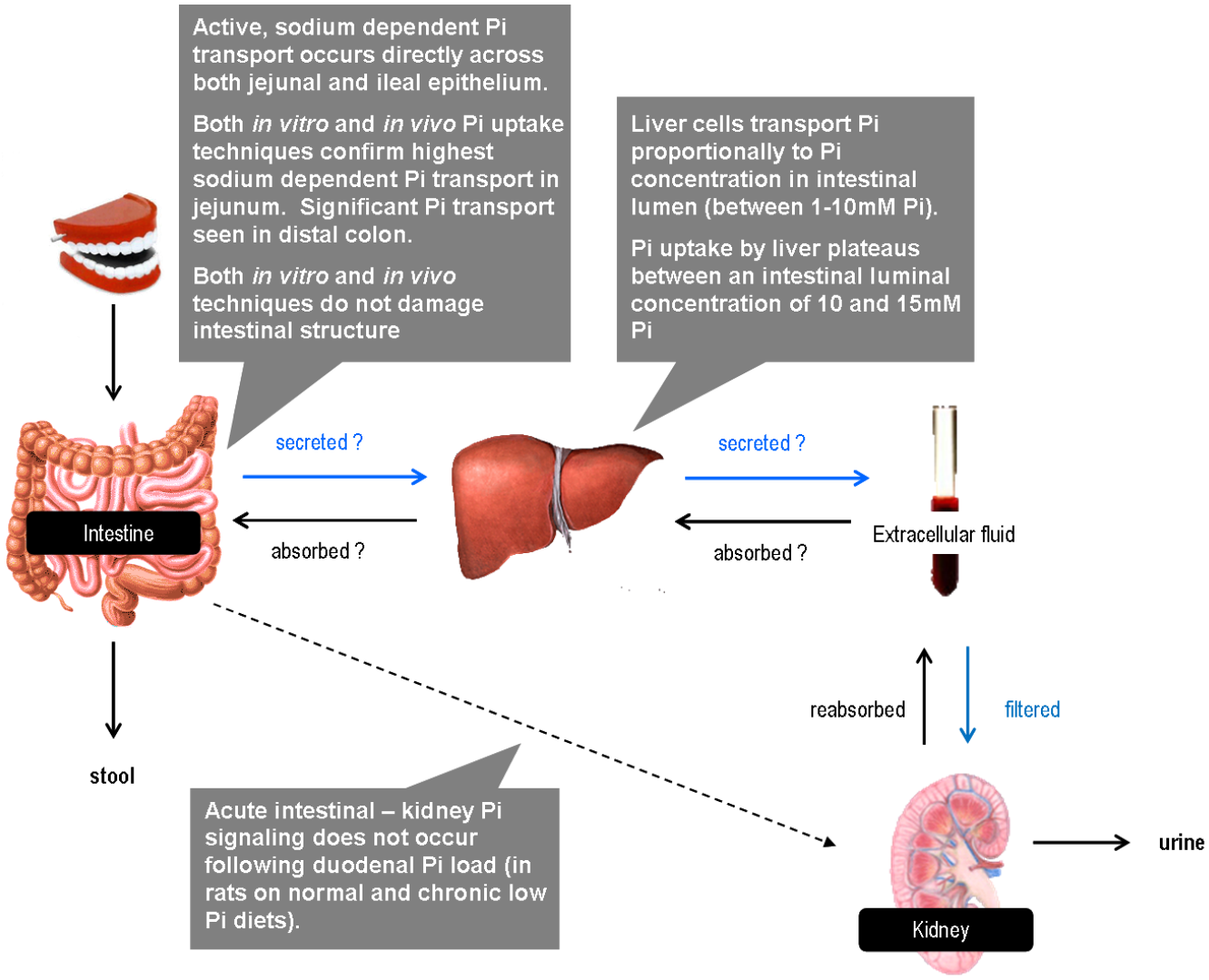


Figure 6.1. Summary of data and potential Pi signalling pathways between the small intestine, kidney, and the liver

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Abstracts, publications, and funding

Abstracts

Nephrology Day 2013 (London, UK) poster presentation: An acute duodenal phosphate load following chronic low phosphate diet does not induce phosphaturia in rats.

Physiological Society annual meeting 2012 (Edinburgh, UK) poster presentation: An acute duodenal phosphate load does not downregulate sodium phosphate co-transporter.

Publications

Lee GJ, Marks J, Debnam ES, Unwin RJ. An acute duodenal phosphate load does not induce acute phosphaturia in Sprague Dawley rats. (manuscript in preparation for Experimental Physiology)

Marks J, Lee GJ […] Unwin RJ. Regional variations in Na+-dependent and Na+-independent phosphate transport along the rat small intestine and colon. (manuscript in preparation for American Journal of Physiology)

Lee GJ, Marks J. Intestinal phosphate transport: a therapeutic target for CKD and beyond? Pediatr Nephrol. Feb 5 (2014)

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