REGULATION OF PATHWAYS INVOLVED IN INTESTINAL PHOSPHATE TRANSPORT

by

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I, Sobiya Prathusha Nadaraja, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
ABSTRACT

Despite the importance of extracellular phosphate, the mechanisms and control of intestinal phosphate transport remain unclear. The present study used *in vivo* and *in vitro* methods to compare the extent of Na\(^+\)-dependent versus Na\(^+\)-independent phosphate transport along the rat small intestine and colon at different luminal phosphate concentrations. Na\(^+\)-dependent and Na\(^+\)-independent phosphate transport and genomic expression of type II (NaPi-II) and type III (PiT) transporters in young (3-week old) and adult (8- and 16-week old) animals fed a control or low phosphate diet have also been quantified. mRNA levels of Na\(^+\)-dependent phosphate transporters have been analysed in the 5/6 nephrectomy model of chronic renal failure and following treatment with matrix extracellular phosphoglycoprotein (MEPE). The acute effects of altered luminal phosphate concentration on intestinal phosphate transport and renal phosphate transporter expression was also assessed.

The findings confirm the jejunum to be the main site of Na\(^+\)-dependent phosphate absorption under both normal and low dietary phosphate conditions. Low phosphate diet upregulates Na\(^+\)-dependent and Na\(^+\)-independent phosphate transport in both duodenum and jejunum, whereas age only affects Na\(^+\)-dependent component in the duodenum and jejunum. When luminal phosphate concentrations are in the physiological (millimolar) range, absorption in the jejunum displays less Na\(^+\)-dependency and is unlikely to be mediated exclusively by the Na\(^+\)-dependent NaPi-IIb cotransporter. In the ileum, again using millimolar luminal phosphate concentrations, significant Na\(^+\)-dependent phosphate transport was detected, but the rate of phosphate absorption was lower than in the jejunum. Since NaPi-IIb is not expressed at the rat ileal brush-border membrane (BBM), the presence of significant Na\(^+\)-dependent phosphate transport suggests an alternative phosphate uptake pathway in the ileum. At millimolar luminal phosphate concentrations in the distal colon, only Na\(^+\)-independent phosphate
absorption was detected. Thus, at concentrations of phosphate normally present in the intestinal lumen, Na\(^{+}\)-independent pathways of phosphate absorption are present in the proximal small intestine and distal colon, as well as a non-NaPi-IIb-mediated Na\(^{+}\)-dependent pathway in the distal small intestine.

PiT2 assumes a dominant role in phosphate transport in the jejunum of pre-weaned animals, and in the kidneys of animals fed a low phosphate diet. PiT1 transporter is not regulated by age or low phosphate diet at the genomic level. Chronic renal failure reduces intestinal and renal expression of the major transporter NaPi-II, and also PiT1 and PiT2. However, MEPE reduces intestinal expression of NaPi-Iib and PiT2 alone in chronic renal failure animals. Acute duodenal instillation of either 15 mM or 1.3 M phosphate did not affect the renal BBM protein expression of NaPi-Iia and NaPi-Iic. Duodenal instillation of 1.3 M, but not 15 mM, phosphate increased phosphate uptake in the duodenum only; however, the small intestine is unlikely to encounter phosphate concentrations in the molar range.
ACKNOWLEDGEMENTS

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I also wish to thank my parents and my partner Milaskar Jesudasan who were always there with advice and patience to encourage me through this period.

I am grateful to my PhD colleague Havovi Chichger for her support and I offer my kindest regards to all of those who supported me in any respect during the completion of this project.
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## ABBREVIATION

<table>
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<th>Abbreviation</th>
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<tbody>
<tr>
<td>ASARM</td>
<td>Acidic serine-aspartate-rich MEPE-associated motif</td>
</tr>
<tr>
<td>BBM</td>
<td>Brush-border membrane</td>
</tr>
<tr>
<td>BLM</td>
<td>Basolateral membrane</td>
</tr>
<tr>
<td>CaSR</td>
<td>Calcium-sensing receptor</td>
</tr>
<tr>
<td>CRF</td>
<td>Chronic renal failure</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>FGF-23</td>
<td>Fibroblast growth factor -23</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>HPO$_4^{2-}$</td>
<td>Divalent phosphate anion</td>
</tr>
<tr>
<td>H$_2$PO$_4^{-}$</td>
<td>Monovalent phosphate anion</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>$K_t$</td>
<td>Affinity constant</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEPE</td>
<td>Matrix extracellular phosphoglycoprotein</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>NHERF</td>
<td>Na/H exchange regulatory factors</td>
</tr>
<tr>
<td>OHO</td>
<td>Oncogenic hypophosphataemic osteomalacia</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>Phosphonoformic acid</td>
</tr>
<tr>
<td>PHEX</td>
<td>Phosphate-regulating gene with homologies to endopeptidases on the X chromosome</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic phosphorus</td>
</tr>
<tr>
<td>PO$_4$</td>
<td>Phosphate anion</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>sec</td>
<td>Seconds</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>sFRP-4</td>
<td>secreted frizzled-related protein-4</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute carrier family</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cells</td>
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1.0. Introduction to phosphate, its metabolism, physiology and pathology
1.1. Phosphate

Inorganic phosphorus is vital for processes such as cell signalling, skeletal mineralisation, energy metabolism, membrane function and nucleic acid synthesis (Berndt & Kumar 2007; Tenenhouse, 2007). Phosphorus is the second most abundant mineral in all human tissues and it is critical that its homeostasis is maintained, since it can affect the system of almost any organ. Inorganic phosphorus exists predominantly as phosphate anion ($\text{PO}_4^{3-}$) in the form of $\text{H}_2\text{PO}_4^{-}$ and $\text{HPO}_4^{2-}$, with a 1:4 ratio, respectively at a physiological pH of 7.5.

1.2. Homeostasis of body phosphate

Phosphate balance is maintained according to dietary phosphate intake and body need. The serum phosphate concentration is maintained within a narrow range through a complex interplay between intestinal absorption, exchange with intracellular and bone storage pools, and renal tubular reabsorption. Most of the phosphate within the body is in bone (85%) complexed with calcium, whilst the remainder is essentially distributed in soft tissue (Crook & Swaminathan, 1996; Hansen et al., 1976).

Serum phosphate concentrations are highest at the neonatal stage (1.9 – 2.4 mM), and lower in childhood (1.5 – 1.8 mM) and lowest in adulthood (0.9 – 1.4 mM) (Chan et al., 1981; Crawford et al., 1950; McCrory et al., 1952). The small intestine and kidney are the two main organs primarily involved in phosphate homeostasis. The small intestine absorbs, in a regulated manner, phosphate from the chyme passed from the stomach. The amount of phosphate absorbed across the small intestine in man is approx 0.9 g per day (Fig. 1.1). This represents almost 70% absorption of the dietary phosphate, which is more phosphate than is required by the body (Cramer, 1961). Plasma unbound phosphate is freely filtered by the kidney and then reabsorbed (~80%) in the proximal tubules (Cheng & Sacktor, 1981; Dousa & Kempson, 1982). The amount of excreted
(urinary) phosphate is equivalent to phosphate absorbed by the small intestine, and remainder of the phosphate is excreted in the faeces (Berner & Shike, 1988; Harrison HE & Harrison HC, 1941).

The kidneys represent the major control site for whole body phosphate homeostasis and it can increase or decrease its phosphate reabsorptive capacity to accommodate the phosphate need (Crawford et al., 1950). In man, the diurnal fluctuation in rate of phosphate excretion is associated with a parallel change in plasma phosphate concentration (Fiske, 1921; Stanbury & Thomson, 1951; Mills & Stanbury, 1955). This response of the kidney to changes in plasma phosphate concentration is dependent on maximum tubular reabsorption \( (T_m) \) (Schiesse et al., 1948).
Figure 1.1. Maintenance of phosphate balance. The small intestine and kidney are the major regulators of phosphate homeostasis and regulate absorption of phosphate according to dietary phosphate intake and body need. Absorbed phosphate enters the extracellular pool and moves in and out of the bone as required, which determines serum phosphate concentration. Excess phosphate is primarily excreted in the urine and the remainder in the faeces. In adults, phosphate used from the exchangeable phosphorus pool for bone formation is generally equal to phosphate release into the exchangeable pool due to bone resorption. Adapted from Berndt & Kumar (2007).
1.3. Phosphate absorption

Phosphate absorption across the brush-border membrane (BBM) of the small intestine and kidney occurs against an electrochemical potential difference. This active process involves the transfer of the phosphate anion from lumen to interstitium and is inhibited by metabolic inhibitors (Berner *et al.*, 1976; Gullans *et al.*, 1982). Transport of phosphate is coupled to the flux of sodium ions; this process is secondary active, and driven by the Na\(^+\) gradient (Baumann *et al.*, 1975; Harrison HE & Harrison HC, 1963). The Na\(^+\) concentration in luminal fluid is greater than that in the cytosol, thus providing a concentration gradient to drive Na\(^+\)-dependent phosphate transport. The Na\(^+\) gradient is the rate-limiting step for intestinal and renal Na\(^+\)-dependent phosphate transport. Protons or univalent cations such as K\(^+\), Li\(^+\) and Cs\(^+\) cannot substitute for Na\(^+\) in the active transport of phosphate (Sacktor & Cheng, 1981; Berner *et al.*, 1976).

Phosphate transport across intestinal enterocytes and proximal tubule cells of the kidney involves two further steps: phosphate transfer to the serosal side of the cell, followed by phosphate moving across the basolateral membrane (BLM) and entering the extracellular space (Matsumoto *et al.*, 1980). BBM vesicles represent a suitable experimental tool for the *in vitro* analysis of the first stage of epithelial phosphate transport, without the complications of cell metabolism. This is the reason why many previous studies have used BBM vesicles. Using BBM vesicles, phosphate transport in the small intestine and kidney of rodents was shown to be largely Na\(^+\)-dependent with an affinity constant (K\(_t\)) of ~0.1 mM (Berner *et al.*, 1976; Lee *et al*., 1986b; Loghman-Adham *et al.*, 1993). Phosphate absorption in other species such as chicks, rainbow trout and ruminants has also been studied. However, their phosphate transport characteristics differ from that observed in rodents and humans.

Na\(^+\)-dependent phosphate transport in the small intestine and kidney saturates at Na\(^+\) concentrations of 100 mM, demonstrated using BBM vesicles, oocytes and Ussing apparatus (Berner *et al.*, 1976; Hartmann *et al*., 1976).
al., 1995; Lee et al., 1986b; Quamme, 1985). Therefore, the majority of renal and intestinal phosphate uptake studies have used 0.1 mM phosphate and 100 mM Na\(^+\) concentrations. Phosphate absorption across the small intestine occurs via both passive and active routes, in contrast to kidney where phosphate absorption is thought to be mediated solely via the Na\(^+\)-dependent transcellular pathway (Kempson et al., 1985; Szczepanska-Konkel et al., 1986; Ullrich & Murer, 1982).

1.4. Paracellular phosphate absorption

The evidence behind the contribution of Na\(^+\)-independent paracellular transport to total phosphate absorption by the small intestine is contradictory. Some studies imply that Na\(^+\)-independent component is responsible for 40-50\% of total phosphate absorption (Cross et al., 1990; Murer et al., 1976; Katai et al., 1999). Debiec & Lorenc (1985) showed, using intestinal BBM vesicles, that in younger rats Na\(^+\)-dependent phosphate transport is predominant whereas in adults paracellular transport is the major pathway for phosphate transport. More recent studies confirmed this in adult rats, both in vitro and in vivo (Douard et al., 2010; Williams & DeLuca, 2007). The study of Debiec & Lorenc (1985) proposed that the contribution of Na\(^+\)-dependent and Na\(^+\)-independent component in rats is age-dependent.

Other studies proposed that paracellular transport of phosphate is dependent on dietary phosphate intake and paracellular phosphate transport may play a larger role under normal dietary conditions (Danisi et al., 1980; Walton & Gray, 1979; Williams & DeLuca, 2007). Following ingestion of a phosphate-containing meal, postprandial increases in phosphate concentration would likely favour the paracellular pathway. This corresponds with the majority of the in vivo findings that demonstrated total phosphate absorption is Na\(^+\)-independent under normal dietary conditions (Williams & DeLuca, 2007; McHardy, 1956; Davis et al., 1983; Aloia & Yeh, 1985). In vivo studies in man indicate that phosphate absorption is mediated by active Na\(^+\)-dependent transport at
luminal concentrations below 2 mM and by passive diffusion when over 2 mM phosphate (Davis et al., 1983). Intracellular phosphate concentration is difficult to determine due to the rapid turnover of various organic phosphate species (Dennis, 1996). The phosphate concentrations in rat and human intestinal luminal fluid are thought to vary between 2 to 12 mM (Davis et al., 1983; Kirchner et al., 2008). Some studies showed linear, non-saturated phosphate absorption with increasing luminal phosphate (Berner et al., 1976; Davis et al., 1983; Lee et al., 1986b; McHardy, 1956; Walton & Gray, 1979). However, the small intestine is also claimed to have a low permeability to the phosphate anion (Cross et al., 1990). Therefore, the role of the passive pathway in intestinal phosphate absorption is unclear.

1.5. Transcellular phosphate absorption

Transcellular phosphate uptake in the small intestine and kidney is initiated by Na\(^+\)-dependent phosphate transporters present at the BBM (Figs. 1.2 and 1.3). These transporters utilise the Na\(^+\) gradient established by the Na\(^+\)/K\(^-\)-ATPase at the BLM (Forster et al., 2006). So far, three different Na\(^+\)-dependent phosphate transporter families have been identified. These transporters differ in their amino acid sequence, affinity for phosphate and mechanisms controlling their activity and tissue expression. It is the type II transporters that are thought to play a key role in phosphate transport across the small intestine and kidney.
1.6. Na\(^+\)-dependent phosphate transporters

1.6.1. Type I
Type I transporters belong to the solute carrier family 17 (SLC17) and are expressed in the kidney, liver and brain (Fernandes et al., 1999). In kidney, type I transporter (NaPi-I) is expressed at the proximal tubules and to a lesser extent in distal tubules (Chong et al., 1995; Murer & Biber, 1997). When type I transporters were expressed in oocytes and Madin-Darby Canine Kidney cell line, they mediated Na\(^+\)-dependent phosphate absorption.
transport (Werner et al., 1991; Werner et al., 1990; Quabius et al., 1995).
However, Na\(^+\)-dependency was found to be weak in the
electrophysiological studies and type I transporters such as NaPi-I and
BNP1 were linked to increased permeability of chloride and other anions
(Busch et al., 1996; Uchino et al., 2000). None of the type I transporters
characterised to date are critical for phosphate absorption in any organ.
Thus, the identified type I transporters are not involved in Na\(^+\)-dependent
phosphate transport, and their role and importance in phosphate
homeostasis is unclear (Virkki et al., 2007). Type I transporters share
20% amino acid sequence homology to type II transporters (Custer et al.,

1.6.2. Type II
Type II family of Na\(^+\)-dependent phosphate transporters belong to the
SLC34 family and comprise both electrogenic and electroneutral NaPi-II
isoforms, expressed in epithelial cells. So far isoforms NaPi-IIa
(SLC34A1), NaPi-IIb (SLC34A2) and NaPi-IIc (SLC34A3) have been
identified. In addition to the small intestine and kidney, these transporters
are also expressed in other organs such as lungs, colon, salivary and
mammary glands, liver and testes (Hilfiker et al., 1998; Virkki et al.,
2007). Amino acid sequences of type II isoforms are similar except in the
main extracellular loop and N- and C-terminal regions (Virkki et al.,
2007). NaPi-II structure consists of several transmembrane-spanning domains
with N and C terminus positioned intracellularly (Virkki et al., 2007). NaPi-
II transporters have a \(K_t\) for phosphate of ~0.1 mM and are Na\(^+\)-
dependent, with a \(K_t\) for Na\(^+\) of 50 mM (Busch et al., 1995; Forster et al.,
1998). The activity of type II transporters is higher at neutral or alkaline
pH and they preferentially transport the divalent phosphate species
(Forster et al., 1999; Virkki et al., 2005; Xu et al., 2002b). Physiological
regulation of phosphate is mediated by changes in the expression of
phosphate transporter proteins, and this is related to changes in the
maximum velocity (\(V_{\text{max}}\)) of phosphate cotransport activity.
1.6.2.1. NaPi-IIa

This was the first family member of SLC34 to be identified and cloned (Magagnin et al., 1993). Multimeric NaPi-IIa cotransporter consists of eight transmembrane domains and several potential phosphorylation sites for protein kinase C (Murer et al., 2000). Mouse and rat NaPi-IIa have a sequence homology of 90.9 and 91.2%, respectively to the human NaPi-IIa isoform (Berndt & Kumar, 2007). Earlier immunohistochemical evidence showed that NaPi-IIa was expressed mainly along the apical membrane of rat proximal tubules, in S1-S3 segments, with highest protein expression in S1 segment (Collins & Ghishan, 1994; Custer et al., 1994; Murer et al., 2000). Approximately 60-70% of total phosphate reabsorption occurs in the proximal convoluted tubule, whilst 15-20% in proximal straight tubule and the remaining 5-10% in the distal convoluted tubule (Knox & Haramati, 1981). Although NaPi-IIa is widely expressed in other tissues such as brain (Mulroney et al., 2004), osteoclasts (Khadeer et al., 2003) and chondrocytes (Mansfield et al., 2001), the protein is present at lower levels compared with kidney. NaPi-IIa is not expressed in the small intestine (Hilfiker et al., 1998).

NaPi-IIa-mediated transport is electrogenic, since it transports phosphate with a 3:1 Na\(^+\):HPO\(_{4}^{2-}\) stoichiometry. NaPi-IIa protein possesses a hydrophilic loop which contains two N-linked glycosylation sites at Asn-298 and Asn-328. These sites are thought to be critical for BBM expression of NaPi-IIa (Hayes et al., 1994). The loss of NaPi-IIa gene function in mice impaired renal phosphate reabsorption by about 70% compared with wild type (Beck et al., 1998; Tenenhouse & Murer, 2003; Tomoe et al., 2010), implying that NaPi-IIa mediates 70% of total phosphate reabsorption in the kidney. NaPi-IIa is a major regulator of phosphate homeostasis and, therefore, crucial for normal skeletal development.

NaPi-IIa is a highly regulated protein and expression of NaPi-IIa at the BBM can be reduced within minutes, as seen in response to parathyroid...
hormone (PTH) (Bacic et al., 2006). Tertiary structure of NaPi-IIa consists of KR (Lys-Arg) motif, which is a signal recognised by PTH (Virkki et al., 2007). NaPi-IIa also responds rapidly to changes in dietary phosphate load within 2 hours (Capuano et al., 2005; Villa-Bellosta et al., 2009b). This adaptation of NaPi-IIa is facilitated by modifying protein-protein interactions that stabilise NaPi-IIa protein at the BBM and by the activation of molecular motifs within the NaPi-IIa protein that mediates its endocytotic retrieval or exocytosis (reviewed in detail in Biber et al., 2009; Murer et al., 2000; Murer et al., 2003).

Renal phosphate reabsorption is an age-regulated process; Na\(^+\)-dependent phosphate transport is much higher in younger animals and it progressively declines as animals get older (Sorribas et al., 1996; Silverstein et al., 1997). This is reflected in the age-dependent decline in expression of NaPi-IIa proteins (Arar et al., 1999; Sorribas et al., 1996). NaPi-IIa is a very important phosphate transporter in phosphate homeostasis as it cannot be compensated by other Na\(^+\)-dependent phosphate transporters in the kidney such as NaPi-IIc (Beck et al., 1998; Tenenhouse & Murer, 2003; Tomoe et al., 2010). Changes in BBM expression of NaPi-IIa proteins parallel altered renal phosphate handling, highlighting its physiological importance (reviewed in detail in Biber et al., 2009; Murer et al., 2000; Murer et al., 2003). NaPi-IIa is regulated by many physiological factors which are discussed in detail in the following sections of this chapter.

1.6.2.2. NaPi-IIc

The expression of NaPi-IIc occurs exclusively in the kidney and NaPi-IIc has been suggested to be a growth-related factor. Unlike NaPi-IIa, NaPi-IIc protein (75 kDa) is expressed exclusively in S1 (convoluted) segment of the proximal tubules (Nowik et al., 2008; Segawa et al., 2002). NaPi-IIc is thought to be responsible for about 30% of the total renal phosphate reabsorption (Beck et al., 1998). Although NaPi-IIc protein level was increased to compensate for NaPi-IIa loss in NaPi-IIa knockout mice studies, NaPi-IIc did not successfully substitute for NaPi-IIa-mediated
transport (Beck et al., 1998). NaPi-IIc-mediated transport is an electroneutral process, since it transports phosphate with a 2:1 \(\text{Na}^+:\text{HPO}_4^{2-}\) stoichiometry (Bacconi et al., 2005; Segawa et al., 2002). Similar to NaPi-IIa, NaPi-IIc preferentially transports the divalent phosphate species, but with a lower \(K_t\) of \(~0.33\) mM (Ghezzi et al., 2009; Villa-Bellosta & Sorribas, 2010b). However, NaPi-IIc functions efficiently owing to its electroneutrality (Bacconi et al., 2005; Segawa et al., 2002). As NaPi-IIc transports only two \(\text{Na}^+\) ions, compared with NaPi-IIa and NaPi-IIb that transport three \(\text{Na}^+\) ions, the energetic cost of transporting phosphate across the cell is smaller. Thus, fewer \(\text{Na}^+\) ions are loaded into the cell during NaPi-IIc-mediated transport, and therefore less energy is needed to maintain the negative membrane potential of the cell. The electroneutrality of NaPi-IIc is mediated by the replacement of an aspartic acid residue (found in NaPi-IIa and NaPi-IIb) with a glycine residue in a sodium-binding site within a conserved amino acid cluster in NaPi-IIc (Bacconi et al., 2005). The amino acids WLHSL in the cytoplasmic C terminus are thought to be important for the apical expression of NaPi-IIc (Ito et al., 2010).

The studies on rodents have shown that expression of NaPi-IIc is age-dependent; the highest NaPi-IIc level is found in weaning animals and expression decreases with age (Segawa et al., 2002; Silverstein et al., 1997). Regulation of NaPi-IIc is not as well studied as NaPi-IIa, but it has been shown to be regulated by dietary phosphate levels (Segawa et al., 2005; Ohkido et al., 2003; Segawa et al., 2002). However, regulation of NaPi-IIc by phosphate diet occurs over a longer time course of \(>24\) hours, compared with \(<1-2\) hours applicable for NaPi-IIa (Villa-Bellosta et al., 2009b; Segawa et al., 2005; Moe, 2009). Recently emerging evidence demonstrate that NaPi-IIc is also regulated by metabolic acidosis, dietary magnesium, the phosphatonin Fibroblast growth factor-23 (FGF-23) and possibly PTH, although the evidence is so far contradictory (Miyamoto et al., 2007; Nowik et al., 2008; Segawa et al., 2003; Segawa et al., 2007; Thumfart et al., 2008). Studies using NaPi-IIc knockout mice suggest that NaPi-IIc is involved in the calcium/vitamin D axis (Segawa et al., 2009b).
The same study demonstrated hypercalcaemia, hypercalciuria, and augmented levels of plasma 1,25-dihydroxyvitamin D$_3$ in NaPi-IIc knockout mice. However, NaPi-IIc knockout mice do not develop hypophosphataemia, hyperphosphaturia, renal calcification or rickets, in contrast to NaPi-IIa knockout mice (Breusegem et al., 2009; Segawa et al., 2009b; Tomoe et al., 2010). Renal phosphate transport in NaPi-IIc knockout mice was not different from normal mice (Segawa et al., 2009b; Tomoe et al., 2010). Thus, NaPi-IIc is not considered to have a major role in phosphate reabsorption in rodents. Additional studies on the differential regulation of renal NaPi-IIa and NaPi-IIc provides further evidence that, in contrast to the complete internalisation of NaPi-IIa following a high phosphate diet, NaPi-IIc is only partially internalised to the subapical compartment (Segawa et al., 2005).

Although NaPi-IIc only plays a minor role in phosphate homeostasis in rodents (Segawa et al., 2009b), the transporter is proposed to be much more important in man where it has a more widespread tissue distribution (Virkki et al., 2007). Mutations in the NaPi-IIc gene in humans cause hereditary hypophosphataemic rickets with hypercalciuria which is characterised by renal phosphate wasting leading to hypophosphataemia and secondary rickets or osteomalacia (Ichikawa et al., 2006; Bergwitz et al., 2006). Studies based on this genetic condition suggest that NaPi-IIc plays a more significant role in humans, in the control of renal phosphate reabsorption, bone mineralisation and plasma phosphate concentrations (Segawa et al., 2009a; Segawa et al., 2009b).

### 1.6.2.3. NaPi-IIb

The NaPi-IIb transporter electrogenically transports phosphate with a 3:1 Na$^+$:HPO$_4^{2-}$ stoichiometry. NaPi-IIb was characterised in *Xenopus oocytes* as Na$^+$-dependent with a $K_t$ of 10-50 µM for phosphate (Hilfiker et al., 1998; Forster et al., 2006). This high affinity $K_t$ suggests that NaPi-IIb-mediated transport would be saturated under normal dietary conditions and NaPi-IIb probably serves a bigger role in the small
intestine under phosphate-restricted conditions and during hypophosphatemia (Douard et al., 2010; Hilfiker et al., 1998; Marks et al., 2010; Williams & DeLuca, 2007). NaPi-IIb is also highly expressed in the lungs where it plays a role in the synthesis of surfactant in the alveoli (Huqun et al., 2007; Corut et al., 2006; Hashimoto et al., 2000). NaPi-IIb is also found in the colon (Capuano et al., 2009), testis (Xu et al., 2003a), liver (Frei et al., 2005), and mammary and salivary glands (Homann et al., 2005; Huber et al., 2007), but it plays a more significant role in the small intestine. NaPi-IIb is not expressed in the kidney (Hilfiker et al., 1998). NaPi-IIb is regulated by several physiological factors, including 1,25-dihydroxyvitamin D$_3$ levels and chronic and acute changes in the level of dietary phosphate. The regulation by these factors is both species- and region-specific in the small intestine, since different regions of the small intestine (duodenum, jejunum and ileum) express distinct levels of NaPi-IIb across species (Borowitz & Granrud, 1992; Huber et al., 2002; Huber et al., 2006; Marks et al., 2006; Radanovic et al., 2005; Schroder et al., 1998).

NaPi-IIb expression is also essential for phosphate absorption during early embryonic development, since deletion of the NaPi-IIb gene in mice causes an embryonic lethal phenotype due to abnormalities in phosphate transfer from the maternal circulation (Shibasaki et al., 2009). Intestinal expression of NaPi-IIb is age-dependent with higher mRNA transcripts and proteins present in sucklings, corresponding with the maximal intestinal Na$^+$-dependent phosphate absorption seen in these animals (Arima et al., 2002; Xu et al., 2002a). In mice, NaPi-IIb is a key player in total phosphate absorption, since NaPi-IIb knockout mice fed a low phosphate diet followed by an acute phosphate load showed approximately 50% less phosphate absorption than wild type controls, and in these experiments NaPi-IIb accounted for >90% of intestinal Na$^+$-dependent phosphate transport (Sabbagh et al., 2009).

The NaPi-IIb cotransporter is an N-linked glycoprotein that is glycosylated during the suckling/weaning transition, a process thought to be important
for its plasma membrane expression (Arima et al., 2002; Hayes et al., 1994). The fully glycosylated NaPi-IIb, containing complex oligosaccharides, has a molecular mass of approximately 108 kDa and is found at the BBM of enterocytes and at the apical pole of alveolar type II cells (Hilfiker et al., 1998; Traebert et al., 1999). There is no evidence of the presence of non-glycosylated NaPi-IIa transporters at any age, highlighting the differential structure-specific regulation of transporters within the SLC34 (type II) family.

Type II transporters have been extensively characterised, in contrast to type III transporters.

1.6.3. Type III

Type III transporters PiT1 and PiT2 were initially isolated as cell surface receptors for gibbon ape leukemia virus (GALV) in mouse and humans and amphotrophic murine retrovirus (A_MuLV) in rats, respectively (Miller et al., 1994; Tatsumi et al., 1998; Bai et al., 2000). They are members of the SLC20 family and are expressed ubiquitously (Kavanaugh et al., 1994). PiT transporters only share about 60% amino acid sequence homology to mammalian type I and type II transporters (Zhao et al., 2006; Tatsumi et al., 1998). PiT1 and PiT2 phosphate transporters were originally thought to play a housekeeping role in phosphate absorption at the BLM by absorbing phosphate from interstitial fluid for normal cellular functions (Bottger & Pedersen, 2002; Collins et al., 2004; Ravera et al., 2007). PiT-mediated phosphate transport in prokaryotes and plants is coupled to the H⁺-gradient (Harris et al., 2001; Daram et al., 1999), in contrast to the Na⁺-dependency seen in animals.

In vitro studies have demonstrated PiT1 and PiT2 to be active Na⁺-dependent phosphate transporters that are expressed in several tissues. Phosphate uptake studies in *Xenopus laevis* oocytes displayed Na⁺-dependent phosphate uptake when PiT1 was expressed, with a $K_t$ for phosphate of 89 µM when expressed in the rat and 333 µM when expressed in man (Bottger et al., 2006; Tatsumi et al., 1998). Similarly,
PiT2-mediated transport was also Na\textsuperscript{+}-dependent with a $K_{i}$ of $\sim$25 µM when expressed in oocytes and 200 µM when expressed in mammalian cells (Bai et al., 2000; Kavanaugh et al., 1994; Bottger et al., 2006). PiT transporters are also capable of transporting the phosphate analogue arsenate, but with a much lower affinity ($K_{i}$ of 1-5 mM) (Villa-Bellosta & Sorribas, 2008b; Villa-Bellosta & Sorribas, 2010a). However, it is important to realise that uptake studies using oocytes is not necessarily specific or accurate in reflecting the behaviour in mammalian cells expressing the same gene.

PiT1 and PiT2 transporters are widely expressed; including the small intestine, heart, kidney, parathyroid, distal colon, bone and vascular smooth muscles (Bai et al., 2000; Capuano et al., 2009; Giral et al., 2009; Kavanaugh & Kabat, 1996; Villa-Bellosta et al., 2009a; Villa-Bellosta et al., 2009b; Tatsumi et al., 1998). PiT1 is thought to play an important role in bone remodelling, as it is expressed in osteoclasts and osteoblasts (Khadeer et al., 2003; Tatsumi et al., 1998). PiT transporters are also imperative for phosphate absorption in vascular smooth muscle cells (VSMC) (Virki et al., 2007; Villa-Bellosta et al., 2007; Gonzalez et al., 2009). Indeed, there is a strong interest in the role of PiT transporters, especially PiT1, in vascular calcification, since they are thought to be more highly expressed in VSMCs under this condition (Li et al., 2006). In contrast, minimal levels of NaPi-II transporters are present in VSMCs (Villa-Bellosta et al., 2007). The implication of PiT transporters in vascular calcification is discussed later in this chapter. Similar to NaPi-II transporters, inter-organ PiT expression also varies between species; in mice highest expression of PiT2 was found in the jejunal region of the small intestine (Bai et al., 2000) whereas in rats minimal PiT2 levels were reported along the small intestine (Giral et al., 2009). Regarding the kidney, PiT2 is exclusively localised in the S1 segment of the rat proximal convoluted tubules under normal dietary conditions (Villa-Bellosta et al., 2009b; Villa-Bellosta & Sorribas, 2010b).
PiT transporters preferentially transport monobasic phosphate species with a 2:1 Na⁺:H₂PO₄⁻ stoichiometry. Thus, PiT-mediated transport is electrogenic and transport activity decreases when the pH is increased, in contrast to type II transporters (Bai et al., 2000; Villa-Bellosta et al., 2007; Zhao et al., 2006). Earlier studies reported that monovalent phosphate species is preferentially transported across the intestinal BBM, whereas divalent phosphate (the NaPi-II substrate) is the predominantly transported phosphate species across the renal BBM (Hammerman, 1986; Berner et al., 1976). Additionally, phosphate uptake studies using intestinal BBM vesicles displayed 4-fold higher transport activity when pH was decreased from 7.4 to 6, proposing a more important role for PiT transporters in intestinal epithelium (Berner et al., 1976). In renal BBM vesicles PiT2-mediated uptake also varied with pH, contributing from approximately 3 to 40% of total phosphate transport at pH values of 7.5 and 6.0, respectively (Villa-Bellosta & Sorribas, 2010b). However, the pH in the proximal tubular fluid does not fall below 6.6, even during metabolic acidosis, thus raising uncertainties in the contribution of PiT2 towards overall renal phosphate reabsorption (Gottschalk et al., 1960; DuBose et al., 1979).

The relative abundance of PiT1 and PiT2 mRNA in the mouse kidney is very low, about 2% of that for NaPi-IIa (Tenenhouse et al., 1998). In wild type mice, the genomic expression of PiT1 and PiT2 is not regulated by dietary phosphate restriction and PiT transporters also do not compensate for NaPi-IIa in NaPi-IIa knockout mice fed a chronic low phosphate diet (Hoag et al., 1999; Tenenhouse et al., 1998). However, in double knockout mice for NaPi-IIa and NaPi-IIc, there remains residual renal phosphate reabsorption, which is proposed to be PiT-mediated (Segawa et al., 2009a). Further evidence of the role of PiT transporters in renal phosphate reabsorption is supported by the finding that PiT1, but not PiT2, mRNA level is specifically increased by growth hormone and IGF-1, both important stimulators of Na⁺-dependent phosphate transport (Hammerman et al., 1980; Caverzasio et al., 1981; Tenenhouse et al., 1998; Zoidis et al., 2004). Growth hormone also stimulates the production
of IGF-1. PiT1 is also important for normal liver growth as disruption of the PiT1 gene in mice leads to mid-gestation lethality due to severe defects in liver development (Beck et al., 2010). In these mice, PiT2 cannot compensate for the loss of PiT1.

In NaPi-IIb knockout mice, residual Na\(^+\)-dependent phosphate transport was not evident in the small intestine, indicating that PiT1 and PiT2 transporters are not likely to play an important role in intestinal phosphate absorption in mice (Sabbagh et al., 2009). In rats, PiT transporters are implicated to play an important role in phosphate absorption across both the small intestine and kidney. PiT expression in rats is regulated by factors that classically affect renal phosphate reabsorption; so far upregulation of renal PiT1 and PiT2 has been demonstrated during phosphate deprivation and metabolic acidosis, and PiT2 only during dietary potassium deficiency (Villa-Bellosta et al., 2009b; Villa-Bellosta & Sorribas, 2010b; Nowik et al., 2008; Breusegem et al., 2009). In contrast to NaPi-IIc, renal PiT2 expression is not age-dependent (Leung et al., 2005). In rat small intestine, PiT1 and PiT2 are not regulated by changes in dietary phosphate (Giral et al., 2009), although PiT2 was shown to be regulated by 1,25-dihydroxyvitamin D\(_3\) in vitro (Katai et al., 1999). The exact physiological role of PiT transporters in intestinal phosphate absorption remains uncharacterised.

1.7. Inhibitors of Na\(^+\)-dependent phosphate transport
Arsenate is a toxic anion structurally analogous to phosphate anion and has been shown to be a competitive inhibitor of Na\(^+\)-dependent phosphate transport in the small intestine (Villa-Bellosta & Sorribas, 2008b; Villa-Bellosta & Sorribas, 2009c; Villa-Bellosta & Sorribas, 2010a). Arsenate specifically inhibits NaPi-IIb-mediated transport whereas renal proteins NaPi-IIa, NaPi-IIc, PiT1 and PiT2 show low affinity for arsenate (Villa-Bellosta & Sorribas 2008b; Villa-Bellosta & Sorribas, 2010a). Thus, arsenate is not a suitable agent for inhibiting Na\(^+\)-dependent phosphate transport in the kidney. Although arsenate can be used to inhibit Na\(^+\)-
dependent phosphate transport in isolated small intestine, it is also a potent metabolic inhibitor, deeming it unsuitable for in vivo studies. Phosphonoformic acid (PFA) is equally as effective as arsenate and inhibits Na\(^+\)-dependent phosphate transport in both small intestine and kidney (Villa-Bellosta & Sorribas, 2009a; Loghman-Adham et al., 1987; Loghman-Adham, 1996; Forster et al., 1998). PFA is an inhibitor of NaPi-II, but not PiT, mediated transport (Villa-Bellosta et al., 2007; Ravera et al., 2007). PFA and arsenate have no effect on BBM alkaline phosphatase activity or the transport of solutes across the BBM, demonstrating the specificity of these agents for phosphate transport (Yusufi et al., 1986). PFA acts at a site located on the luminal surface of the BBM and its binding to NaPi-II transporters is mainly dependent on sodium concentration, in contrast to arsenate that acts as a solute analogue of PO\(_4^{3-}\) (Szczepanska-Konkel et al., 1987; Villa-Bellosta & Sorribas, 2009a; Loghman-Adham et al., 1987).

Nicotinamide is another compound that dose-dependently inhibits NaPi-IIa- and NaPi-IIb-mediated transport across the BBM (Wu et al., 1988). In rats with renal failure, administration of nicotinamide inhibits intestinal phosphate transport and NaPi-IIb expression across the jejunal BBM, thus preventing the progressive increase in serum phosphate levels (Eto et al., 2005). Similarly, clinical studies in haemodialysis patients showed that nicotinamide was able to reduce and keep serum phosphorus under a similar control level that is seen with current phosphate binders (Takahashi et al., 2004). However, nicotinamide is thought to have some serious side-effects, making it unsuitable for use in man (Takahashi et al., 2004).

1.8. Phosphate efflux across the basolateral membrane

Phosphate efflux across the BLM of absorptive cells in the small intestine and kidney has yet to be characterised, although a number of theories have been proposed such as: an anion exchange mechanism, a type 3 transporter and an "unspecific" phosphate leak pathway (Tenenhouse & Murer, 2003; Tenenhouse, 2007). Basolateral phosphate transport is
bidirectional and has two important functions; when luminal phosphate entry exceeds cellular phosphate requirements phosphate is transported across the BLM; if BBM phosphate uptake is insufficient then basolateral phosphate influx from the extracellular fluid occurs to meet cellular phosphate needs (Murer et al., 2000).

1.9. Kidney

1.9.1. Phosphate absorption in the kidney
Phosphate homeostasis is largely dependent on the process involving the renal reabsorption of phosphate against an electrochemical gradient. This is mediated by the Na\(^+\)-dependent phosphate transporter NaPi-IIa and NaPi-IIc, and potentially PiT1 and PiT2, expressed at the BBM, which act as the rate-limiting step for phosphate entry (Fig. 1.3). Phosphate excretion in the kidney is the balance between free glomerular filtration and renal tubular reabsorption. The kidney specifically expresses NaPi-IIa at the apical membrane of proximal tubular cells, which is largely responsible for the maintenance of phosphate homeostasis and to a lesser importance NaPi-IIc and NaPi-I transporters (Murer et al., 2000; Murer et al., 2004; Tenenhouse & Murer, 2003). This corresponds to high Na\(^+\)-dependent phosphate transport in the proximal tubules, especially in the early segments S\(_1\) and S\(_2\) (Greger et al., 1977; Kayne et al., 1993; Baumann et al., 1975). The experimental techniques of micropuncture, in situ microperfusion and primary cell cultures revealed that Na\(^+\)-dependent phosphate transport is higher in the proximal convoluted tubules, compared with proximal straight tubules and distal regions of the kidney,
Figure 1.3. Renal phosphate reabsorption. At the BBM of proximal tubule cells, four different Na\(^+\)-dependent phosphate transporters, designated as NaPi-IIa, NaPi-IIc, PiT1 and PiT2, mediate phosphate uptake from the lumen. The phosphate efflux pathway at the BLM is so far uncharacterised.

and this was ascribed to a higher \(V_{\text{max}}\) of the Na\(^+\)-dependent phosphate transport system (Berndt & Knox, 1992). Inter- and intra-nephron expression of NaPi-IIa relies on the phosphate status of the body. Short-term regulation of renal phosphate reabsorption is mediated by shuttling mechanisms involving the movement of NaPi-IIa and NaPi-IIc protein-containing vesicles between the cytoplasm and the BBM (Segawa et al., 2005; Picard et al., 2010; Levi et al., 1994; Kempson et al., 1995).
Phosphate reabsorption by the kidney is predominantly regulated by PTH, dietary phosphate intake, serum phosphate levels and phosphatonin, and their effects are mediated through changes in the abundance of NaPi-IIa and NaPi-IIc proteins at the BBM. Thus, physiological and pathophysiological regulation of renal phosphate reabsorption is associated with changes in the expression of NaPi-IIa, and sometimes NaPi-IIc, at the BBM.

Proximal tubular phosphate reabsorption is increased at higher pH (>7), due to the preferential transport of the HPO$_4^{2-}$ phosphate species by NaPi-II transporters (Amstutz et al., 1985; Magagnin et al., 1993). Type I and type III transporters are not thought to play an important role in renal phosphate reabsorption, since their expressions are not increased to compensate for NaPi-IIa loss in NaPi-IIa knockout mice (Hoag et al., 1999). A recent report has shown immunohistochemical evidence of localisation of PiT2 protein at the BBM of the rat kidney (Villa-Bellosta et al., 2009b). The abundance of PiT2 proteins at the renal BBM is regulated by dietary phosphate; Villa-Bellosta et al (2009b) showed the induction of PiT2 protein expression in all segments of the proximal tubules upon dietary phosphate restriction. The time course of regulation of renal PiT2 transporter in response to changes in diet is about 8 hours, which is different from the time taken for NaPi-IIa and NaPi-IIc, suggesting that the regulatory pathway involved in PiT regulation is different to that involved in NaPi-II transporters (Villa-Bellosta et al., 2009b). PiT transporters are physically associated with the actin cytoskeleton network and forms high molecular weight complexes (Salaun et al., 2001; Salaun et al., 2004; Rodrigues & Heard, 1999). Acute post-translational changes in PiT transporter expression at the BBM can occur, e.g. in response to extracellular phosphate concentrations (Rodrigues & Heard, 1999). However, the processes involved are unknown.
In contrast to the slow adaptations seen in small intestine, the kidney is capable of changes in Na\(^+\)-dependent phosphate transport within minutes or hours under altered physiological conditions.

1.9.2 Regulation of renal phosphate reabsorption

1.9.2.1. Dietary phosphate level
A low phosphate diet increases renal phosphate absorption to the maximal rate (Trohler et al., 1976). Thus, in mice a low phosphate diet enhances Na\(^+\)-dependent phosphate transport in the proximal tubules. This adaptation involves changes in NaPi-IIa at protein, but not mRNA, level (Madjdpour et al., 2004). In contrast, both protein and mRNA expression of NaPi-IIc at the BBM is increased (Madjdpour et al., 2004). In rats however, NaPi-IIa protein and mRNA expression are both upregulated (Villa-Bellosta et al., 2009b; Marks et al., 2007; Giral et al., 2009), highlighting again the importance of species differences in the control of phosphate absorption. Dietary phosphate levels control the recruitment of NaPi-IIa and NaPi-IIc proteins to the BBM acutely, within a few hours, in a microtubular-dependent mechanism (Madjdpour et al., 2004; Bacic et al., 2006; Keusch et al., 1998). High phosphate levels stimulate secretion and gene transcription of PTH indirectly (by decreasing 1,25-dihydroxyvitamin D\(_3\) production), and directly (independent of 1,25-dihydroxyvitamin D\(_3\) and calcium levels) (Almaden et al., 1998; Tallon et al., 1996; Hernandez et al., 1996; Keusch et al., 1998). Conversely, dietary phosphate restriction causes a type of “resistance” to the phosphaturic effect of PTH, therefore stimulating renal phosphate reabsorption (Harris & Seely, 1979).

An *in vitro* study showed rapid upregulation of phosphate transport in cultured proximal tubule cells exposed to low phosphate concentrations, indicating an intrinsic ability of these cells to sense and adapt to phosphate levels without the involvement of PTH (Markovich et al., 1995). Furthermore, this ‘phosphate sensing’ effect at the BBM of proximal convoluted tubule may be linked to a calcium sensor and/or
cytosolic calcium concentrations, since changes in phosphate and calcium concentrations are interdependent (Murer et al., 2000).

1.9.2.2. Parathyroid hormone

Regulation of PTH levels is dependent upon two processes: the release of PTH already stored in secretory granules within the parathyroid gland and the other involves the synthesis of new PTH (Fox & Heath, 1981; Habener et al., 1984). Rapid changes in serum PTH concentrations occur through alterations in the release of PTH from secretory granules, which are controlled by the binding of calcium to the calcium-sensing receptor (CaSR) in the parathyroid gland (Pollak et al., 1994; Pollak et al., 1993; Muff et al., 1988). More long-term changes in PTH release rely on the synthesis of new PTH.

1,25-dihydroxyvitamin D₃ inhibits PTH gene transcription and secretion in the parathyroid glands and suppresses parathyroid cell growth (Silver et al., 1986; Cantley et al., 1985; Russell et al., 1984; Silver et al., 1985; Kremer et al., 1989; Szabo et al., 1989). 1,25-dihydroxyvitamin D₃ is thought to act on the PTH gene by increasing Vitamin D receptor (VDR) mRNA in the parathyroid gland (Okazaki et al., 1988). VDR heterodimerises with retinol X receptor (RXR) and binds to vitamin D-response elements within the PTH gene, thus repressing its transcription. VDR mRNA levels in the parathyroid glands is equivalent to the VDR levels present in the duodenum, a main tissue targeted by 1,25-dihydroxyvitamin D₃ (Dupret et al., 1987; Liel et al., 1999).

PTH causes phosphaturia by inhibiting Na⁺-dependent phosphate transport at the renal BBM (Murer et al., 2000). Plasma PTH levels are rapidly regulated, in some cases within minutes. Such regulation was demonstrated in vivo during duodenal gavage of a low and high phosphate diet, which caused changes in plasma PTH levels within 10-15 minutes (Martin et al., 2005). This effect is hypothesised to be mediated
through an unidentified ‘intestinal phosphatonin’, which efficiently links changes in intestinal phosphate levels to altered secretion PTH.

PTH inhibits NaPi-IIa-mediated transport by inducing endocytic retrieval of NaPi-IIa from the renal BBM into the late endosomal/lysosomal compartment, signaling via type I PTH receptor (PTHR1) (Zhao & Tenenhouse, 2000; Bacic et al., 2006). PTH stimulates several signal transduction pathways including extracellular signal-regulated kinases (ERK), which inhibits renal phosphate reabsorption independent of changes in NaPi-IIa expression (Lederer et al., 2000). PTH stimulates ERK, one of the mitogen-activated protein kinase (MAPK) cascades, through two signal transduction pathways: one involving the tyrosine kinase and phosphoinositide kinase-3 (PI3K) and the other pathway dependent on protein kinase C. Thus, PTH inhibits Na⁺-dependent phosphate reabsorption via two distinct mechanisms, one involving the internalisation of NaPi-IIa from the BBM and the other relating to the ERK pathway. PTH bound PTH receptor 1 activates Gα(S)/adenyllyl cyclase/cAMP/PKA (cAMP/PKA) and Gα(q/11)/phospholipase C/phosphatidylinositol 1,4,5-trisphosphate (IP₃/PKC) signaling pathways to mediate its inhibitory action on NaPi-IIa (Nagai et al., 2011). The direct phosphorylation of NaPi-IIa has not yet been demonstrated (Murer et al., 2003). Further events following the activation of the cAMP/PKA and IP₃/PKC pathways are not yet known. PTH also decreases activity of the Na⁺/K⁺-ATPase at the BLM and this may indirectly reduce phosphate transport across the BBM via a reduced sodium electrochemical gradient (Ribeiro & Mandel, 1992).

1.9.2.3. Vitamin D₃
The active form of vitamin D, 1,25-dihydroxyvitamin D₃, is produced in the proximal tubule cells and it stimulates phosphate absorption in the proximal tubule (Berndt & Knox, 1984, 1992). In vitamin D-deficient rats, the genomic and protein expression of NaPi-IIa is significantly decreased in the juxtamedullary kidney cortex, and following 1,25-dihydroxyvitamin D₃ administration, renal Na⁺-dependent phosphate transport is increased
in parallel with elevated NaPi-IIa mRNA and protein levels (Taketani et al., 1998).

1,25-dihydroxyvitamin D$_3$ is converted from the precursor 25-hydroxyvitamin D$_3$ through the activity of 25-hydroxyvitamin D$_3$-1α-hydroxylase (1α-hydroxylase) enzyme, which adds a hydroxyl group to circulating 25-hydroxycholecalciferol. This enzyme is stimulated by PTH, an effect involving the recruitment of the protein kinase A/protein kinase C signalling pathways (Ro et al., 1992; Janulis et al., 1993). Thus, an endocrine feedback loop regulates the synthesis of PTH and 1,25-dihydroxyvitamin D$_3$.

Surprisingly, Mühlbauer et al (1981) showed that 1,25-dihydroxyvitamin D$_3$ inhibits renal phosphate reabsorption in thyroparathyroidectomised (TPTX) rats. Later work showed that this effect of 1,25-dihydroxyvitamin D$_3$ was mediated via an effect on serum levels of a phosphatonin called Fibroblast Growth Factor-23 (FGF-23) (Sommer et al., 2007; Shimada et al., 2004a). Administration of FGF-23 decreases serum phosphate and 1,25-dihydroxyvitamin D$_3$ levels in both normal and TPTX rats (Shimada et al., 2004a). FGF-23 inhibits 1,25-dihydroxyvitamin D$_3$ production by regulating renal expressions of main enzymes involved in the vitamin D metabolism, independently of PTH (Sommer et al., 2007; Shimada et al., 2004a; Shimada et al., 2004b). Evidently, serum levels of 1,25-dihydroxyvitamin D$_3$ is higher in FGF-23 knockout mice (Sommer et al., 2007). Further details are discussed in section 1.12.1.

1.9.2.4. Metabolic acidosis
Phosphate plays a role in the compensation of metabolic acidosis, acting as a buffer in extracellular fluid and more importantly in urine (Stauber et al., 2005). Increases in the release of phosphate from bone, together with renal excretion of protons and absorption of bicarbonate are the major mechanisms involved in restoring acid-base balance during metabolic acidosis (Hamm & Simon, 1987; DuBose & Alpern, 2001).
During metabolic acidosis, renal excretion of phosphate and ammonia is increased to preserve bicarbonate and to excrete excess protons (Tanner, 1984; Guntupalli et al., 1982; Nowik et al., 2008). The increase in phosphate excretion is moderate (from 1.8 to 2.8 millimoles), compared with ammonia excretion (from 1.4 to 5.7 millimoles) (Nowik et al., 2008). Increased urinary phosphate excretion is mediated by inhibition of renal phosphate reabsorption, as supported by the evidence that Na\(^+\)-dependent phosphate transport in renal BBM vesicles is decreased during metabolic acidosis, in parallel with reduced levels of NaPi-IIa mRNA and protein in rats (Ambuhl et al., 1998). This effect is independent of PTH, extracellular fluid volume, natriuresis, plasma phosphate or changes to the phosphate balance (Guntupalli et al., 1982; Ambuhl et al., 1998). Similarly, in mice, metabolic acidosis increases urinary phosphate excretion. However, neither Na\(^+\)-dependent phosphate uptake nor NaPi-IIa and NaPi-IIc protein expression is decreased during metabolic acidosis in mice (Nowik et al., 2008), demonstrating the differential regulation of renal phosphate reabsorption between species. Interestingly, metabolic acidosis increases NaPi-IIc and PiT2 levels in both rat and mice (Nowik et al., 2008; Villa-Bellosta & Sorribas, 2010b) (Fig. 1.4).

1.9.2.5. Other factors influencing renal phosphate transport

In addition to PTH and the vitamin D-endocrine system, bone-derived phosphaturic factors called phosphatonins also play an important role in the regulation of phosphate reabsorption in the kidney (Berndt et al., 2005; Marks et al., 2008; Rowe et al., 2000; Shaikh et al., 2008). These phosphatonins, which are discussed later in this chapter (section 1.12), were identified from studies on a number of diseases associated with hypophosphataemia.

Renal phosphate reabsorption and NaPi-IIa expression are also regulated by other factors, including epidermal growth factor (EGF), glucocorticoids, growth-related hormone and thyroid (Alcalde et al., 1999;
Taketani et al., 1998; Sorribas et al., 1995; Guner et al., 1999; Arar et al., 1999; Arar et al., 1995).

EGF is an important regulator of renal phosphate reabsorption in addition to its importance in DNA synthesis, tissue proliferation and nutrient absorption (Xu et al., 2001). EGF is a polypeptide secreted predominantly by salivary glands and to a lesser extent by the kidney. Inhibition of renal phosphate reabsorption by EGF in vitro and in vivo is mediated by regulation of NaPi-IIa expression at the protein and genomic level (Xu et al., 2001; Han et al., 2003; Arar et al., 1999).
Figure 1.4. Main factors controlling type II and type III phosphate (Pi) transporters in the proximal tubule. Type II and type III transporters are differentially regulated.
The signalling pathway used by EGF in regulating phosphate transport is unknown; it is independent of cyclic adenosine monophosphate (cAMP) but may involve tyrosine kinases (Han et al., 2003).

Insulin-like growth factor 1 (IGF-1) stimulates proximal tubular Na\(^{+}\)-dependent phosphate reabsorption, an effect mediated through the binding of IGF-1 to growth hormone receptors present at the BLM of proximal tubules and subsequent activation of phospholipase C and/or tyrosine kinase pathway (Mulroney et al., 1989; Caverzasio et al., 1990; Hammerman et al., 1980).

Thyroid hormone is another regulator of renal phosphate transport. Thyroid hormone stimulates phosphate reabsorption in the proximal tubules by increasing the genomic and protein expression of NaPi-IIa (Alcalde et al., 1999). The increase in renal phosphate reabsorption is significant enough to increase serum phosphate concentration. Unlike PTH and dietary phosphate, the regulation of phosphate transport by thyroid hormone is more long-term (Alcalde et al., 1999). Thyroid hormone also influences Na\(^{+}\)-dependent phosphate transport in the kidneys of weaning animals, an effect that involves changes in the K\(_t\) and V\(_{\text{max}}\) (Euzet et al., 1995; Euzet et al., 1996; Alcalde et al., 1999). In rats, the stimulatory effect of thyroid hormone is reduced in older rats (Alcalde et al., 1999), which is likely to be related to altered expression of NaPi-IIa.

Glucocorticoids are regulators of a number of responses including immunologic and inflammatory responses, and these hormones also play a role in the regulation of renal phosphate reabsorption. Glucocorticoids act on the proximal tubules and their effect is independent of PTH (Levi et al., 1995). Administration of glucocorticoids increases urinary phosphate excretion whilst decreasing serum phosphate levels (Komiya et al., 1992). This effect involves a reduction in the V\(_{\text{max}}\) of Na\(^{+}\)-dependent phosphate transport in adults only, complemented by a decrease in the
protein and genomic expression of NaPi-IIa (Guner et al., 1999; Levi et al., 1995).

The CaSR is also thought to play a role in regulating renal phosphate reabsorption although the mechanism is unknown. Expression of CaSR occurs in the kidney, and interestingly its BBM expression in the proximal convoluted tubule was altered in parallel with NaPi-IIa expression following changes in dietary phosphate (Riccardi et al., 2000; Brown et al., 1995). Thus, it was proposed that expression of NaPi-IIa and CaSR may be co-regulated during renal phosphate handling. The potential importance of CaSR in phosphate homeostasis was further highlighted by the findings that 1,25-dihydroxyvitamin D₃ alters renal expression of CaSR and activation of CaSR in turn prevents inhibition of PTH-sensitive renal phosphate reabsorption (Ba et al., 2003; Brown et al., 1996).

Renal phosphate reabsorption is also regulated by other hormonal factors such as: 1) PTH-related peptide (PTH analog), which is produced by tumours and causes phosphaturia identical to the PTH pathway (Berndt, 1992), 2) glucagon, which directly, through glucagon receptors expressed in the kidney, and indirectly causes phosphaturia by increasing cAMP concentrations in the plasma (Brubaker & Drucker, 2002; Ahloulay et al., 1996), and 3) prostaglandins, which is produced in the kidney and antagonises the phosphaturia caused by different physiological conditions such as increased PTH levels (Berndt, 1992). Other effects include those by nerve transmitters, since acute renal denervation increases renal phosphate excretion independent of PTH levels (Bello-Reuss et al., 1975; Mann et al., 1992).

1.9.3. Mechanisms involved in cellular redistribution of NaPi-IIa

The internalization of NaPi-IIa protein at the BBM in response to phosphaturic signals occurs by highly specific pathways. As mentioned previously, increases in PTH levels or the feeding of high phosphate diets causes rapid internalisation of NaPi-IIa from the BBM and subsequent degradation in the lysosomes (Murer et al., 2000). For PTH, the hormone
initially binds to its receptor (PTHR), at both BBM and BLM, activating protein kinase A, protein kinase C and cAMP-dependent signalling pathways (Tenenhouse & Murer, 2003). Internalisation occurs at intermicrovillar clefts involving clathrin and adapter protein (AP-2) (Traebert et al., 2000). MAPK and ERK1/2 are also thought to take part in this pathway, since NaPi-IIa internalisation is prevented by ERK1/2 inhibition (Hernando et al., 2005; Tenenhouse & Murer, 2003). FGF-23 inhibits NaPi-IIa-mediated phosphate transport via activation of MAPK (Yamashita et al., 2002). The downstream targets for ERK/MAPK-mediated phosphorylation are unknown.

The last three amino acids (TRL) in the C terminus tail of NaPi-IIa are important for its expression at the BBM (Hernando et al., 2002; Gisler et al., 2001; Gisler et al., 2003). Single PDZ (post synaptic density protein) domains of the Na/H exchange regulatory factors (NHERF1, NHERF2, NHERF3 and NHERF4) interact with TRL residues and play a critical role in ensuring the trafficking of NaPi-IIa transporter to the BBM. Additionally, NHERF1/2 interact with MERM (merlin–ezrin–radixin–moesin) in order to provide a link to the cytoskeleton (Hernando et al., 2010). Further mechanistic details behind the involvement of these proteins have not yet been established.

Besides NHERF proteins, PDZ domains of Shank2E (splice form of Shank2) and GABA$_A$-receptor associated protein (Gabarap) are also thought to play a role in the apical expression of NaPi-IIa (Reining et al., 2007; McWilliams et al., 2005). Shank2E is found at the BBM of proximal tubules (McWilliams et al., 2005). In Gabarap-null mice, NaPi-IIa, but not NaPi-IIc, expression is increased, in parallel with increased Na$^+$-dependent phosphate transport (Reining et al., 2007; McWilliams et al., 2005). Despite the pronounced effect seen in these animal models, the mechanisms behind Gabarap involvement in apical expression of NaPi-IIa are not yet known.
Deletion studies and other work on the involvement of proteins at intermicrovillar clefts such as the microtubular network and clathrin, have shown two amino acids in intracellular loop 3 domain of NaPi-IIa to be important for BBM retrieval (Tenenhouse & Murer, 2003). The microtubular network does not partake in the initial internalization step, but it is pivotal for the transfer of NaPi-IIa containing vesicles to the lysosomes for degradation (Pfister et al., 1998; Lotscher et al., 1999; Keusch et al., 1998). On the contrary, insertion of NaPi-IIa transporter requires an intact microtubular network (Lotscher et al., 1999). NaPi-IIa is not thought to be recycled, but this process cannot be ruled out as a “fine-tuning system” for rapid adaptations to minor fluctuations in PTH and FGF-23 levels (Tenenhouse & Murer, 2003).

The signalling cascade of events leading to insertion or retrieval of NaPi-IIa transporters from the BBM is not completely understood. Interacting proteins involved in the ubiquitination of NaPi-IIa proteins following their retrieval may also play a role in the membrane redistribution of NaPi-IIa. It is not known whether internalisation of NaPi-IIa is preceded by transporter inhibition, as seen with PTH-dependent inhibition of the NHE3 (Tenenhouse 2003). In contrast, mechanisms and proteins involved in BBM expression of NaPi-IIC and PiT2 are not known. One study proposed the interaction of NaPi-IIC with NHERF1 and NHERF3 (Villa-Bellosta et al., 2008a); however the link between this interaction with apical expression of NaPi-IIC was not established.

1.10. Small intestine

1.10.1. Intestinal phosphate absorption
Despite the plethora of knowledge on the mechanisms and regulation of renal phosphate transport, less is known about intestinal phosphate absorption. This is because, in contrast to the kidney, hormonal regulation of intestinal phosphate transport was thought to play only a minor role in phosphate homeostasis.
Enterocytes undergo a process of maturation during transit from the crypt of Lieberkühn to villus tip. Only those enterocytes located in the mid to upper region of the villi are responsible for phosphate uptake (Marks et al., 2007). These cells express NaPi-IIb transporters at the BBM and this process is thought to be the rate-limiting step for phosphate absorption (Berndt et al., 2007; Marks et al., 2006 & 2007). A recent report has shown immunohistochemical evidence of localisation of PiT1 protein at the rat small intestinal BBM (Giral et al., 2009). However, the role of PiT transporters in BBM phosphate absorption remains unclear.

Na\(^+\)-dependent phosphate absorption across the intestinal BBM has been reported to decrease with age in several mammalian species, and NaPi-IIb gene and protein expression also decreases proportionally with age (Xu et al., 2002a; Arima et al., 2002). Na\(^+\)-dependent phosphate transport across the intestinal BBM is higher in suckling animals compared with weanlings and adults (Borowitz et al., 1985; Arima et al., 2002). In contrast, renal BBM Na\(^+\)-dependent phosphate transport is lowest in suckling rats, highest in weanling rats, and lower in adults (Taufiq et al., 1997). This might be attributed to the rapid maturation of the intestinal mucosa during the suckling/weaning transition (Henning et al., 1994), in combination with the change of diet from milk to solid food.

Phosphate absorption across the rat small intestine shows similar regional features to that in the human small intestine in that the highest rate of phosphate transport is seen in the duodenum and jejunum and least in the ileal region (Walton & Gray 1979; Borowitz & Ghishan 1989; Marks et al., 2006). In contrast, in mice maximal phosphate absorption occurs in the ileum with small amounts absorbed in the jejunum and duodenum (Marks et al., 2006; Radanovic et al., 2005). This makes the rat a potentially suitable model for the study of intestinal phosphate transport in man. However, it is important to note that results from in vitro and ex vivo based studies may be biased, since the natural movement of food contents along the intestinal tract does not occur under these conditions. A compartmental mathematical analysis based on an in vivo
technique showed that although phosphate absorption in rats is generally
greater in the duodenum, the ileum is responsible for the largest
proportion of phosphate absorption, about 40% of total absorption,
compared with other regions of the small intestine due the increased
segment length and thus longer transit time (Kayne et al., 1993). The ileal
membrane contains Na⁺/H⁺ exchangers that permit net Na⁺ entry, thus
contributing to the serosa positive potential difference (Kikuchi et al.,
1988). However, no known phosphate transporter proteins are present in
the rat ileum to mediate favoured entry of the negatively charged
phosphate. The study of Kayne et al (1993) defined distal jejunal segment
as ileum in their analysis, thus making their prediction slightly inaccurate.
These studies together present a slightly confused understanding of
region-specific regulation of intestinal phosphate transport. Despite this, it
is now generally accepted that the majority of phosphate is absorbed in
the proximal small intestine of rats (Giral et al., 2009; Marks et al., 2006,

Intestinal phosphate transport is controlled by genomic and protein
changes in the Na⁺-dependent phosphate transporters expressed at the
BBM. NaPi-IIb plays a main role in phosphate absorption across the
small intestine; in mice NaPi-IIb is responsible for over 90% of total active
phosphate absorption (Sabbagh et al., 2009). The absence of NaPi-IIb
gene in NaPi-IIb knockout mice triggers compensatory mechanisms
whereby renal NaPi-IIa expression is significantly upregulated (Sabbagh
et al., 2009). Inhibition of NaPi-IIb in hyperphosphataemic late stage
chronic renal failure successfully reduces serum phosphate levels, thus
proving intestinal NaPi-IIb as a promising target for interventions in
phosphate conditions associated with loss of kidney function (Eto et al.,
2005). NaPi-IIb is regulated predominantly by phosphate diet and 1,25-
dihydroxyvitamin D₃ (Marks et al., 2006; Tenenhouse, 2007; Katai et al.,
1999). Although PTH is a major regulator of NaPi-IIa expression in the
kidney, there is no evidence of any direct effect of PTH on intestinal
expression of NaPi-IIb (Murer et al., 2001). Thus, PTH affects intestinal
phosphate absorption indirectly via its stimulatory effect on renal 1,25-dihydroxyvitamin D$_3$ synthesis.

1.10.2. Regulation of intestinal phosphate absorption

1.10.2.1. Phosphate diet
Low dietary phosphate intake increases the synthesis of 1,25-dihydroxyvitamin D$_3$ via stimulation of the renal 1α-hydroxylase enzyme (Brown et al., 1999; Capuano et al., 2005). Increased 1,25-dihydroxyvitamin D$_3$ levels in turn stimulate Na$^+$-dependent phosphate absorption across the small intestine by increasing expression of NaPi-IIb. In mice, a low phosphate diet increases the protein and gene expression of NaPi-IIb transporter, whereas in rats only NaPi-IIb protein expression is increased (Brown et al., 1999; Capuano et al., 2005) implying control of the transporter at post-transcriptional level.

Studies indicate that a low phosphate diet can upregulate Na$^+$-dependent phosphate transport across the small intestine independently of 1,25-dihydroxyvitamin D$_3$ levels, since vitamin D receptor knockout mice, 1α-hydroxylase knockout mice and 1α-hydroxylase-deficient mice all showed increased intestinal phosphate uptake in response to a low phosphate diet (Capuano et al., 2005; Segawa et al., 2004).

1.10.2.2. Vitamin D$_3$
Several studies have shown that 1,25-dihydroxyvitamin D$_3$ increases Na$^+$-dependent phosphate absorption across the small intestine (Danisi et al., 1980; Fuchs & Peterlik, 1980; Lee et al., 1986b; Matsumoto et al., 1980; Xu et al., 2002a). However, vitamin D deficiency does not cause a large effect on total intestinal phosphate absorption in adult rats, suggesting that 1,25-dihydroxyvitamin D$_3$ plays a minor role in regulating intestinal phosphate handling (Walling, 1977; Lee et al., 1986a; Katai et al., 1999).

In contrast to the genomic effect of 1,25-dihydroxyvitamin D$_3$ on renal NaPi-IIa expression, the effect on the small intestine of adult rats involves
post-transcriptional increases in NaPi-IIb proteins (Hattenhauer et al., 1999; Katai et al., 1999). Interestingly, PiT2 mRNA levels were increased by 1,25-dihydroxyvitamin D₃ in vitro (Katai et al., 1999); the significance of this effect is unknown. In suckling rats however, 1,25-dihydroxyvitamin D₃ treatment increases NaPi-IIb mRNA expression, indicating that the genomic effect of 1,25-dihydroxyvitamin D₃ is age-dependent (Xu et al., 2002a). The responsive region in the promoter and the factors involved in 1,25-dihydroxyvitamin D₃ regulation of the NaPi-IIb gene are so far unidentified.

1.10.2.3. Metabolic acidosis

Compared to the well characterised response of renal phosphate reabsorption during metabolic acidosis, intestinal phosphate absorption has received much less consideration. The study of Gafter et al (1986) showed that metabolic acidosis in rats increased intestinal Na⁺-dependent phosphate uptake, an effect mediated by a concomitant increase in the abundance of NaPi-IIb protein at the BBM. They claimed that metabolic acidosis caused this effect by enhancing the stimulatory effect of 1,25-dihydroxyvitamin D₃ on intestinal phosphate absorption. In mice, the increase in Na⁺-dependent phosphate transport and NaPi-IIb protein expression during metabolic acidosis is seen in the ileum, the region responsible for maximal phosphate absorption in this species (Marks et al., 2006; Stauber et al., 2005). However, in both the rat and mouse, the adaptation to metabolic acidosis does not extend to changes in genomic expression of NaPi-IIb. The importance of intestinal phosphate absorption during metabolic acidosis is not clearly established but increased phosphate transport would help buffer the excess protons and compensate for the loss of phosphate from bone during acidosis.

1.10.2.4. Other physiological factors

In addition to dietary phosphate and 1,25-dihydroxyvitamin D₃, intestinal phosphate absorption is also regulated by other factors such as phosphatoninins (see section 1.12), EGF and glucocorticoids, factors that
also regulate renal phosphate reabsorption. EGF is proposed to be an important part of a system involved in gut morphogenesis and maintenance (Chaillet & Menard, 1999). High affinity EGF receptors are expressed along the gastrointestinal tract and EGF treatment in rats decreases NaPi-IIb mRNA levels by approximately 50% (Xu et al., 2001; Chaillet & Menard, 1999). This transcriptional regulation involves a reduction in the NaPi-IIb gene promoter activity. In some conditions, such as intestinal ischemia or injury-induced hyperphosphataemia, EGF levels are significantly increased as is the tissue utilization of endogenous EGF (Thompson, 1999), implying an important role in the regulation of intestinal phosphate absorption.

Glucocorticoids are also important physiological regulators of intestinal maturation, and plasma levels of glucocorticoids are age-dependent (Henning, 1978). The inhibitory effect of glucocorticoids on intestinal phosphate transport is also age-dependent with higher inhibition of Na⁺-dependent phosphate transport in sucklings. This is in contrast to the effect of glucocorticoids on renal phosphate reabsorption that occurs in adult rats only (Arima et al., 2002). The inhibitory effect of glucocorticoids on intestinal phosphate uptake is mediated through changes in genomic and protein expression of NaPi-IIb.

NaPi-IIb expression is stimulated by glucocorticoid-inducible kinase 1 (SGK1), an effect partly mediated by phosphorylation and therefore inhibition of Nedd4-2 (Neural precursor cell expressed, developmentally down-regulated 4) (Palmada et al., 2004). SGK1 is highly expressed in enterocytes and is activated by IGF-1 and insulin (Kobayashi et al., 1999; Kobayashi & Cohen, 1999). The significance of SGK1 in intestinal phosphate regulation is unclear.

Oestrogen is important in the maintenance of calcium homeostasis. It plays a role in regulating calcium absorption, bone density and 1,25-dihydroxyvitamin D₃ synthesis (Xu et al., 2003b). Oestrogen stimulates intestinal Na⁺-dependent phosphate transport, an effect mediated through
changes in genomic and protein expression of NaPi-IIb (Xu et al., 2003b). Studies have established the presence of oestrogen receptors in the intestinal epithelium, implying the ability of the hormone to regulate gene transcription of proteins involved in \( \text{Na}^+ \)-dependent enterocyte phosphate absorption (Thomas et al., 1993).

CaSR is expressed along the rat and human small intestine but its role in intestinal phosphate absorption is not yet known (Gama et al., 1997; Chattopadhyay et al., 1998).

1.11. Disturbances of phosphate homeostasis

1.11.1. Phosphate deficiency

Abnormal phosphate homeostasis occurs in many characterised clinical disorders. Hypophosphataemia is an important consequence of malnutrition or malabsorption of phosphate, or inherited disorders affecting renal phosphate reabsorption such as X-linked hypophosphataemia and tumour-induced osteomalacia (Bielesz et al., 2004; Amanzadeh & Reilly, 2006). Tumour-induced osteomalacia is an acquired rare syndrome characterised by excessive urinary phosphate excretion, hypophosphataemia, reduced 1,25-dihydroxyvitamin \( \text{D}_3 \) levels, and osteomalacia (Kumar, 2000). Children with the Tumour-induced osteomalacia condition exhibit defective bone and cartilage mineralisation, whereas in adults only bone mineralisation defect is obvious (Drezner, 2001). X-linked hypophosphataemia syndrome has a similar biochemical phenotype to tumour-induced osteomalacia (Kumar, 2000). These disorders are linked in the long term to further clinical features such as bone demineralisation, nephrolithiasis, haemolysis, and reduced myocardial contractility (Amanzadeh & Reilly, 2006; Page et al., 2008; Prie et al., 2002).

A common cause of moderate hypophosphataemia is hyperparathyroidism (excessive PTH secretion), whereas PTH-independent renal phosphate wasting is usually associated with more
severe hypophosphataemic conditions and is either a result of intrinsic proximal tubular defects (Fanconi syndrome) or due to abnormal levels of phosphatoninins (Bando et al., 2009; Gore et al., 2009; Amanzadeh & Reilly, 2006; Pande et al., 2006; Higgins et al., 1990).

1.11.2. Phosphate overload
Chronic renal failure results in a progressive loss of renal function; glomerular filtration rate (GFR) gradually decreases as time progresses. Renal phosphate handling is altered by the progressive decline in the fraction of filtered phosphate that is reabsorbed, normally ~70%. In patients with chronic renal failure, PTH secretion is increased to enhance calcium release from bone. The increase in PTH secretion is triggered by the decrease in ionised serum calcium concentrations, detected by the CaSR, due to high phosphate levels complexing with serum calcium (Brown & MacLeod, 2001). High PTH levels in turn increase urinary phosphate excretion to remove the excess phosphate.

Chronic renal failure suppresses renal 1α-hydroxylase enzyme, thus decreasing 1,25-dihydroxyvitamin D₃ levels in chronic renal failure patients (LaClair et al., 2005). The following mechanisms are responsible for the downregulation of this enzyme in chronic renal failure:

- decreasing functional renal mass, which means lower levels of 1α-hydroxylase is available,
- declining GFR, which may limit the distribution of the substrate 25-hydroxyvitamin D₃ to 1α-hydroxylase (Nykjaer et al., 2001; Willnow & Nykjaer, 2002; Hilpert et al., 2002),
- phosphate retention/hyperphosphataemia (Dusso et al., 2005)
- increasing FGF-23 levels seen in chronic renal failure patients (see section 1.12.1), since FGF-23 directly inhibits transcription of 1α-hydroxylase (Perwad et al., 2007; Shimada et al., 2004a).

Additionally, PTH fragments (N-terminally truncated or C-terminal) are also thought to decrease 1α-hydroxylase enzyme activity (Usatii et al., 2007). Inhibition of 1,25-dihydroxyvitamin D₃ in turn decreases intestinal absorption of calcium and renal reabsorption of calcium and phosphate,
thus impairing bone mineralisation. Since 1,25-dihydroxyvitamin D₃ normally inhibits PTH, the loss of 1,25-dihydroxyvitamin D₃ in chronic renal failure contributes towards the overproduction of PTH.

As renal failure reaches end-stage, where GFR falls below 25 to 40 ml/min and the number of functioning nephrons is very low, renal phosphate reabsorption rate drops to as low as 15% and the failure to excrete the required amount of absorbed/ingested phosphate leads to hyperphosphataemia, hypocalcaemia and vascular calcification (see section 1.11.3 for details on vascular calcification). As hyperphosphataemia stimulates PTH release, over-secretion of PTH eventually leads to hyperparathyroidism as a secondary consequence (Fig. 1.5) (Slatopolsky et al., 1984; Yalcindag et al., 1999; Stanbury, 1981).

Additionally, decrease in CaSR expression in parathyroid glands of chronic renal failure patients is thought to contribute towards secondary hyperparathyroidism (Block et al., 2004; Gogusev et al., 1997). Secondary hyperparathyroidism also now contributes towards hyperphosphataemia by continuing to stimulate the release of calcium phosphate from bone (Levin et al., 2007). Although PTH increases renal phosphate excretion, as renal failure progresses the kidney's ability to excrete phosphate continues to worsen.
Figure 1.5. A model for progression of events in chronic renal failure: a pre FGF-23 paradigm. Arrows ↑ and ↓ indicate increase and decrease. Adapted from Kopple & Massry (2004).

Current treatments for hyperphosphataemia to prevent mortality include dietary phosphate restriction and oral phosphate binders, which helps to reduce plasma levels of PTH and phosphate, and this delays the progression of this condition (Miyamoto et al., 2003; Locatelli et al., 2002; Loghman-Adham, 1999; Loghman-Adham, 2003; Qunibi et al., 2004). However, these treatments are not without side-effects and may even contribute to accelerated vascular calcification, which leads to cardiovascular morbidity (Albaaj & Hutchison, 2003; Loghman-Adham, 1999; Locatelli et al., 2002). For example, intake of aluminium-containing phosphate binders leads to accumulation of aluminium following its
absorption across the small intestine, and causes toxic side effects such as osteomalacia and microcytic anaemia (Cannata-Andia & Rodriguez-Garcia, 2002; Loghman-Adham, 1999). Calcium-containing binders can lead to vascular calcification due to the excessive calcium absorbed (Bushinsky, 2006). Dietary phosphate restriction in uraemic patients, although successful at reducing phosphate retention, may induce protein malnutrition.

Unlike the kidney, chronic renal failure does not affect the intestinal handling of phosphate (Douard et al., 2010; Marks et al., 2007). When dietary phosphate intake was restricted in chronic renal failure rats, circulating PTH levels were lowered and consequently renal NaPi-IIa expression was increased. In contrast, there was no change in intestinal phosphate absorption or NaPi-IIb mRNA level (Douard et al., 2010; Marks et al., 2007). Thus, the small intestine is a promising target in the prevention and treatment of hyperphosphataemia (Marks et al., 2007), making the search for an effective blocker of intestinal phosphate transport important for treating chronic renal failure. However, this search is restricted by our limited knowledge of the processes involved in phosphate absorption across the intestinal tract.

1.11.3. Vascular Calcification
Vascular calcification is one of the common complications of chronic renal failure. It involves ossification (deposition of calcium phosphate products), primarily within the arterial vessel wall, causing them to harden. Vascular calcification contributes towards accelerated cardiovascular disease, which is known to be responsible for the largest cause of death in end-stage renal disease patients. Calcification of VSMCs is stimulated by elevated extracellular levels of phosphate, calcium and PTH, commonly seen in chronic kidney disease (Hayden et al., 2005). The process of calcification during hyperphosphataemia involves osteogenic differentiation of VSMCs into osteoblast-like cells, promoted by the transcription factor Runx2/Cbfa1 (runt-related
transcription factor 2/core binding factor alpha1), which promotes expression of a broad range of genes related to the osteoblast phenotype such as bone Gla protein (BGP) and alkaline phosphatase (Moe & Chen, 2004; Shanahan et al., 1999). An elevated level of phosphate in blood is responsible for driving this phenotypic change (Lau et al., 2010).

Phosphate uptake by VSMCs encompasses both Na⁺-dependent and, to a lesser extent, Na⁺-independent components (Villa-Bellosta et al., 2007). The saturable Na⁺-dependent phosphate transport is mediated via the type III transporters PiT1 and PiT2 (Villa-Bellosta et al., 2007; Villa-Bellosta et al., 2009a; Li et al., 2006). In contrast to the small intestine and kidney, genomic expression of type II transporters in VSMCs is minimal (Villa-Bellosta et al., 2007). In these VSMCs, levels of PiT1 mRNA were slightly higher than those of PiT2, in agreement with previous results (Lau et al., 2010). Increased expression of PiT1 and PiT2 transporters is thought to mediate vascular calcification during chronic renal failure, by permitting the increased entry of phosphate into VSMCs and causing subsequent VSMC osteochondrogenic phenotype change and calcification (Fig. 1.6) (Lau et al., 2010).

Vascular calcification is a dynamic regulated pathological event where many factors are implicated. For example, inflammatory cytokine interleukin-6 and tumour necrosis factor alpha (TNF-α) are of particular importance in vascular calcification because they promote osteogenic differentiation and calcification of vascular cells (Boström, 2005). FGF-23 phosphatonin and Klotho are thought to play an important role in promoting vascular calcification in hyperphosphataemic model, since FGF-23 or Klotho gene disruption is associated with accelerated vascular calcification (Memon et al., 2008). However, it is not yet known whether the effect of FGF-23/Klotho is direct or indirect.
Figure 1.6. The effects of high phosphate concentration on VSMCs. Na\(^+\)-dependent phosphate transporters PiT1 and PiT2 present at the plasma membrane of VSMCs allow cellular phosphate uptake and play a key role in vascular calcification. The potential inhibitory effects of FGF-23/Klotho are indicated.

1.12. Phosphatonins

PTH was considered the most important physiological regulator of renal phosphate excretion until the discovery of phosphatonin in mesenchymal tumours from patients with tumour-induced osteomalacia (Econs & Drezner, 1994; Cai et al., 1994). Phosphatonin, produced primarily by osteoblasts (cells responsible for bone formation), are now known to act as humoral regulators of phosphate metabolism (Berndt & Kumar, 2007; Berndt et al., 2005; Bowe et al., 2001; Kumar, 2002; Yuan et al., 2008). They help circumvent the complications of phosphate overload. Based on a large number of clinical studies, phosphatonin may be crucial for the prevention or reversal of the complications associated with chronic renal failure and hyperphosphataemia.

Phosphatonin are phosphaturic factors that cause increased renal excretion of phosphate, and are linked to phosphate wasting diseases such as tumour-induced osteomalacia, X-linked hypophosphataemia and autosomal dominant hypophosphataemic rickets. These phosphatonin-
related disorders may be genetic (X-linked hypophosphataemia and autosomal dominant hypophosphataemic rickets) or acquired (tumour-induced osteomalacia). In these disorders, phosphatoninns such as FGF-23, FGF-7, secreted frizzled-related protein-4 (sFRP-4) and matrix extracellular phosphoglycoprotein (MEPE) are found to be aberrantly expressed and resistant to inactivation by a number of proteases. These four phosphatoninns inhibit proximal tubular phosphate reabsorption in vitro and in vivo, partly mediated by a decrease in the abundance of NaPi-IIa proteins at the BBM (Berndt & Kumar, 2007; Berndt et al., 2005; Marks et al., 2008). However, only FGF-23 and MEPE have been shown to inhibit phosphate absorption across the small intestine (Marks et al., 2008 & 2010; Miyamoto et al., 2005).

The following sections in this chapter will focus on the linkage of phosphatoninns to the well-studied bone diseases X-linked hypophosphataemia, tumour-induced osteomalacia, autosomal dominant hypophosphataemic rickets, hereditary hypophosphataemic rickets with hypercalciuria and oncogenic hypophosphataemic osteomalacia (OHO), which are all associated with renal phosphate wasting (Tenenhouse & Murer, 2003).

1.12.1. Fibroblast growth factor-23

FGF-23 is a naturally occurring peptide produced in several tissues, including the small intestine, parathyroid, heart, liver and bone (White et al., 2006). In mice, the highest genomic expression of FGF-23 is found in bone (Mirams et al, 2004; Liu et al, 2006). FGF-23 is thought to be important in the regulation of normal renal phosphate handling, since serum levels of FGF-23 are rapidly altered in response to dietary phosphate loading in both rodents and humans (Burnett et al., 2006; Ferrari et al., 2005; Perwad et al., 2005).

Clinical studies showed that in early stage of chronic renal failure, the higher levels of circulating FGF-23 levels prevented the development of hyperphosphataemia by inducing phosphaturia (Juppner et al., 2010).
However, FGF-23 also reduced plasma levels of 1,25-dihydroxyvitamin D$_3$ (discussed later in this chapter), thus contributing to the development of secondary hyperparathyroidism. Furthermore, in patients with end-stage renal disease FGF-23 levels increased with increasing plasma phosphate, and the elevated levels of FGF-23 were linked to bone mineralisation, vascular calcification, left ventricular hypertrophy and mortality (Juppner et al., 2010). An abnormally elevated FGF-23 level is associated with several inherited and acquired hypophosphataemic disorders such as autosomal dominant hypophosphataemic rickets, X-linked hypophosphataemia and tumour-induced osteomalacia, respectively.

Autosomal dominant hypophosphataemic rickets is a rare inherited disorder characterised by renal phosphate wasting, hypophosphataemia, abnormally low or normal Vitamin D$_3$ levels, and rickets or osteomalacia (Imel et al., 2007). Autosomal dominant hypophosphataemic rickets is caused by heterozygous FGF-23 missense mutations in the amino acid residues 176 or 179 (Imel et al., 2007). These residues form part of a protease cleavage site for subtilisin-like proprotein convertase, thus mutations cause resistance of FGF-23 to intracellular cleavage processing. The gain-of-function mutations in FGF-23 gene consequently cause severe hypophosphataemia in autosomal dominant hypophosphataemic rickets patients due to its phosphaturic actions.

X-linked hypophosphataemia is another inherited disorder characterised by impaired renal phosphate handling caused by inactivating mutations in PHEX (phosphate-regulating gene with homologies to endopeptidases on the X chromosome), which encodes for a protein with homology to M13 zinc metallopeptidases (Dixon et al., 1998; Juppner et al., 2010). The physiological substrate of the PHEX gene product is unknown. FGF-23 is one of the main phosphatonin responsible for mediating renal phosphate wasting in X-linked hypophosphataemia. The loss-of-function mutation in PHEX indirectly causes a reduction in the proteolytic degradation of full-length FGF-23; full-length non-processed FGF-23 inhibits renal
phosphate reabsorption (Benet-Pages et al., 2004). Furthermore, inactivating mutations in PHEX have also been linked to elevated proteolytic release of a carboxy-terminal peptide called ASARM (acidic serine-aspartate-rich MEPE-associated motif) from MEPE, and ASARM inhibits bone mineralization (David et al., 2009).

Tumour-induced osteomalacia is an acquired disorder caused by small tumours that express augmented levels of FGF-23 protein and mRNA. Hence patients display severe hypophosphataemia due to the phosphaturic actions of FGF-23 (Jüppner et al., 2010).

In addition to PHEX, DMP1 (dentin matrix protein 1), and ENPP1 (ectonucleotide pyrophosphatase phosphodiesterase 1) are also thought to be upstream regulators of FGF-23 synthesis (Juppner et al., 2010). FGF-23 exerts its inhibitory effect on renal phosphate absorption by binding to FGF receptors (FGFR) that are associated with its co-receptor Klotho (see section 1.12.2), leading to increased phosphate excretion due to decreased expression of NaPi-IIa and NaPi-IIc (Juppner et al., 2010; Shimada et al., 2004c; Larsson et al., 2004). FGF receptors have a widespread distribution, and, therefore, their activation is controlled by the tissue-specific expression of Klotho. FGFR1, and to a lesser extent FGFR4, are the main receptors that mediate the phosphaturic effect of FGF-23 (Gattineni et al., 2009). FGF-23 injection stimulates rapid phosphorylation of MAPK and the expression of the early growth-response gene 1 (Egr-1) in the distal tubules, where Klotho is mainly expressed. These changes are associated, via an unknown pathway, with decreased NaPi-IIa expression in neighbouring proximal tubules (Kuro-o et al., 1997; Farrow et al., 2009).

FGF-23 decreases 1,25-dihydroxyvitamin D₃ levels by increasing renal 24-hydroxylase (enzyme responsible for clearance of 1,25-dihydroxyvitamin D₃ by converting it into 24,25-dihydroxyvitamin D₃) whilst decreasing 1α-hydroxylase (Saito et al., 2003; Kolek et al., 2005; Barthel et al., 2007). In patients with X-linked hypophosphataemia,
abnormally low circulatory levels of 1,25-dihydroxyvitamin D$_3$ are present evident due to elevated FGF-23. The low plasma levels of 1,25-dihydroxyvitamin D$_3$ contributes further towards hypophosphataemia, since 1,25-dihydroxyvitamin D$_3$ is a stimulator of both renal and intestinal phosphate absorption (Palmada et al., 2004; Berndt et al., 2003; Berndt & Kumar, 2007; Bowe et al., 2001; Dobbie et al., 2008; Kumar, 2002; Marks et al., 2008). FGF-23 treatment of wild type mice reduced plasma levels of 1,25-dihydroxyvitamin D$_3$ and inhibited intestinal phosphate transport and NaPi-IIb protein expression (Miyamoto et al., 2005). This action by FGF-23 is dependent on the VDR, since the same response is not elicited in VDR-null mice (Miyamoto et al., 2005). Interestingly, administration of 1,25-dihydroxyvitamin D$_3$ in mice is directly linked to increases in serum FGF-23 levels (Kolek et al., 2005). High dietary phosphate and 1,25-dihydroxyvitamin D$_3$ are, therefore, the two most important stimulators of FGF-23 synthesis (Juppner et al., 2010). Chronic changes in intestinal phosphate transport can also influence circulating FGF-23 levels (Sabbagh et al., 2009). Thus, this feedback loop between FGF-23 and 1,25-dihydroxyvitamin D$_3$ not only links phosphate absorption across the small intestine and kidney together (gut-renal axis), but also proposes that FGF-23 is the main hormone affected by long-term changes in intestinal phosphate transport. However, the processes underlying the intestinal control of circulating FGF-23 are not yet known.

FGF-23 is also an important promising biomarker in uraemic patients, since levels are an accurate diagnostic tool for analysing phosphate homeostasis in normal and pathophysiological conditions. FGF-23 may form an important part of therapeutic interventions in the different stages of chronic renal failure.

1.12.2. Klotho

FGF-23 mediates its effects through FGFR-Klotho complex. At normal physiological concentrations, FGF-23 has very low affinity to FGFR and the FGF-23/FGFR receptor interaction requires the presence of co-receptor Klotho (Yu et al., 2005). Thus, Klotho is essential for facilitating
the phosphaturic effect of FGF-23. The link between FGF-23 and Klotho provide strong evidence for a bone-kidney endocrine axis in the maintenance of phosphate homeostasis (Kuro-o, 2008; Kuro-o et al., 1997). The loss of klotho gene causes resistance to FGF-23, since klotho-deficient mice were resistant to FGF-23 although they expressed augmented serum FGF-23 levels (Tsujikawa et al., 2003; Yoshida et al., 2002).

Only recently the importance of Klotho protein in phosphate metabolism was realised, aided by the revelation that the phenotypes of Klotho-deficient mice were similar to mice deficient in FGF-23 (Kuro-o, 2010; Shimada et al., 2004b). Mice deficient in FGF-23 or Klotho exhibit hyperphosphataemia, soft tissue calcification and premature-aging syndrome (DeLuca et al., 2008; Tsujikawa et al., 2003; Yoshida et al., 2002; Utsugi et al., 2000; Razzaque et al., 2006; Shimada et al., 2004b). These studies revealed an unanticipated link between phosphate homeostasis and the aging process.

The klotho gene is also thought to function as an aging suppressor (Kurosu et al., 2005; Kuro-o, 2010). There are two forms of the Klotho protein; membrane-bound Klotho and secreted Klotho, and they have differential functions. The secreted Klotho acts as a humoral factor responsible for regulating the activity of multiple glycoproteins on the cell surface, such as ion channels and growth factor receptors (i.e. insulin/IGF-1 receptors), while membrane-bound Klotho acts as a co-receptor for FGF-23 (Kuro-o, 2010).

A summary of the linkage between 1,25-dihydroxyvitamin D₃, FGF-23 and Klotho is as follows:
- 1,25-dihydroxyvitamin D₃ binds to VDR forming a heterodimer with another nuclear receptor RXR (Fig. 1.7).
- The VDR-RXR heterodimer then binds to the FGF-23 gene promoter and activates its expression.
- Secreted FGF-23 binds to the Klotho–FGF receptor complex in the kidney and conveys a signal to inhibit the synthesis of 1,25-dihydroxyvitamin D$_3$ and stimulate inactivation of 1,25-dihydroxyvitamin D$_3$.

- In the parathyroid gland, secreted FGF-23 inhibits PTH production and secretion. PTH stimulates 1,25-dihydroxyvitamin D$_3$ levels in the kidney. Therefore, inhibition of PTH by FGF-23 is another route by which FGF-23 reduces serum levels of 1,25-dihydroxyvitamin D$_3$. 
Figure 1.7. A schematic diagram of the gut-renal-bone axis in the maintenance of phosphate (P<sub>i</sub>) homeostasis, mediated by FGF-23, Klotho and 1,25-dihydroxyvitamin D<sub>3</sub>. The active form of Vitamin D (1,25-dihydroxyvitamin D<sub>3</sub>) binds to vitamin D receptor (VDR) and nuclear receptor retinol X receptor (RXR) in bone cells and transactivates the FGF-23 gene promoter activity. FGF-23 secreted from bone binds to Klotho-FGFR complex in the kidney and suppresses 1,25-dihydroxyvitamin D<sub>3</sub> synthesis, by downregulating and upregulating expression of 1α-hydroxylase (Cyp27b1 gene) and 24-hydroxylase (Cyp24 gene) enzyme, respectively at the same time. 1,25-dihydroxyvitamin D<sub>3</sub> stimulates intestinal phosphate absorption by increasing the abundance of NaPi-IIb and possibly PiT2 proteins. Adapted from Kuro-o (2010).
This regulation forms a negative feedback loop that is essential for vitamin D-regulated phosphate homeostasis, since abnormalities in either FGF-23 or Klotho can lead to augmented levels of $1,25\text{-dihydroxyvitamin D}_3$.

Recent work by Hu et al (2010) showed that Klotho caused phosphaturia and inhibited NaPi-IIa in FGF-23 null mice. Hu et al (2010) also demonstrated direct dose-dependent inhibition of NaPi-IIa-mediated transport in vitro, in renal BBM vesicles and opossum kidney (OK) cells. Thus, Klotho has a direct, FGF-23-independent, phosphaturic effect on renal phosphate reabsorption, via a mechanism so far unknown.

1.12.3 Matrix extracellular phosphoglycoprotein

MEPE, a member of the SIBLING (Small Integrin Binding Ligand N-Glycoprotein) family, is abundantly expressed in bone and its phosphaturic effect is mediated by the C-terminal peptide ASARM that is released following proteolysis by cathepsin (Rowe et al., 2004; Fisher & Fedarko, 2003).

Zn-metalloendopeptidase PHEX protects MEPE from cleavage by preventing cathepsin B/protease cleavage (Rowe, 2004). Phex knockout mice displayed increased synthesis and serum levels of MEPE and FGF-23 (Yuan et al., 2008). MEPE was initially cloned from a tumour resected from a tumour-induced osteomalacia patient (Rowe et al., 2000). MEPE is normally expressed in osteoblasts, osteocytes and odontoblasts, with increased expression in Hyp (murine X-linked hypophosphataemia homologue) osteoblasts (as PHEX is defective) and OHO tumours (Rowe, 2004). MEPE is thought to contribute towards pathogenesis of hypophosphataemia in patients with tumour-induced osteomalacia (Rowe et al., 2000 & 2004). MEPE is present in healthy humans and their levels decline with age, correlating positively with serum phosphate and negatively with PTH concentrations (Jain et al., 2004). Thus, MEPE protein is thought to be important for bone and tooth mineralisation in a
normal population. In mice, MEPE knockout causes age-dependent accelerated bone mineralisation and bone formation although serum phosphate and calcium levels are normal (Rowe et al., 2000; Jain et al., 2004; Gowen et al., 2003), whereas MEPE overexpression causes defects in growth and mineralisation (David et al., 2009; Rowe et al., 2000). Administration of human derived-MEPE in normal mice causes dose-dependent hypophosphataemia and hyperphosphaturia (Rowe et al., 2004).

MEPE was demonstrated to dose-dependently suppress renal phosphate reabsorption in vivo, and this correlated with increased phosphate excretion and reduced expression of NaPi-IIa in proximal convoluted tubules (Dobbie et al., 2008; Marks et al., 2008; Rowe et al., 2004). Overexpression of MEPE in mice increases serum phosphate levels by stimulating the expression of renal NaPi-IIa (David et al., 2009). Short-term infusion of MEPE in rats inhibited in vivo phosphate absorption in the jejunum, but not duodenum (Marks et al., 2008). This effect was independent of changes in the blood levels of PTH, 1,25-dihydroxyvitamin D₃ and FGF-23. This study of Marks et al (2008) contradicts a previous report that demonstrated increased serum 1,25-dihydroxyvitamin D₃ levels in mice following treatment with human-derived MEPE (Rowe et al., 2004). Thus, it was proposed that differences in MEPE exposure time causes differential effects (Marks et al., 2008). Additionally, MEPE mRNA was detected in the small intestine and kidney, with higher expression levels in the BBM of proximal convoluted tubules and duodenum (Marks et al., 2010; Ogbureke & Fisher, 2005; Rowe et al., 2000). Thus, MEPE may be secreted into the tubular and intestinal lumen, where it binds to its (so far unidentified) cell surface receptor and cause an autocrine or paracrine effect. A local effect in bone was demonstrated in a recent in vivo study where MEPE acted in an autocrine manner in osteoblasts to inhibit bone resorption following its secretion (David et al., 2009).
Findings from studies using rodents suggest that MEPE may be involved in a complex interplay with FGF-23 and 1,25-dihydroxyvitamin D₃. Changes in serum MEPE levels was reported in healthy subjects who consumed high phosphate diets (Allen et al., 2003). This might be related to 1,25-dihydroxyvitamin D₃ levels, since studies in rodents showed that 1,25-dihydroxyvitamin D₃, which is decreased by a high phosphate diet, inhibits MEPE expression (Argiro et al., 2001). Furthermore, VDR null-mice express augmented levels of MEPE mRNA (Okana T et al., 2003). MEPE expression by osteoblasts is also suppressed by 1,25-dihydroxyvitamin D₃ (Rowe et al., 2004). MEPE also increases FGF-23 production in bone marrow stromal cells (Liu et al., 2007; Argiro et al., 2001; Rowe et al., 2005).

1.12.4. Secreted frizzled-related protein-4
sFRP-4 is a phosphatonin found highly expressed in tumours associated with renal phosphate wasting and osteomalacia (De Beur et al., 2002). sFRP-4 protein causes phosphaturia and hypophosphataemia by decreasing renal phosphate reabsorption and by suppressing the compensatory increases in 1α-hydroxylase enzyme (Berndt et al., 2003). This effect on renal phosphate handling is directly related to a decrease in Na⁺-dependent phosphate transport at the renal BBM as well a decrease in NaPi-IIa protein expression (Berndt et al., 2006). sFRP-4 is expressed in many tissues, including the kidney (Berndt et al., 2003). Augmented levels of sFRP-4 is found in bone and serum of Hyp mice and Phex knockout mice (Yuan et al., 2008).

sFRP-4 is thought to play a role in the long-term regulation of renal phosphate reabsorption following a high dietary phosphate intake, since sFRP-4 protein levels were upregulated in the kidneys of rats fed a chronic high phosphate diet (Sommer et al., 2007). In contrast, levels of sFRP-4 were not altered in animals fed a chronic low phosphate diet.

sFRPs are antagonists for the Wnt proteins, which play a role in the activation of cellular events upon binding to frizzled receptors and
changing the levels of the intracellular messenger β-catenin (Berndt et al., 2003). Based on two studies by Berndt et al (2003 & 2006), sFRP-4 is proposed to function by altering Wnt signalling pathways in proximal tubule cells.

### 1.12.5 Fibroblast growth factor-7

FGF-7, also called keratinocyte growth factor (KGF), was identified in tumours from tumour-induced osteomalacia patients where it was found to be overly expressed (Carpenter et al., 2005). FGF-7 stimulates wound healing and is thought to be a potential tumour marker (Carpenter et al., 2005). FGF-7 is highly expressed in keratinocytes and is also found in the gastrointestinal epithelium, transitional urothelial cells, and type II pneumocytes (Danilenko, 1999; Beer et al., 2000). FGF-7 inhibits Na\(^+\)-dependent phosphate transport in opossum kidney cells, and causes phosphaturia in vivo (Carpenter et al., 2005). However, details behind the mechanism of FGF-7 action are unknown.

### 1.13. Choice of animal

Sprague Dawley rats (SD) were chosen to study intestinal phosphate absorption for several reasons. SD rats are a good model to study developmental changes, since young rats are of manageable size and the small intestine is more easily manipulated compared with mice, where the intestine is very fragile for manipulation of the tissue for uptake studies. The quantity of mucosa that can be retrieved from small intestine of mice for western blotting and other studies is also much less in mice compared with rats.

### 1.14. Aims

- To employ suitable in vitro and in vivo techniques for measuring Na\(^+\)-dependent and Na\(^+\)-independent phosphate transport across the BBM of the rat small intestine.
- To determine the normal luminal unbound phosphate concentrations along the intestinal tract of rats.

- To measure Na\(^+\)-dependent and Na\(^+\)-independent transport in the duodenum, jejunum and ileum under normal luminal phosphate concentrations found in rats, with the use of the validated \textit{in vitro} and \textit{in vivo} techniques.

- To measure Na\(^+\)-dependent and Na\(^+\)-independent transport in the proximal and distal colon under normal luminal phosphate concentrations.

- To determine the effect of a chronic low phosphate diet, aging, chronic renal failure and MEPE treatment on mRNA expression of PiT transporters in the small intestine and kidney.

- To investigate whether the release of a proposed duodenal ‘phosphaturic factor’, following exposure to a high luminal phosphate concentration, causes local effects on intestinal phosphate absorption and to determine whether increased phosphate excretion, as observed by Berndt \textit{et al} (2007), is mediated through changes in BBM abundance of renal NaPi-II transporters.
2.0. Measurement of intestinal phosphate transport using \textit{in vitro} and \textit{in vivo} techniques
2.1. Introduction

There are conflicting views concerning mechanisms involved in intestinal phosphate uptake and these are likely in part to reflect the wide variety of techniques and experimental conditions used. Another confounding factor arises from important species differences in the primary location of phosphate absorption along the intestinal tract. In the rat, phosphate transport occurs maximally in the proximal small intestine, which is similar to the profile in human small intestine (Armbrecht, 1986; Davis et al., 1983; McHardy, 1956; Marks et al., 2006). In contrast, in mice the ileum displays the highest rate of phosphate absorption; only small amounts are absorbed in the jejunum and duodenum (Marks et al., 2006; Radanovic et al., 2005).

Intestinal phosphate absorption occurs via two distinct pathways: paracellular transport involving tight junctions, and transcellular transport. Early studies using brush-border membrane (BBM) vesicles revealed a phosphate transport system that was critically dependent on sodium, with a $K_t$ for phosphate of around 0.1 mM (Berner et al., 1976; Lee et al., 1986b; Loghman-Adham et al., 1987). The type II sodium phosphate cotransporter, NaPi-IIb, was later cloned and characterised, and proposed to be responsible for active, saturable phosphate transport across the intestinal BBM (Hilfiker et al., 1998). However, in vivo studies found no evidence of this Na$^+$-dependent process (McHardy, 1956; Williams & DeLuca, 2007). Similarly, using the everted sac technique, Douard et al (2010) showed no Na$^+$-dependency for phosphate uptake. Furthermore, reports, based mainly on studies using BBM vesicles, indicate that the Na$^+$-dependent transport process is regulated by dietary phosphate level and by aging (Danisi et al., 1980; Giral et al., 2009; Kirchner et al., 2008; Xu et al., 2002a). Figure 2.1 summarises the overall changes induced by a low or high phosphate diet for maintenance of serum phosphate levels within the normal range. The detailed regional-specific adaptation induced under both of these conditions is, however,
not well established in the small intestine, including the regulation of Na\textsuperscript{+}-independent transport.

The aim of this study was to determine the extent of Na\textsuperscript{+}-dependency for phosphate transport in the three regions of the small intestine: duodenum, jejunum and ileum. To achieve this, in vitro and in vivo techniques were employed to measure Na\textsuperscript{+}-dependent phosphate transport at 0.1 mM phosphate. These two techniques were validated by using chronic low phosphate diet and aging, two physiological conditions classically known to regulate intestinal phosphate absorption. Uptake data are of great importance, since there are currently no reliable available antibodies for the detection of NaPi-IIb protein in rats. Therefore, a number of potential antibodies were tested, using tissues from young and adult rats.
Figure 2.1. Diagram demonstrating mechanisms for maintenance of serum phosphate (Pi) levels in conditions of high or low phosphate diet. Adapted from Berndt & Kumar (2007).
2.2. Materials and Methods
Experiments were performed in accordance with national and local ethical guidelines.
During experimentation, animals under anaesthesia were constantly checked for pedal withdrawal to a pinch and/or eye-blink reflex, and 45 mg/kg of the original anaesthetic dose of pentobarbitone sodium was given intraperitoneally to maintain the animal under a deep level of anaesthesia. Rats were killed at end of experiment by using an overdose of anaesthetic (60 mg/kg of pentobarbitone sodium).

2.2.1. Animals and diet
Male Sprague Dawley (SD) rats aged 21 days, 8 weeks, and 16 weeks were obtained from Charles River Laboratories (UK) and used in accordance with the Animals (Scientific Procedures) Act 1986. Control rats were allowed ad libitum access to a standard rodent chow containing 0.52% phosphate (Diet RM1, SDS Ltd, Witham, Essex, UK), and water.

Rats on normal diet were switched to a low phosphate diet containing 0.02% phosphate (Harlan Tekland, Indianapolis, USA) for 7 days prior to experimentation.

2.2.2. Phosphate uptake

2.2.2.1. Phosphate uptake by everted intestinal sleeves
The everted sleeve technique exposes the mucosal surface to the incubation buffer containing radiolabelled phosphate, therefore allowing direct measurement of phosphate uptake across the intestinal BBM. In comparison with in vivo techniques, there is a great reduction of unstirred water layer thickness, owing to vigorous stirring rates of mucosal solutions. The validation of this preparation and optimal conditions for in vitro measurement of solute uptake has been described in detail by Karasov & Diamond (1983). Rats were anaesthetised with an intraperitoneal (i.p) injection of pentobarbitone sodium (45 mg/kg)
Regions of intestine (2-4 cm) representing duodenum (1 cm from stomach), jejunum (beginning at the ligament of Treitz), ileum (5 cm proximal to the caecum), proximal colon (4 cm distal to the caecum) and distal colon (2 cm distal to the caecum) were then removed from the animal. The lumen was flushed through with saline (154 mM NaCl) at room temperature to remove contents, everted on a glass rod and the everted tissue secured to the rod with ligature thread before pre-incubation for 5 min pre-incubation buffer (pH 7.4) containing, in mM: 16 HEPES, 3.5 KCl, 10 MgSO\textsubscript{4}, 1 CaCl\textsubscript{2} and 125 NaCl, which was oxygenated with 100% O\textsubscript{2} and stirred continually at room temperature. The tissue was then incubated at room temperature for 2 min in 2 ml of oxygenated and stirred uptake buffer (Fig. 2.2) containing pre-incubation buffer, with the addition of 10 mM glucose, 0.1 mM KH\textsubscript{2}PO\textsubscript{4} and 0.74 MBq \textsuperscript{32}P (PerkinElmer, Bucks, UK). A period of 2 min was chosen for phosphate uptake, since this time was on the constant phase of uptake (Fig. 2.3) and long enough to allow for adequate uptake across the BBM and equilibration of the adherent fluid, as well as to minimise the efflux of \textsuperscript{32}P to the serosa. Both buffers were rapidly stirred to minimise the effects of static water layers at the mucosal surface. Choline chloride (ChCl) was used as an iso-osmotic substitute for NaCl for sodium-free phosphate uptake solutions (pre-incubation buffer, incubation buffer and wash solutions) and intestinal tissues were flushed with 150 mM ChCl before mounting on glass rods. For some experiments 1 or 10 mM phosphonoformic acid (PFA) was added to the pre-incubation buffer and uptake solution to inhibit NaPi-IIb-mediated phosphate cotransport (Bai et al., 2000; Collins et al., 2004; Ravera et al., 2007; Virkki et al., 2007). Uptake was then terminated by washing the tissue with 10-fold (when phosphate concentration in the uptake solution was less than 1 mM) or 20-fold cold excess phosphate (when phosphate concentration was greater than 1 mM) in 150 mM NaCl or ChCl for 10 min, followed by PBS (phosphate-buffered saline) for 5 min at room temperature. These two washes were sufficient to displace most bound \textsuperscript{32}P on the mucosal surface and at the same time retain absorbed radiolabelled \textsuperscript{32}P in the cells (Karasov & Diamond, 1983; Marks et al., 2007). Incubated tissue
(~100 mg) was weighed and digested overnight in Solvable (Perkin Elmer, MA, USA) and counted in Ultima-Gold using a scintillation counter (Beckman LS2000, Beckman-Coulter Research, Buckinghamshire, UK). $^{32}$P activity in digested samples and in the initial uptake solution was used to calculate tissue phosphate retention in nmoles/100 mg mucosa. For each rat, two sleeves were used from the same region of small intestine for measuring uptake rates in the presence and absence of sodium at 0.1 mM phosphate concentration.

During experimentation, animals were constantly checked for pedal withdrawal and/or eye-blink reflex to a pinch, and 1:4 diluted 45 mg/kg of the original anaesthetic dose of pentobarbitone sodium was given intraperitoneally to maintain the animal under a deep level of anaesthesia. Rats were killed at the end of experiment by using an overdose of anaesthetic (60 mg/kg) of pentobarbitone sodium.
Figure 2.2. Diagram showing *in vitro* preparation for measurement of intestinal phosphate uptake.

Figure 2.3. Time-dependence of intestinal phosphate uptake using the everted sleeve technique. 0.1 mM phosphate was used in the uptake buffer.
2.2.2.2. Transepithelial phosphate absorption

The *in situ* loop method was used to measure transepithelial phosphate absorption, since it has been previously shown to be suitable for measurement of transepithelial absorption of phosphate (Aloia & Yeh, 1985; Marks *et al.*, 2006, 2007, 2008). Marks *et al* (2007) have demonstrated that a 30 min incubation period is suitable for measurement of duodenal and jejunal phosphate absorption. Rats were anaesthetised with an i.p injection of pentobarbitone sodium (45 mg/kg) and their body temperature maintained at 37°C using a thermostatically controlled heating blanket. A 5 cm long segment of duodenum, jejunum, ileum, proximal colon or distal colon was selected (location was as described above for the everted sleeve technique) and cannulated and flushed with 150 mM NaCl or ChCl (at 37°C) to remove contents, followed by air. 500 µl of uptake buffer containing, in mM: 16 Na-HEPES or Na-free HEPES, 140 NaCl or ChCl, 3.5 KCl, 0.1 KH$_2$PO$_4$ and 0.37 MBq $^{32}$P, was instilled into the lumen without causing distension and both ends of the segment were tied off, to achieve a closed loop (Fig. 2.4) and returned to the abdominal cavity. In some experiments 1 or 10 mM PFA was added to the buffer to inhibit NaPi-IIb-mediated transport. Blood was collected after 30 min by cardiac puncture and centrifuged at 1,500 g for 10 min to obtain plasma. The intestinal segment was removed from the animal and mucosal bound $^{32}$P was displaced by flushing through quickly with 40 ml of 10-fold (when phosphate concentration in the uptake solution was less than 1 mM) and 20-fold excess phosphate (when phosphate concentration was over 1 mM) and 40 ml saline at room temperature, using a 20 ml syringe. The mucosa was removed by scraping with a microscopic slide and weighed and digested with Protosol (Perkin Elmer, MA, USA). The radioactivities of both mucosa and plasma were counted using Ultima Gold scintillation fluid. The rates of mucosal phosphate retention and transepithelial transport ($^{32}$P appearance in plasma) were calculated taking into account the initial $^{32}$P activity in the uptake buffer. All uptake experiments were repeated a minimum six times.
2.2.3. Phosphate assay

Blood samples, collected from animals fed a normal and low phosphate diet for 7 days, were centrifuged at 1,500 g for 10 min. Plasma phosphate levels were determined using a Quanichrom phosphate assay kit (BioAssay Systems, Hayward, CA) and used according to the manufacturer’s instructions.
2.2.4. Membrane vesicles

2.2.4.1. Intestinal brush-border membrane (BBM) vesicles
Rats were terminally anaesthetised and three regions of small intestine were removed as follows: 5 cm duodenum (1 cm from the pylorus), 10 cm upper jejunum (3 cm from the ligament of Treitz) and 10 cm ileum (last 10 cm proximal to caecum). All subsequent steps were carried out at 4°C and the methods used for the preparation of vesicles followed those described previously by (Kessler et al., 1978). This technique allows the removal of subcellular contaminants for the separation of intact BBM. The intestinal regions were flushed through with ice-cold saline to remove contents and then opened longitudinally on an ice-cold glass surface, and the mucosa was scraped away using glass microscope slides. The mucosa was added to homogenising buffer (pH 7.1), 28 ml/g mucosa, containing, in mM: 50 mannitol, 2 HEPES (4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid), and 0.25 PMSF (phenylmethane sulphonylfluoride), and homogenised (Ultra Turrax homogeniser, Janke & Kunkel, FRG) at setting 5 for three times at 20 s with a 5 s interval in between. MgCl₂ was added to a final concentration of 10 mM, and stirred on ice for 20 min. The suspension was centrifuged at 3,000 g for 20 min and the resulting supernatant further centrifuged for 30 min at 27,000 g. The pellet was resuspended using a 21-gauge needle in resuspension buffer (pH 7.2) containing, in mM: 300 mannitol, 20 HEPES, 0.1 MgSO₄ and 0.25 PMSF. The suspension was centrifuged at 6,000 g for 15 min and the supernatant further centrifuged at 27,000 g for 30 min. The final pellet represented purified BBM vesicles and was resuspended with the same resuspension buffer (50-150 µl) by 6 passes using a 21-gauge needle and syringe to obtain a final protein concentration of 5-9 mg/ml. When weaning animals were used less mucosal scrapes were available, so samples were pooled. Aliquots of BBM vesicles were immediately frozen in liquid nitrogen and stored at -70°C.
2.2.4.2. Lung membrane vesicles
Lung tissue sections were harvested from normal rats and were used for extraction of a crude membrane fraction, as described by Traebert et al., (1999). All steps were carried out at 4°C. Tissue was homogenized with an Ultra Turrax homogeniser at setting 5 for 1 min in 15 ml of buffer A (pH 7.1) containing, in mM: 300 mannitol, 5 EGTA, and 12 Tris/HCl and 0.5 PMSF. The suspension was centrifuged at 1,000 g for 10 min and the resulting supernatant was diluted with 10 ml of deionised water and further centrifuged at 27,000 g for 30 min. The pellet obtained was resuspended in 20 ml of the same buffer and centrifuged at 27,000 g for 30 min. The final pellet was resuspended in about 2 ml of buffer A by 6 passages through a 21-gauge needle fitted to a syringe to obtain a protein concentration of ~7-9 mg/ml.

2.2.4.3. Determination of protein concentration
Protein concentration was measured using the Bradford assay (Bradford, 1976), designed to assess binding of proteins to Coomassie Brilliant Blue G-250 dye. A calibration curve was obtained by measuring known concentrations of bovine serum albumin (BSA), ranging from 15-90 mg/ml, in 0.1 M sodium hydroxide (NaOH). Protein binding causes the absorption maximum of the dye to shift from 465 to 595 nm, measured using a spectrophotometer (Beckman 650, Beckman-Coulter Research, Buckinghamshire, UK), and the increase is proportional to the protein concentration. Samples were initially diluted 1:10 in 0.1 M NaOH before adding the Bradford reagent.

2.2.4.4. Determination of intestinal purity
Alkaline phosphatase assay was used to calculate an enrichment value for the enzyme in the final BBM vesicles suspension, relative to the initial homogenate, as a measure of purity. It was important to establish that vesicles were highly purified and largely free of basolateral membrane and subcellular components. Alkaline phosphatase activity was measured in the initial homogenate (1:5 dilution) and the final purified BBM vesicles (1:50 dilution) extracted, using the method described by
Forstner et al (1968). The assay is based on the rate of formation of the yellow product p-nitrophenol (p-NP), which is produced by hydrolysis of p-nitrophenylphosphate (p-NPP) in alkaline solution.

As a standard 4-nitrophenol was used, ranging from 50-300 nM concentration; following the necessary dilution of samples and standard with distilled water, 100 µl was aliquoted into fresh tubes to which 500 µl of alkaline buffer (pH 9.4) was added that contained, in mM: 0.05 glycine, 5 MgCl₂ and 1 Zn(O₂CCH₃)₂. After preincubation for 30 min at 37°C, the reaction was terminated by adding 2.5 ml of 1 M NaOH. The change in absorbance resulting from the production of p-NP was read spectrophotometrically at 405 nm. Enzyme activity was expressed as units/mg protein and calculated as shown below:

\[
\frac{\text{BBM alkaline phosphatase activity (nmol)}}{\text{BBM [protein]}} \times \frac{\text{Homogenate}}{\text{BBM alkaline phosphatase activity (nmol)}}
\]

Intestinal BBM vesicles typically showed an alkaline phosphatase enrichment of: Duodenum 10.3 ± 2.1, Jejunum 11.1 ± 2.9, and Ileum 9.9 ± 1.9. There were no segmental differences in the enrichment values (P=0.78, using one-way ANOVA).

2.2.5. Detection of NaPi-IIb protein

2.2.5.1. Western Blotting

BBM vesicle samples (15-100 µg protein) were solubilised in 2 x Laemmli sample buffer (Bio-Rad, CA, USA) containing 5% sodium dodecyl sulphate (SDS – Sigma, Dorset, UK) and/or 5% mercaptoethanol, depending on the degree of denaturation needed. Loaded samples and Kaleidoscope pre-stained molecular weight marker (Bio-Rad, Hemel Hempstead, UK) were electrophoresed on a 10% SDS polyacrylamide gel in running buffer containing, in mM: 25 Tris, 192 glycine and 0.1 % (w/v) SDS-running buffer, at 20 mA for about 1 h. Separated proteins were transferred to Sequi-blot PVDF nitrocellulose membrane (Bio-Rad)
by semi-dry electrophoretic blotting for 1 h at a fixed current of 110-130 mA for 1-1½ h. The membrane was then blocked in 6% skimmed milk in 0.1% PBS-Tween for 1 h at room temperature to remove non-specific protein binding. Following two quick washes with PBS-Tween, membranes were incubated with a number of potential NaPi-IIb primary antibodies overnight on a shaker at 4°C, as listed in Table 2.1. Primary antibody β-Actin only needed 1 h incubation at room temperature.

The membrane blots were washed in PBS-Tween once for 5 min and then twice for 10 min, and incubated with secondary antibody IgG conjugated to horseradish peroxidase for 1 h at room temperature. After washing with PBS-Tween as above, bound antibodies were detected using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech UK Ltd, Bucks, UK) and visualised using a Fluor-S Multimager System (Biorad, Hertfordshire, UK).
Table 2.1: Primary and their secondary antibodies used in Western Blotting

<table>
<thead>
<tr>
<th>No</th>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Species</th>
<th>Regional specificity of epitope</th>
<th>Species-specific epitope</th>
<th>Source</th>
<th>Secondary Antibody</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NaPi-IIb</td>
<td>1:100-1:2000</td>
<td>Rabbit</td>
<td>N terminus</td>
<td>Mouse</td>
<td>Biber J (Institute of Physiology), University Zürich, Switzerland</td>
<td>Donkey anti-rabbit</td>
<td>1:1000-1:2000</td>
<td>GE Healthcare, Buckinghamshire, UK</td>
</tr>
<tr>
<td>2</td>
<td>NaPi-IIb</td>
<td>1:100-1:2000</td>
<td>Rabbit</td>
<td>C terminus</td>
<td>Mouse</td>
<td>Biber J (Institute of Physiology), University Zürich, Switzerland</td>
<td>Donkey anti-rabbit</td>
<td>1:1000-1:2000</td>
<td>GE Healthcare, Buckinghamshire, UK</td>
</tr>
<tr>
<td>4A &amp; B</td>
<td>NaPi-IIb</td>
<td>1:100-1:2000</td>
<td>Chicken</td>
<td>2 of N terminus</td>
<td>Rat</td>
<td>Acologix, California, USA</td>
<td>Rabbit anti-chicken</td>
<td>1:1000-1:2000</td>
<td>Zymed Laboratories, CA, USA</td>
</tr>
<tr>
<td>5</td>
<td>β-Actin</td>
<td>1:5000</td>
<td>Mouse</td>
<td>C terminus</td>
<td>Xenopus laevis</td>
<td>Abcam</td>
<td>Mouse anti-mouse</td>
<td>1:5000</td>
<td>Sigma, UK</td>
</tr>
</tbody>
</table>
Table 2.2: Comparison of protein sequences of primary antibodies from Table 2.1 against rat sequences.

<table>
<thead>
<tr>
<th>No. (from Table 2.1)</th>
<th>Species</th>
<th>Sequences</th>
<th>No. of residues different</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mouse¹</td>
<td>MAPWPELENAQPNGK (1-16)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Rat²</td>
<td>MAPWPELENAHNPNGK (1-16)</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Mouse</td>
<td>QDEGKGQVEVLSMKA (676-690)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>QDEGKGQVEVLMKA (674-688)</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Human³</td>
<td>RSEFRRAGATVHDFFNW (206-225)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>RNEFRRAGATVHDFFNW (207-225)</td>
<td>0</td>
</tr>
<tr>
<td>4A</td>
<td>Rat</td>
<td>TFSQNGEDAPDILKVTD (251-269)</td>
<td>0</td>
</tr>
<tr>
<td>4B</td>
<td>Rat</td>
<td>CKTISVIEENVTPSPDN (303-321)</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>X. laevis⁴</td>
<td>MEDDIALVVDNLSGMCKAGF (1-21)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Rat⁵</td>
<td>MDDDIALVVDNLSGMCKAGF (1-21)</td>
<td>0</td>
</tr>
</tbody>
</table>

¹ Accession number NP_035532
² Accession number NP_445832
³ Accession number NP_001171469
⁴ *Xenopus laevis*, Accession number NP_001082422
⁵ Accession number NP_112406
The membrane was stripped of antibodies bound to the protein, using stripping buffer (Fisher Scientific, Leicestershire, UK) for 5-8 min and washed twice with PBS for 5 min. The membrane was treated as before, starting with the milk blocking step and probed for β-actin.

2.2.5.2. Dot blot
The dot blot technique is based on the same principles as western blotting, but differs in that protein samples are not separated electrophoretically and are spotted directly onto the nitrocellulose membrane. Therefore, it is an easier and less time consuming way of testing antibodies. Two filter papers were initially soaked in PBS and the nitrocellulose membrane in methanol for a few minutes, followed by PBS. Wet membrane was placed directly above the filter papers. Protein samples (50-100 µg), prepared using the same conditions as those described for western blotting (without the coloured loading dye), were applied directly onto the membrane and left to dry overnight. The rest of the procedure followed was the same as that used for western blotting, starting at blocking with milk. NaPi-IIb antibodies were tested using this method.

2.2.6. Statistical analysis
Data are presented as means ± SEM. Unpaired t-test or a one-way ANOVA, as appropriate, was used and group differences were considered statistically significant if P <0.05; n represents the number of samples per group. Post hoc analysis was carried out within groups by using the Bonferroni correction,
2.3. Results

2.3.1. Sodium-dependency of intestinal phosphate transport
Na\(^+\)-dependent phosphate transport in the three regions of small intestine was measured by two techniques; *in vitro* using the everted sleeve method and *in vivo* via blood appearance of radiolabelled phosphate using the *in situ* closed loop technique. For both approaches, experiments were carried out with or without sodium in the uptake buffer. Initially, a concentration of 0.1 mM phosphate was chosen for uptake solutions, in keeping with the known kinetic parameters for NaPi-IIb-mediated phosphate transport. The increase in phosphate uptake using everted sleeves showed a constant relation with time for the initial 2 minutes (Fig. 2.3). Therefore, 2 minutes was chosen as the incubation period for *in vitro* uptake of phosphate. The optimal instillation period for measurement of *in vivo* transepithelial phosphate absorption was previously established as 30 minutes (Marks *et al.*, 2008).

As previously reported, phosphate absorption occurred predominantly in the jejunum (Figs. 2.5 and 2.6); minimal phosphate transport was seen in the ileum, which was not Na\(^+\)-dependent. In the jejunum, Na\(^+\)-dependent transport accounted for 84% of total transport measured *in vitro* (Fig. 2.5) and 67% *in vivo* (Fig. 2.6). In the duodenum, 61% of transport *in vitro* was Na\(^+\)-dependent, but surprisingly Na\(^+\)-dependent phosphate transport was not evident when measured *in vivo*. This is the first *in vivo* observation of Na\(^+\)-dependency of intestinal phosphate absorption. Under Na\(^+\)-free conditions *in vivo*, there is a possibility of back-leak of Na\(^+\) into the lumen, therefore driving Na\(^+\)-dependent phosphate transport under ‘nominally’ Na\(^+\)-free conditions. For this reason, in Chapter 3 the Na\(^+\) concentration in the lumen under Na\(^+\)-free conditions following the 30 minute instillation period was measured.

2.3.2. Inhibition of phosphate transport by PFA
PFA is an established competitive inhibitor of NaPi-IIb-mediated transport. Na\(^+\)-dependent phosphate transport in the jejunum was 84% of
total transport and 10 mM PFA inhibited 66% of the total transport in vitro (Fig. 2.7), in broad agreement with that previously reported (Loghman-Adham et al., 1987). This means that the remaining 18% of Na\(^+\)-dependent transport was PFA-insensitive.

Figure 2.5. Transport of 0.1 mM \(^{32}\)P measured in vitro using duodenal, jejunal and ileal regions of small intestine. Na\(^+\)-dependent (shaded bars) and –independent (open bars) transport was measured. Data are means ± SEM. ***P <0.005 compared with absorption in the same region in the presence of sodium using unpaired t-test. n = 6-9.
Figure 2.6. Transepithelial phosphate absorption measured in vivo by the A: plasma appearance of $^{32}$P and B: mucosal retention of $^{32}$P following 30 min instillation of buffer containing 0.1 mM $^{32}$P into cannulated loops of duodenum, jejunum and ileum. Na$^+$-dependent (shaded bars) and Na$^+$-independent (open bars) transport were measured. Data are means ± SEM. ***P <0.005 compared with absorption in the same region in the presence of sodium using unpaired t-test. n = 6-9.
Figure 2.7. Inhibition of Na\(^+\)-dependent phosphate uptake by PFA, measured in vitro using uptake buffers containing 0.1 mM \(^{32}\)P. Na\(^+\)-dependent (shaded bars) and Na\(^+\)-independent (open bars) transport were measured in the jejunum. Phosphate uptake was also measured in the presence of 1 and 10 mM PFA (hatched bars), which was added to the pre-incubation and incubation buffers. Data are means ± SEM. ***P <0.005 compared with absorption in the absence of PFA and ###P <0.005 compared with absorption in the absence of sodium using unpaired t-test. n = 6-9.
Using the *in situ* loop technique, 67% of the total transepithelial phosphate absorption in the jejunum was Na\(^+\)-dependent and 1 and 10 mM PFA completely inhibited this component (Fig. 2.8). There was no PFA-insensitive component of phosphate uptake evident *in vivo*. Past studies have generally used 10 mM PFA to inhibit NaPi-II mediated transport (Eto *et al.*, 2006; Loghman-Adham *et al.*, 1987). The apparent K\(_i\) for PFA, established from jejunal BBM vesicles, is 1.3 mM (Loghman-Adham *et al.*, 1987). In the study of Loghman-Adham *et al.* (1987), although there was a progressive decrease in Na\(^+\)-dependent transport, maximal reduction in intestinal Na\(^+\)-dependent phosphate transport was achieved using 10 mM PFA. Similarly, a recent study used increasing concentrations of PFA to inhibit Na\(^+\)-dependent phosphate transport in renal BBM vesicles, again measured using 0.1 mM phosphate (Picard *et al.*, 2010). They demonstrated the EC\(_{50}\) for PFA to be in the low millimolar range (~3 mM). However, the present study results show that 1 mM PFA caused the same percentage of inhibition as 10 mM PFA both *in vitro* and *in vivo*.

2.3.3. Response to chronic low phosphate diet

A low phosphate diet increases Na\(^+\)-dependent phosphate absorption across the small intestine via increased NaPi-IIb protein expression (Giral *et al.*, 2009; Hattenhauer *et al.*, 1999). To assess whether the everted sleeve technique was suitable for evaluating the adaptation of intestinal phosphate uptake, experiments were carried out using 0.1 mM phosphate in the uptake buffer with or without sodium, using intestine from rats fed a normal or low phosphate diet for 7 days. A low phosphate diet upregulated Na\(^+\)-dependent phosphate transport in the duodenum and jejunum (Fig. 2.9). Na\(^+\)-dependent phosphate transport adaptations were proportionally greater in the jejunum than duodenum. Interestingly, a low phosphate diet also promoted Na\(^+\)-independent phosphate transport in the duodenum and jejunum by 2 and 3-fold, respectively. Plasma phosphate levels, measured from blood collected from rats fed a normal or chronic low phosphate diet, were similar in the two groups (Table 2.3).
Figure 2.8. Inhibition of Na⁺-dependent phosphate absorption by PFA, measured in vivo using instillation buffer containing 0.1 mM $^{32}$P. Na⁺-dependent (shaded bars) and Na⁺-independent (open bars) absorption were measured in the jejunum. A: plasma appearance of $^{32}$P and B: mucosal retention of $^{32}$P were determined following 30 min instillation period. Phosphate absorption was also measured in the presence of 1 and 10 mM PFA (hatched), which was added to the instillation buffers. Data are means ± SEM. ***P <0.005 compared with absorption in the presence of Na⁺ and PFA using unpaired t-test. n = 6.
Figure 2.9. *In vitro* phosphate uptake by duodenum and jejunum using buffer containing 0.1 mM $^{32}$P with (+) or without (-) sodium. Tissue was taken from rats maintained on a normal (open bars) or low phosphate diet (shaded bars) for 7 days. Results are mean ± SEM. ***P <0.005 compared with normal diet and ###P <0.005 compared with uptake in the presence of sodium under low phosphate diet. *n* = 6.
Table 2.3. Plasma phosphate levels in animals fed a normal or low phosphate diet for 7 days. Results are mean ± SEM. n = 5-6.

<table>
<thead>
<tr>
<th></th>
<th>Plasma phosphate (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal diet</td>
<td>1.09 ± 1.33</td>
</tr>
<tr>
<td>Low phosphate diet</td>
<td>0.98 ± 1.39</td>
</tr>
</tbody>
</table>

Therefore, changes in intestinal phosphate handling in rats fed a chronic low phosphate diet were independent of plasma phosphate levels. Body weight was also similar in both group of animals (normal 255 ± 15 g and low phosphate diet 260 ± 18 g).

2.3.4. Response to aging

Na\(^+\)-dependent phosphate transport and NaPi-IIb expression dramatically decreases with age (Xu et al., 2002a; Kirchner et al., 2008; Arima et al., 2002), whereas the Na\(^+\)-independent component has been reported to increase (Debiec & Lorenc, 1985), or remain unchanged (Armbrecht et al., 1980). The everted sleeve technique was further assessed for its suitability in detecting age-dependent adaptation of phosphate transport. Animals of three age groups were used for the study: 3-week (suckling), 8-week, and 16-week old rats. Na\(^+\)-dependent phosphate transport in duodenum and jejunum were maximal during suckling (Fig. 2.10A and 2.10B), which is consistent with a previous observation of Kirchner et al (2008), in which they reported Na\(^+\)-dependent phosphate transport in weaning rats with a K\(_i\) of 2 mM. Na\(^+\)-dependent phosphate transport in both duodenum and jejunum declined significantly during development to the adult stage, but once animals reached adulthood, no further decline was apparent. In contrast, ileal transport was minimal and transport rates were similar in all three age groups (Fig. 2.10C), demonstrating that this region is not regulated by age. There was no Na\(^+\)-dependent component in the ileum (P=0.6 at 3 weeks, P=0.3 at 8 weeks and P=0.9 at 16
weeks). In addition, age had no significant effect on Na\textsuperscript{+}-independent phosphate transport in any of the three intestinal regions.

Figure 2.10. Na\textsuperscript{+}-dependent (+) and Na\textsuperscript{+}-independent (-) phosphate uptake across A: duodenum, B: jejunum and C: ileum of young (3 week), adult (8 week) and aged (16 week) rats. Experiments were carried out in vitro using 0.1 mM \textsuperscript{32}P in the uptake buffer. Results are mean ± SEM. **P <0.01 and ***P <0.005 compared with Na\textsuperscript{+}-dependent absorption in weaned animals, using unpaired t-test. n = 6.
2.3.5. Detection of NaPi-IIb protein

BBM vesicles represent microvillus membrane prepared free of subcellular contaminants and fibrillar material (Forstner et al., 1968). Intestinal BBM vesicles were prepared for the detection of NaPi-IIb, since the protein is known to be expressed at the apical membrane (Giral et al., 2009). BBM preparations need to be of high purity; alkaline phosphatase (a brush-border marker enzyme) assay was performed to monitor purity. The enrichment values for this enzyme in intestinal BBM preparations were 10 to 18-fold.

A number of different rat antibodies were tested for the detection of NaPi-IIb protein. Both antibodies from Acologix (Fig. 2.11A) were specifically designed against rat NaPi-IIb sequences, whereas all other antibodies were specific for mouse or human NaPi-IIb sequences and were highly homologous to rat, except for 1 or 2 differences in some residues (Table 2.2). Three types of tissues were tested: adult lung membrane, since lungs highly express NaPi-IIb compared with other organs (Traebert et al., 1999), and small intestine from young (Fig. 2.11B) and adult rats.
Young animals express the highest level of age-dependent NaPi-IIb protein in the small intestine (Xu et al., 2002a). The only available commercial NaPi-IIb antibody reported to work in rats was obtained from US Biological. The remainder of the antibodies were gifts from institutes or organisations (Table 2.1) that have been successfully used to detect NaPi-IIb protein in mice.

Denaturing is a common approach used for western blot detection of proteins. The extent of denaturing conditions used can, in some cases, improve the detection of the protein of interest. The different denaturing conditions that protein samples were subjected to prior to electrophoresis are: 5% SDS, 5% SDS and heating at 95°C (2 minutes), 5% Mercaptoethanol with 5% SDS or 5% Mercaptoethanol with 5% SDS and heating at 95°C (2 minutes). The latter method is the severest denaturing condition imposed on the native NaPi-IIb protein. Furthermore, BBM vesicles that have been stored for months at low temperatures and that have undergone repeated freeze-thawing cycles are likely to be degraded. Therefore, fresh BBM vesicles prepared on the day were also used for comparison with those stored at -20°C (Fig. 2.11C). However, clear bands using both western blotting (Fig. 2.11) and dot blot techniques (data not shown) were undetectable with all of the above tissues, antibodies and conditions used.
Figure 2.11. Western Blotting analysis of NaPi-IIb protein in membrane vesicles prepared from lungs and specific regions of the rat small intestine. A: Detection of NaPi-IIb protein in BBM vesicles prepared from intestinal mucosa of adult jejunum using two dilutions of the antibody from Acologix. Samples were heated in SDS and Mercaptoethanol prior to loading. B: Detection of NaPi-IIb protein in lung (L) membrane vesicles, duodenal (3wd) and jejunal (3wj) BBM vesicles from 3 week old sucklings, using 1:1000 dilution of primary antibody obtained from US Biological. Samples were heated in SDS prior to loading. C: Detection of NaPi-IIb in jejunal (J), duodenal (D) and ileal (I) BBM vesicles prepared and stored at -20°C for approximately a month (1) and in BBM vesicles freshly prepared on the day (2) using 1:2000 dilution factor of antibody supplied by J Biber (University of Zurich) directed against the C terminus. β-actin protein was used as loading control. Samples were loaded with SDS.
2.4. Discussion

Hyperphosphataemia is a severe disturbance in phosphate balance, most commonly caused by chronic renal failure. In this disease setting, expression levels of NaPi-IIa in the kidney are significantly decreased, both at mRNA and protein level (Elhalel et al., 2004; Marks et al., 2007), and although a low phosphate diet is commonly used to reduce plasma phosphate levels, this approach to treating the disease is limited by the progressive loss in renal function. In contrast, intestinal phosphate absorption and NaPi-IIb expression have been shown to be unaffected in chronic renal failure (Marks et al., 2007; Douard et al., 2010), making the small intestine a promising target for the treatment of hyperphosphataemia. However, the mechanisms involved in phosphate absorption are still not well understood, partly due to species differences and the limitations of the techniques used for assessing uptake. To date, information on the regulation of phosphate transport has employed mainly in vitro techniques to study changes in Na\(^+\)-dependent phosphate transport. In vivo studies are needed to confirm that regulatory changes at the BBM correspond to overall changes in transepithelial phosphate absorption. The aim of this study was to characterise further the relative contribution of Na\(^+\)-dependent and Na\(^+\)-independent phosphate transport in different regions of the rat small intestine using both in vitro and in vivo approaches. The results reveal that, in keeping with previous reports, the majority of phosphate transport occurs in the jejunum, both in vitro and in vivo, and compared with other regions of the small intestine the jejunum exhibits by far the greatest adaptation to a chronic low phosphate diet and to aging.

Studies using intestinal BBM vesicles revealed a phosphate transport system critically dependent on sodium, with a K\(_t\) for phosphate of around 0.1 mM (Danisi et al., 1980; Lee et al., 1986b; Loghman-Adham et al., 1987; Harrison HE & Harrison HC, 1963). Therefore, most studies to date have used phosphate concentrations of around 0.1 mM to investigate
features of the transport process. The present study also used this phosphate concentration in uptake buffer, so that the results can be compared with previous work. This has enabled conclusions to be made on the suitability of the in vitro everted sleeve and in vivo in situ closed loop technique for uptake studies.

Phosphate absorption in rats occurs in the proximal small intestine, the duodenum and jejunum, as also observed in man (Walton & Gray, 1979; Marks et al., 2006; Davis et al., 1983). Data presented in this chapter show that both in vitro and in vivo the duodenum absorbs significant levels of phosphate, although maximal phosphate absorption does not occur in this region of the small intestine, in contrast to the in vivo observation by Marks et al (2006). The in vitro technique used in this chapter shows that duodenal phosphate transport is a combination of Na⁺-dependent and Na⁺-independent pathways, as established previously (Debiec & Lorenc, 1985; Giral et al., 2009; Loghman-Adham et al., 1987). Conversely, the in vivo technique illustrates that duodenal transepithelial phosphate absorption is completely Na⁺-independent. The reason for this discrepancy is not clear. Unstirred water layer effects and/or sodium ion secretion in the duodenum may have influenced Na⁺-dependent transport under nominally Na⁺-free conditions (Dugas et al., 1975). Previous results have consistently reported that the rat ileum has little or no capacity to absorb phosphate (Marks et al., 2006; Peters & Binswanger, 1988). In agreement, data presented in this chapter show minimal ileal phosphate transport, which importantly is not Na⁺-dependent. In contrast, the jejunum is responsible for maximal phosphate absorption, which is Na⁺-dependent, as established previously in BBM vesicle systems (Loghman-Adham et al., 1987). This correlates with the detection of the highest level of NaPi-IIb mRNA in the jejunum (Giral 2009). Thus, both in vitro and in vivo techniques give very similar results for phosphate uptake, except in the case of the duodenum.

PFA is a competitive and specific inhibitor of NaPi-IIb-mediated phosphate transport (Bai et al., 2000; Loghman-Adham et al., 1987 &
1993; Ravera et al., 2007). This study used PFA to dissect the contribution of NaPi-IIb and PiT transporters, as some claim that the latter proteins are insensitive to PFA (Collins et al., 2004; Ravera et al., 2007; Villa-Belostoa & Sorribas, 2009b).

A potential role of PiT transporters in phosphate handling by the small intestine has only just started to emerge. Expression of PiT1 mRNA in the small intestine and PiT1 protein at the intestinal BBM have been reported in rats (Giral et al., 2009). In contrast, expression of PiT2 mRNA is minimal along the rat small intestine (Giral et al., 2009), but a regional profile remains to be established. The $K_t$ for both PiT1 and PiT2 has been estimated to be between 25-89 µM (Bai et al., 2000; Kavanaugh et al., 1994; Tatsumi et al., 1998), but a functional role for these proteins in intestinal phosphate absorption has not been confirmed. The *in vitro* and *in vivo* experiments of the present study were designed to test two concentrations of PFA, 1 and 10 mM, that have been used in past work to inhibit Na$^+$-dependent phosphate transport using 0.1 mM luminal phosphate in uptake buffer (Szczepanska-Konkel et al., 1986; Shillingford et al., 1996; Picard et al., 2010; Loghman-Adham et al., 1987; Loghman-Adham et al., 1992). Data presented in the present study show that 1 and 10 mM PFA reduced NaPi-IIb-mediated transport by the same amount *in vitro* and *in vivo*. This finding is in contrast to the progressive decline in Na$^+$-dependent phosphate cotransport observed by Loghman-Adham *et al* (1987) using jejunal BBM vesicles. Past studies using PFA have consistently reported that 20-30% of total transport under *in vitro* condition is not inhibitable by PFA, regardless of the PFA concentration used (Szczepanska-Konkel *et al*., 1987; Picard *et al*., 2010; Loghman-Adham *et al*., 1987). In agreement, the residual PFA-insensitive Na$^+$-dependent phosphate transport apparent *in vitro* in the present study was approximately 18%, which may represent PiT-mediated phosphate transport. However, in contrast, PFA completely inhibits the Na$^+$-dependent component of phosphate absorption measured *in vivo*. Thus, under *in vivo* conditions 1-10 mM PFA may completely inhibit NaPi-IIb-mediated transport.
The effects of age and dietary phosphate restriction on phosphate absorption in different regions of the rat small intestine were studied to confirm the suitability of the everted sleeve technique for assessing changes in phosphate transport. Previous studies indicate that in man and rats, phosphate absorption occurs maximally in the jejunum and that regulation of intestinal phosphate absorption by 1,25-dihydroxyvitamin D₃, MEPE and chronic dietary phosphate manipulation occurs exclusively in this region (Davis et al., 1983; Giral et al., 2009; Jungbluth & Binswanger 1989; Marks et al., 2006, 2007 & 2008; Walling, 1977). Data presented in this chapter, using the everted sleeve technique, confirm and extend these previous findings. In keeping with previous reports, the highest rate of Na⁺-dependent phosphate absorption was seen in the jejunum, and adaptations to chronic dietary manipulation occur maximally in this region. It should also be noted that the slower transit time of contents in the jejunum compared with duodenum (Kayne et al., 1993) would accentuate this regional specificity. Low phosphate diet stimulates Na⁺-dependent phosphate transport by increasing the recruitment of Na⁺-dependent phosphate transporters at the BBM (Katai et al., 1999; Loghman-Adham et al., 1993). Thus, low phosphate diet increases the $V_{\text{max}}$ without affecting the $K_t$ of Na⁺-dependent phosphate transporters involved (Kempson & Dousa, 1979; Cheng et al., 1983; Katai et al., 1999). Giral et al (2009) showed, using BBM vesicles, the duodenum to be capable of adaptation to acute, but not chronic, dietary phosphate manipulation. However, experiments reported in the present study detected adaptation in the duodenum in animals chronically fed a low phosphate diet. Plasma phosphate concentration in these animals was not different from those fed a normal phosphate diet, demonstrating that adaptation of the small intestine to dietary phosphate restriction is independent of changes in plasma phosphate levels, as established previously (Capuano et al., 2005; Katai et al., 1999; Loghman-Adham et al., 1993).
Past studies have demonstrated that the Na\(^+\)-dependent phosphate transport system in the small intestine has similar characteristics, but is also distinct from, the cotransporter system in renal proximal tubules. Phosphate uptake in BBM vesicles independent of Na\(^+\) is much greater in the small intestine compared with that in the kidney (Loghman-Adham et al., 1987). Using BBM vesicles and other in vitro approaches, the Na\(^+\)-independent component of intestinal phosphate transport was until now thought to be unregulated (Douard et al., 2010; Fuchs & Peterlik, 1980; Katai et al., 1999; Lee et al., 1986b; Walling, 1977). Interestingly, the in vitro approach used in our studies revealed that dietary phosphate deprivation increased the Na\(^+\)-independent component of phosphate absorption. The precise identity of this component, which is usually designated in the literature as ‘paracellular’, is uncharacterised, partly due to the relatively minor role it plays in contributing towards phosphate absorption at low (0.1 mM) phosphate concentration (Berner et al., 1976; Loghman-Adham et al., 1987; Sabbagh et al., 2009). It is therefore important to evaluate this component in detail.

BBM vesicle studies have established that Na\(^+\)-dependent phosphate transport is greatest in the small intestine of young animals and progressively declines during the development process (Xu et al., 2002a; Arima et al., 2002). This might reflect growth-related requirements for phosphate. Uptake data is paralleled in the increased expression of NaPi-IIb (Arima et al., 2002; Kirchner et al., 2008; Xu et al., 2002a). Using BBM vesicles from rats of age 3, 4 and 7 weeks, Debiec & Lorenc (1985) demonstrated that Na\(^+\)-dependent phosphate absorption predominated in younger rats, and as the animals got older the Na\(^+\)-dependent component decreased in importance, whilst the Na\(^+\)-independent component became more dominant. They observed in adult rats phosphate absorption was mediated solely via a process that was attributed to diffusion. In contrast, data presented in this chapter show that active Na\(^+\)-dependent phosphate transport predominates at all age groups, from young to older rats, although Na\(^+\)-dependent phosphate transport declined with age. Unlike the study of Debeic & Lorenc (1985), present results were derived from
intact tissue. Furthermore, in the present study aging did not influence the Na\(^+\)-independent component, in keeping with the previous finding that the contribution of Na\(^+\)-independent transport to overall intestinal phosphate absorption in young animals is low (Arima et al., 2002; Kirchner et al., 2008). Importantly, data also show that although Na\(^+\)-dependent phosphate transport was significantly higher in the duodenum and jejunum of younger rats, absorption does not decline further once the animals reach adulthood.

The ileum is generally considered to play only a minor role in phosphate absorption in rats (Marks et al., 2006). Debiec & Lorenc (1985) claimed that ileal BBM vesicles do absorb phosphate and that the process is age-regulated; uptake is predominantly Na\(^+\)-dependent in younger rats, whilst in adults the rate of ileal phosphate transport is much lower and mediated by diffusion. Data presented in this chapter failed to find significant Na\(^+\)-dependent phosphate transport in the ileum of any of the age groups analysed. This contradictory finding to that of Debiec & Lorenc (1985) further highlights the limitations of the BBM vesicle uptake method that has been used almost exclusively to study phosphate transport.

The transport data obtained using the everted sleeve technique in the present study accurately reflects past findings concerning the effects of dietary phosphate restriction and age on phosphate absorption across the small intestine, and extends that knowledge. The technique is a more suitable method for the study of phosphate transport since experimental conditions can be more easily manipulated, in contrast to the in vivo technique, where ion secretion and unstirred water layer effects may pose problems for the interpretation of uptake data (Dugas et al., 1975). However, in vivo studies are needed to obtain a picture of phosphate transport across epithelium under both physiological and pathological situations. Also, since the blood supply is intact, the effect of circulating influences on phosphate uptake can be assessed.
The paracellular pathway may be responsible for Na\(^+\)-independent phosphate absorption detected in the small intestine \textit{in vivo}. The paracellular pathway consists of tight-junctions, which are paracellular ‘pores’ that allow passage of certain molecules based on charge and size. The replacement of sodium with choline, under Na\(^+\)-free conditions in the experiments presented in this thesis, would have changed the transepithelial electrical potential difference across the epithelium due to a positive serosal-to-mucosal Na\(^+\) gradient. As Na\(^+\) moves from serosa-to-mucosa, the serosa becomes increasingly more negative and therefore there is less likelihood for paracellular movement of the negatively charged phosphate ion in the mucosal-to-serosal direction. Thus, the reduction in phosphate transport seen under Na\(^+\)-free conditions may be a combination of both transcellular (because of reduced NaPi-IIb-mediated transport) and paracellular (because of change in epithelial polarity) transport of phosphate. The influence of transepithelial potential difference on the movement of ions has been observed in man (Gustke \textit{et al}., 1981).

A number of tight junction proteins are responsible for controlling the function of the tight epithelia, including claudin-1, claudin-3, claudin-4, claudin-5 and claudin-8 (Furuse \textit{et al}., 2002; Van Itallie \textit{et al}., 2001; Amasheh \textit{et al}., 2005 & 2009). In addition, there are other members of the claudin family, such as claudin-2 and claudin-12, which form a paracellular channel and thus mediate paracellular transport of certain ions and molecules (Amasheh \textit{et al}., 2002). Expression of claudins varies between species (Rahner \textit{et al}., 2001). Claudin-2, claudin-7 and claudin-12 mediate paracellular permeability (Amasheh \textit{et al}., 2002; Fujita \textit{et al}., 2006). The rat colon strongly expresses claudin-1, claudin-3, claudin-4, claudin-5 and claudin-8, whereas ileum has a high expression of claudin-2, claudin-7 and claudin-12, which control paracellular cation permeability (Markov \textit{et al}., 2010). The jejunum expresses claudin-5 and claudin-12, permitting paracellular cation permeability, whereas duodenum highly express claudins associated with “tightening” of the junctions; claudin-1, claudin-3 claudin-4 and claudin-8. Thus, it is not surprising that the
duodenum has a high epithelial resistance compared with other regions of the small intestine. Expression of claudin-2, a paracellular channel that is highly cation-selective, increases distally along the small intestine (Amasheh et al., 2002; Markov et al., 2010). The cation-selective claudin-2 and claudin-12 act as paracellular Ca\(^{2+}\) channels in the small intestine and their expression is regulated by 1,25-dihydroxyvitamin D\(_3\) (Fujita et al., 2008). No anion-selective claudins have yet been analysed to see if they are capable of transporting the phosphate ion.

It is important to establish that changes in gene expression and transport activity are supported by protein levels of corresponding transporters. Although there are several commercial antibodies available to detect NaPi-IIb expression in mice, none are available in rats. A number of potentially suitable NaPi-IIb antibodies were tested in rat BBM. Membrane vesicles were prepared from lungs, since this tissue is known to more highly express NaPi-IIb at the apical membrane than the small intestine (Feild et al., 1999; Hashimoto et al., 2000). Intestinal BBM vesicles from young animals were also used, since they have been reported to express a higher level of NaPi-IIb than adult rats. However, irrespective of the type of tissue used, none of the antibodies tested were effective at detecting NaPi-IIb protein in BBM vesicles using western blotting and dot blot techniques. Although some of these antibodies are known to successfully detect NaPi-IIb in mice (Antibody no 1 and 2 from Table 2.1) and man (Antibody no 3 from Table 2.1), they were unable to detect the same protein in rat BBM. The reasons behind this failure are unclear at the present time.
2.5. Conclusion

The data obtained indicate that the everted sleeve technique is suitable for the study of phosphate transport across the BBM of intact intestine. The *in situ* closed loop technique is also sensitive enough to detect and distinguish between Na\(^+\)-dependent and Na\(^+\)-independent phosphate transport *in vivo*. The disparity in the overall contribution of Na\(^+\)-dependent transport with these *in vitro* and *in vivo* techniques highlight the necessity to use both approaches to study intestinal phosphate transport, and may also explain the discrepancies in the literature regarding the mechanisms of phosphate transport. Results presented suggest that the everted sleeve technique is comparable to using BBM vesicles, in as much as Na\(^+\)-dependent transport is readily measurable and quantifiable. Additional studies are needed to gain insight into the mechanisms and regulation of the Na\(^+\)-independent component in the duodenum and jejunum that is upregulated by a chronic low phosphate diet. This may help develop novel therapeutic agents that could be used to target the different components of the overall transport process to control hyperphosphataemia.
3.0. Variations in Na\(^+\)-dependent and Na\(^+\)-
independent phosphate transport along the small
intestine and colon.
3.1. Introduction

Data presented in Chapter 2 showed that intestinal phosphate absorption involves both Na\(^+\)-dependent and Na\(^+\)-independent components and the latter may involve paracellular transport. Previous conflicting views on the mechanisms involved in phosphate transport are likely to reflect the variety of experimental techniques used for assessing uptake, as well as widely differing phosphate concentrations present in uptake buffers. Indeed, there is very little information concerning the normal postprandial phosphate level in the intestinal lumen.

Although *in vitro* studies using BBM vesicles clearly demonstrated a saturable Na\(^+\)-dependent process with a $K_t$ of 0.1 mM, *in vivo* studies using very high luminal phosphate concentrations (up to 100 mM) revealed a linear relationship between phosphate concentration and its absorption, with no evidence for a saturable process (Aloia & Yeh, 1985; Davis *et al.*, 1983; McHardy, 1956; Williams & DeLuca, 2007; Walton & Gray 1979). These and related studies concluded that paracellular transport was the dominant absorption pathway at phosphate concentrations in the high millimolar range. However, recent studies using NaPi-IIb knockout mice have reported that NaPi-IIb-mediated transport in the ileum, the region responsible for majority of intestinal phosphate transport in mice, accounts for 90% of total absorption (Sabbagh *et al.*, 2009). Interestingly, in rats oral administration of phosphonoformic acid (PFA), a competitive inhibitor of NaPi-IIb, had no effect on BBM phosphate transport using rats maintained on a normal phosphate diet, but it blunted the increased Na\(^+\)-dependent phosphate transport induced by a low phosphate diet (Loghman-Adham *et al.*, 1993). Taken together, these studies raise the question of the identity of the transport pathway responsible for the non-NaPi-IIb component of phosphate absorption in rats.
The colon has hitherto not been considered to play a major role in phosphate absorption; however, paracellular phosphate transport has been reported in the distal colon (Lee et al., 1980; Hu et al., 1997). Hu et al. (1997) demonstrated permeation of phosphate through the paracellular, mannitol permeable pathway using the in vitro Ussing technique. More recently, NaPi-IIb, PiT1 and PiT2 mRNA have been detected in rat distal colon, with expression levels regulated by dietary phosphate content (Capuano et al., 2009). Interestingly, 1,25-dihydroxyvitamin D₃ stimulates active calcium absorption in the proximal colon of vitamin D-replete rats in vitro (Lee et al., 1980), implying the presence of the vitamin D receptor in colonocytes. The fact that in man high phosphate-containing enemas can induce hyperphosphataemia (Carl & Mitchell, 2007; Hu et al., 1997; Hunter et al., 1993) also indicates the existence of an absorptive route for rapid translocation of phosphate across the colonic mucosa.

The aim of the present study was to address some of the discrepancies emerging from previous studies of intestinal phosphate absorption. Since very little is known about the postprandial concentration of phosphate in the intestinal lumen, unbound phosphate levels were measured in luminal contents collected from specific intestinal regions of rats maintained on a normal phosphate diet. Based on these values, in vitro and in vivo methods were then used to determine the contribution of Na⁺-dependent and Na⁺-independent phosphate absorption in defined regions of rat small intestine. The contributions of the colon to phosphate uptake and the pathways involved have also been investigated.
3.2. Materials and Methods

3.2.1. Animal models
Male Sprague Dawley (SD) rats aged 3 weeks, 8 weeks and 16 weeks were obtained from Charles River Laboratories (UK) and used in accordance with the Animals (Scientific Procedures) Act 1986. Control rats were allowed ad libitum access to a standard rodent chow, containing 0.52% phosphate (Diet RM1, SDS Ltd, Witham, Essex, UK), and water.

3.2.2. Phosphate uptake

3.2.2.1. In vitro phosphate uptake using everted intestinal sleeves
The protocol differed from that described in Chapter 2, section 2.2.2.1 in that the incubation buffer contained 0.1-10 mM phosphate. For each rat, adjacent sleeves of the particular segment of small or large intestine were used for measuring uptake in the presence or absence of Na⁺ at each luminal phosphate concentration. 1, 10 or 20 mM PFA in uptake buffer was used to inhibit NaPi-IIb mediated transport.

3.2.2.2. In vivo transepithelial phosphate absorption
The protocol differed in that described in Chapter 2, section 2.2.2.2 only in that the instillation buffer contained phosphate in the concentration range 0.1-5 mM. 1, 10 or 20 mM PFA in uptake buffer was used to inhibit NaPi-IIb-mediated absorption.

3.2.3. Measurement of intestinal passive permeability
Experiments were repeated as described in Chapter 2.2.2.1 and 2.2.2.2 except that Na⁺ in the uptake buffer was substituted with choline chloride (ChCl) to determine Na⁺-independent phosphate uptake. In other experiments, buffer contained trace amounts of tritiated (³H) mannitol (0.74 MBq/1 ml buffer) and 2 mM KH₂PO₄ and 1 mM mannitol. The wash solution contained, in mM: 20 mannitol, 40 KH₂PO₄ and 150 ChCl. Mannitol was used as a passive permeability marker due to its low
molecular weight, non-toxicity, hydrophilic and lipophobic properties (Ukabam & Cooper, 1984; Krugliak et al., 1994).

### 3.2.4. Measurement of sodium concentration in mucosal fluid

This was carried out to assess the extent of Na\(^+\) secretion into initially Na\(^+\)-free buffer. Uptake solutions made Na\(^+\)-free by isosmotic replacement of Na\(^+\) with ChCl and containing 2 mM phosphate were instilled into cannulated sections of jejunum, ileum and colon, as described elsewhere (Chapter 2, section 2.2.2.2) and Na\(^+\) concentration in the luminal solution after 30 min incubation was measured using CoroNa™ Green dye (Invitrogen). A calibration curve was obtained using known concentrations of sodium up to 25 mM, and ChCl-HEPES buffer was used as a blank. 0.25 µM of dye was added to 100 µl of standard and sample solutions, and fluorescence was read at 492 nm using a spectrofluorimeter (SFM 25, Kontron Instruments, UK).

For *in vitro* measurement of the extent of Na\(^+\) secretion, Na\(^+\)-free uptake buffer containing 2 mM phosphate was incubated with everted jejunum, ileum and colon, as described elsewhere (section 2.2.2.1) and Na\(^+\) concentration in the uptake buffer after 2 min incubation was measured using CoroNa™ Green dye.

### 3.2.5. Osmolarity measurements

Osmolarity of uptake solutions were determined using freezing point depression osmometry using a micro-osmometer (Model 3M0, Advanced Instruments Inc, Massachusetts, US). All solutions used for studies of phosphate uptake were isotonic, within the range 290-310 mOsM.

### 3.2.6. Phosphate level in the intestinal lumen.

Intestinal contents were squeezed out of defined regions of the gastrointestinal tract of unfasted adult rats maintained on normal chow. The contents were diluted 1:1 with distilled water and vortexed vigorously. Solid contents from ileum and colon were diluted 1:1 and homogenised for 10 sec (Ultra Turrax homogeniser, Janke & Kunkel,
FRG). Samples were centrifuged at 6,000 rpm for 6 min and an aliquot of the supernatant was used to determine free (unbound) phosphate concentration using a Quantichrom phosphate assay kit (BioAssay Systems, Hayward, CA, USA), according to the manufacturer’s instructions.

3.2.7. Statistical analysis.
Data are presented as means ± SEM. Unpaired t-test or a one-way ANOVA, as appropriate, was used and group differences were considered statistically significant if P <0.05; n, represents the number of samples per group. Post hoc analysis was carried out within groups by using the Bonferroni correction,
3.3. Results

3.3.1. Luminal phosphate concentration along the small and large intestine

Most studies of intestinal phosphate handling have used phosphate concentrations of 0.1 mM. However, other work have used low millimolar or even molar phosphate concentrations, making it difficult to compare the findings of these disparate reports (Williams & DeLuca, 2007; Kirchner et al., 2008; Douard et al., 2010; Berndt et al., 2007). Therefore, studies in this chapter measured the free (unbound) phosphate concentration in luminal contents collected from defined regions of the gastrointestinal tract in rats maintained on a normal phosphate diet. Interestingly, phosphate levels in all regions studied were in the millimolar range (Table 3.1). At these concentrations, NaPi-IIb-mediated transport will be highly saturated, since the $K_t$ for the phosphate uptake process measured \textit{in vitro} has been reported to be around 0.1 mM (Hilfiker et al., 1998; Werner et al., 1998). The mean value for phosphate concentration in small intestinal luminal fluid was 6.3 mM; higher levels were found in the stomach, caecum and distal colon; regions that have not previously been considered to partake in phosphate transport (Berndt et al., 2007; Cramer, 1961; Kayne et al., 1993).

Table 3.1. Unbound phosphate concentration in luminal contents removed from specific regions of the small and large intestine. Animals were allowed food the night before and contents were removed at midday the following day. Values are means ± SEM. $n = 6$-8.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Free phosphate concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>14.84 ± 3.43</td>
</tr>
<tr>
<td>Duodenum</td>
<td>7.04 ± 1.50</td>
</tr>
<tr>
<td>Jejunum</td>
<td>5.69 ± 0.37</td>
</tr>
<tr>
<td>Ileum</td>
<td>6.27 ± 0.86</td>
</tr>
<tr>
<td>Cecum</td>
<td>12.82 ± 1.18</td>
</tr>
<tr>
<td>Distal Colon</td>
<td>11.49 ± 0.62</td>
</tr>
</tbody>
</table>
3.3.2. Concentration dependence of phosphate uptake in duodenum and jejunum in vitro

Based on the above luminal phosphate concentrations, the aim of work presented in this chapter was to examine the effect of increasing phosphate levels from 0.1 mM to a maximum of 10 mM in the uptake buffer on the rate of Na\textsuperscript{+}-dependent and Na\textsuperscript{+}-independent transport in vitro. In contrast to the expected saturable curve at millimolar concentrations, phosphate uptake continued to rise linearly with increasing phosphate concentrations in both the duodenum (Fig. 3.1) and jejunum (Fig. 3.2). The jejunum maintained its position as the region with the maximal transport activity in vitro using millimolar phosphate concentrations. Thus, at 5 and 10 mM luminal phosphate level, total phosphate transport in the jejunum was double that observed in the duodenum (P <0.005).

In both the duodenum and jejunum, uptake was mediated by Na\textsuperscript{+}-dependent and -independent mechanisms. In duodenum, transport was dominated by Na\textsuperscript{+}-independent uptake at phosphate concentrations above 0.5 mM. The Na\textsuperscript{+}-dependent component on the other hand remained constant, indicating complete saturation at millimolar phosphate concentrations, with a mean $K_t$ of 197 $\mu$M. In contrast, the Na\textsuperscript{+}-dependent component in the jejunum increased linearly with increasing luminal phosphate levels, implying the presence of a Na\textsuperscript{+}-dependent transporter other than NaPi-IIb in this region. The non-saturability meant that it was not possible to determine the $K_t$ of this component in the jejunum. In contrast to the duodenum, activity of the Na\textsuperscript{+}-independent transport increased in a similar fashion to Na\textsuperscript{+}-dependent transport at phosphate levels over 2 mM. Na\textsuperscript{+}-dependent transport at higher phosphate levels of 2 and 10 mM corresponded to 65% and 50% respectively, of total phosphate transport in the jejunum compared with 84% at 0.1 mM phosphate.
Figure 3.1. Na\(^+\)-dependent and Na\(^+\)-independent phosphate uptake in the duodenum, measured \textit{in vitro}, at phosphate concentrations up to 10 mM. Na\(^+\)-dependent uptake was calculated as total transport minus that observed following replacement of NaCl with ChCl. Results are mean ± SEM. \(n = 6\). Note that where SEM bars are not visible, they fall within the height of the symbol.
Figure 3.2. Na\textsuperscript{+}-dependent and Na\textsuperscript{+}-independent phosphate uptake in the jejunum, measured \textit{in vitro} at phosphate concentrations up to 10 mM. Na\textsuperscript{+}-dependent uptake was calculated as total transport minus that observed following replacement of NaCl with ChCl. Results are mean ± SEM. \( n = 6 \). Note that where SEM bars are not visible, they fall within the height of the symbol.
3.3.3. Concentration dependence of phosphate absorption across the duodenum and jejunum in vivo

Data representing appearance of $^{32}\text{P}$ in the blood (Fig 3.3A and 3.4A), and mucosal retention (Figs. 3.3B and 3.4B), the amount of $^{32}\text{P}$ retained by enterocytes, were analysed in vivo following instillation of phosphate buffer into defined intestinal regions for 30 minutes. Similar to the results in vitro, in the duodenum and jejunum transepithelial phosphate absorption followed a linear pattern with increasing phosphate concentrations (Figs 3.3 and 3.4, respectively). However, duodenal phosphate absorption in vivo appeared to be mediated by only a Na$^+$-independent pathway at all phosphate concentrations used. In the jejunum, although total and Na$^+$-independent transport rose linearly with phosphate concentration, Na$^+$-dependent transport did not show the linear increase observed in vitro: Na$^+$-dependent transport accounted for 67% of uptake at 0.1 mM, but only 34% and 0% at 2 and 10 mM luminal phosphate, respectively. The Na$^+$-dependent component of phosphate transport fits Michaelis-Menten kinetics (Fig 3.4B) with a $V_{\text{max}}$ of 4.84 nmoles per 100 mg mucosa and an apparent $K_t$ of 245 µM.

Contrary to in vitro observations, total phosphate transport in the jejunum was only moderately higher than that in duodenum at 0.5-2 mM luminal phosphate concentrations, and at luminal phosphate levels above 5 mM transepithelial phosphate absorption was similar in the duodenum. However, transit time in the jejunum is longer than the duodenum (Kayne et al., 1993), and it would, therefore, be expected that the jejunum efficiently transports more phosphate from the diet. Thus, at luminal phosphate concentrations above 0.5 mM (since there is some Na$^+$-dependent transport evident in vitro at phosphate concentrations below 0.5 mM), in vitro and in vivo results agree that phosphate transport in the duodenum is Na$^+$-independent.
Figure 3.3. Phosphate absorption across the duodenum in vivo. A: Blood appearance of phosphate and B: mucosal retention of phosphate; Na⁺-dependent (shaded bars) and Na⁺-independent (open bars). Results are mean ± SEM. ***P < 0.005 compared with absorption at 0.1 mM and 2 mM phosphate using an unpaired t-test. n = 6. Note that where SEM bars are not visible, they fall within the height of the symbol.
Figure 3.4. Phosphate absorption across the jejunum measured *in vivo*. A: Blood appearance of phosphate, B: Na\(^+\)-dependent component from transepithelial data fitted Michaelis-Menten kinetics and C: mucosal retention of phosphate, Results are mean ± SEM. **P <0.01 and *P <0.05 compared with absorption in the presence of Na\(^+\) at the same phosphate concentration, and ###P <0.005 and #P <0.05 compared with absorption at 0.1 mM and 2 mM, respectively using an unpaired t-test n = 6. Note that where SEM bars are not visible, they fall within the height of the symbol.
The $K_t$ of phosphate transport in everted sleeves of weaning rats was reported to be 2 mM using a 2 minutes uptake period (Kirschner et al., 2008). In the present study, total phosphate transport in vitro and in vivo (Figs 3.1 - 3.4) in adult rats could not be separated into saturable and nonsaturable components, since in vitro and in vivo phosphate transport remain linear at the maximum luminal phosphate concentration studied. The $K_t$ measured in vitro is therefore likely to be much higher than that reported previously (of 0.1 mM). Due to the unstirred layers associated with everted sleeve preparations (Karasov & Diamond, 1983), this $K_t$ is likely to be higher than seen using BBM vesicles but is probably closer to the true $K_t$ measured in vivo. Present results show that at the maximal 5 mM luminal phosphate level, transepithelial phosphate absorption does not reach saturation.

### 3.3.4. Inhibition of phosphate transport in vitro by PFA

PFA is a well established inhibitor of NaPi-IIb-mediated phosphate transport (Loghman-Adham et al., 1987, 1992 & 1993; Picard et al., 2010). At 0.1 mM phosphate, Na$^+$-dependent phosphate transport measured in vitro in the jejunum was almost completely inhibited by 1 and 10 mM PFA (Table 3.2 and Fig. 3.5A). Although 67 and 65% of total transport was Na$^+$-dependent at 1 and 2 mM phosphate respectively,
when 1, 10 or 20 mM PFA was included in the uptake buffer BBM transport was inhibited by only 33-37%, suggesting the remaining PFA-insensitive Na\(^+\)-dependent phosphate transport in the jejunum may be PiT-mediated. These studies failed to observe the progressive increase in inhibition of Na\(^+\)-dependent transport with increasing PFA concentrations, as reported in earlier studies (Loghman-Adham et al., 1987; Picard et al., 2010; Szczepanska-Konkel et al., 1986).
Table 3.2. A summary of Na\(^+\)-dependent jejunal phosphate transport *in vitro*, measured using 0.1 mM, 1 mM and 2 mM phosphate in the uptake buffer. Data shows the percentage that is Na\(^+\)-dependent, determined using Na\(^+\)-free buffer (without PFA), and the percentage of total transport inhibited by 1, 10 and 20 mM PFA in the presence of Na\(^+\). Values are mean ± SEM. *n* = 6. *nd*: not determined.

<table>
<thead>
<tr>
<th>Pi concentration</th>
<th>Na(^+)-dependent transport</th>
<th>Percentage inhibition of phosphate transport <em>in vitro</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 mM PFA</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>84 ± 10 %</td>
<td>65 ± 4 %</td>
</tr>
<tr>
<td>1 mM</td>
<td>67 ± 5 %</td>
<td>33 ± 5 %</td>
</tr>
<tr>
<td>2 mM</td>
<td>65 ± 7 %</td>
<td>34 ± 3 %</td>
</tr>
</tbody>
</table>
Figure 3.5. Summary of data from Table 3.2 and Table 3.3A. Percentage of total transport that is $\text{Na}^+$-dependent and $\text{Na}^+$-independent are indicated \textit{in vitro} (A) and \textit{in vivo} (B). $\text{Na}^+$-dependent transport (calculated as total transport minus $\text{Na}^+$-independent transport) was differentiated into PFA-sensitive and –insensitive components. Values represent average inhibition of $\text{Na}^+$-dependent phosphate transport by 1-20 mM PFA using 0.1 and 2 mM phosphate. Note that there is no $\text{Na}^+$-dependent transport in figure B, and $\text{Na}^+$-independent transport was assumed as PFA-insensitive based on no effect seen at 2 mM phosphate concentration under $\text{Na}^+$-free conditions with and without PFA.
To elucidate whether the Na\(^+\)-independent phosphate transport is driven by secretion of Na\(^+\) into the nominally Na\(^+\)-free buffer, the Na\(^+\) concentration in the post-incubation *in vitro* uptake buffer was measured. Na\(^+\) was undetectable in the buffer after 2 minutes incubation (data not shown) and the increasingly dominant Na\(^+\)-independent uptake in this preparation was, therefore, not due to Na\(^+\) secretion during the 2 minutes incubation period.

3.3.5. Inhibition of phosphate absorption *in vivo* by PFA

At 0.1 mM, 67% of the total phosphate absorption in the jejunum was Na\(^+\)-dependent and 1 and 10 mM PFA completely inhibited this component (Table 3.3 and Fig 3.5B). At 2 mM, Na\(^+\)-dependent transport accounted for only 34% of total transport, with PFA again completely inhibiting this component. Under Na\(^+\)-free conditions and with buffer containing 2 mM luminal phosphate, 10 mM PFA was added to analyse if PFA inhibited Na\(^+\)-independent absorption in the jejunum. Results indicated no such inhibition (Control 1.24 ± 0.20 vs 10 mM PFA 0.93 ± 0.15 nmoles per 1 ml plasma/5cm, P=0.15). To determine whether the higher proportion of Na\(^+\)-independent transport seen *in vivo* when compared with *in vitro* was driven by Na\(^+\) entering the luminal uptake buffer from mucosal secretions, the Na\(^+\) concentration of the buffer was measured 30 minutes after instillation of Na\(^+\)-free uptake buffer into jejunal segments *in vivo*. Results indicate that Na\(^+\) levels were too low to support significant Na\(^+\)-dependent phosphate across the jejunal BBM (mean value 11.9 ± 2.8 mM, n=6; reported K\(_m\) for Na\(^+\) ~40 mM (Murer *et al.*, 2004; Virkki *et al.*, 2006). Normal luminal Na\(^+\) concentration is typically about 140 mM; the concentration at which the V\(_{max}\) is achieved with Na\(^+\)-dependent phosphate transport (Berner *et al.*, 1976; Lee *et al.*, 1986b). However, this experiment measured the bulk luminal Na\(^+\) concentration, thus Na\(^+\) concentration in fluid bathing the BBM could be significantly higher. Since 10 mM PFA was equally effective as 1 and 20 mM PFA for inhibition of Na\(^+\)-dependent phosphate transport *in vitro* and *in vivo*, 10 mM PFA was used in subsequent experiments; this concentration was also used in previous studies of phosphate transport.
Table 3.3. A summary of Na\(^+\)-dependent phosphate absorption measured *in vivo* in the jejunum. Transepithelial (A) and mucosal retention (B) of phosphate was measured using 0.1 and 2 mM phosphate in the instillation buffer. Data show the percentage that is Na\(^+\)-dependent, determined using Na\(^+\)-free buffer, and the percentage of total transport inhibited by 1, 10 and 20 mM PFA measured in the presence of Na\(^+\). Values are mean ± SEM. *n* = 6. nd: not determined.

### A

<table>
<thead>
<tr>
<th>Pi concentration</th>
<th>Na(^+)-dependent transport <em>in vivo</em></th>
<th>% inhibition of transepithelial phosphate absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 mM PFA</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>67 ± 6 %</td>
<td>80 ± 3 %</td>
</tr>
<tr>
<td>2 mM</td>
<td>34 ± 10 %</td>
<td>39 ± 4 %</td>
</tr>
</tbody>
</table>

### B

<table>
<thead>
<tr>
<th>Pi concentration</th>
<th>Na(^+)-dependent transport <em>in vivo</em></th>
<th>% inhibition of mucosal retention of phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 mM PFA</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>69 ± 3 %</td>
<td>75 ± 3 %</td>
</tr>
<tr>
<td>2 mM</td>
<td>26 ± 8 %</td>
<td>29 ± 4 %</td>
</tr>
</tbody>
</table>
3.3.6. Ileal phosphate absorption.

Previous transport studies have shown very low values for phosphate transport, and undetectable gene and protein expression for NaPi-IIb in the rat ileum (Giral et al., 2009; Marks et al., 2006). The present study confirms that, using 0.1 mM luminal phosphate, minimal ileal phosphate transport occurred in vitro (Fig. 3.6) and in vivo (Fig. 3.7), with or without Na\(^+\) in the uptake buffer. However, at 2 mM phosphate, some Na\(^+\)-dependent phosphate transport was noted, although it corresponded to only about 35-45% of the total transport observed in the jejunum; at this phosphate concentration, in vitro and in vivo phosphate transport was inhibited by ~60% with the addition of 10 mM PFA in the Na\(^+\)-containing uptake buffer. Transport at 0.1 mM phosphate was not reduced under Na\(^+\)-free conditions (Na\(^+\)-dependent vs Na\(^+\)-independent transport is 0.094 ± 0.011 vs 0.069 ± 0.022 in vitro and 0.014 ± 0.002 vs 0.016 ± 0.003 in vivo), unlike at 2 mM phosphate, suggesting the presence of a Na\(^+\)-dependent phosphate transporter at higher (millimolar) phosphate concentrations but not at lower (micromolar) concentrations. Analysis of Na\(^+\) levels in Na\(^+\)-free uptake buffer following 2 minutes uptake in vitro revealed that no Na\(^+\) was present (data not shown), whereas low levels of Na\(^+\) were present in the in vivo buffer after 30 minutes ileal exposure (mean value 12.89 ± 5.27 mM, n=6). Therefore, under both conditions Na\(^+\) secretion does not reach a level for significant Na\(^+\)-dependent transport to occur under nominally Na\(^+\)-free conditions. However, Na\(^+\) concentration in fluid immediately adjacent to the ileal BBM could be significantly higher than the bulk value of 12.89 mM Na\(^+\) measured in the ileal lumen.

3.3.7. Phosphate absorption in the colon

Experiments were carried out in vitro and in vivo in order to determine whether the colonic epithelium is capable of transporting phosphate. Regardless of the phosphate concentrations used, a low rate of phosphate uptake was noted in the proximal colon in vitro (Fig. 3.8) and in vivo (Fig. 3.10). At 0.1 mM phosphate concentration, transport was minimal in the proximal and distal colon (Figs. 3.8 and 3.9). However, at
higher phosphate concentration (2 mM), absorption was consistently greater in distal compared with proximal colon measured \textit{in vitro} (Figs. 3.8 and 3.9) and \textit{in vivo} (Fig. 3.10). Using 2 mM phosphate, transport in the distal colon was Na$^+$-independent and was approximately 4- and 6-fold higher, measured \textit{in vitro} (Fig. 3.9) and \textit{in vivo} (Fig. 3.10, A) respectively, than in proximal colon. Transport in the distal colon was also insensitive to PFA (Fig. 3.11), further demonstrating that the uptake process is unlikely to be mediated by NaPi-IIb.
Figure 3.6. Ileal phosphate uptake measured *in vitro* using 0.1 mM and 2 mM phosphate in the presence (shaded bars) and absence (open bars) of Na\(^+\). Significant inhibition by 10 mM PFA (hatched bar) indicates Na\(^+\)-dependent phosphate transport at 2 mM phosphate. Results are mean ± SEM. ###P <0.005 compared with Na\(^+\)-dependent uptake at 0.1 mM phosphate. **P <0.01 compared with uptake in the presence of Na\(^+\) and ฿P <0.05 compared with phosphate uptake in the absence of PFA at 2 mM phosphate using one-way analysis of variance with *post hoc* comparisons performed using the Bonferroni multiple comparisons test. *n* = 6.
Figure 3.7. A: Transepithelial absorption and B: Mucosal retention of phosphate in the ileum, measured in vivo using 0.1 mM and 2 mM phosphate in uptake buffer. Experiments were carried out in the presence (shaded bars) and absence (open bars) of Na\(^+\). Significant inhibition by 10 mM PFA (hatched) indicates Na\(^+\)-dependent transport at 2 mM phosphate. Results are mean ± SEM. ###P <0.005 compared with Na\(^+\)-dependent absorption at 0.1 mM phosphate, ***P <0.005 compared with absorption in the presence of Na\(^+\), $$P <0.01$$ and $$P <0.005$$ compared with transport in the absence of PFA at 2 mM phosphate using one-way analysis of variance with post hoc comparisons performed using the Bonferroni multiple comparisons test. n = 6.

**A**

![Appearance of \(^{32}\)P in blood](image)

**B**

![Mucosal retention of \(^{32}\)P](image)
Figure 3.8. Phosphate uptake in the proximal colon, measured \textit{in vitro} using 0.1 mM, 0.5 mM and 2 mM phosphate in Na\textsuperscript{+}-containing uptake buffer. Results are mean ± SEM. ***P <0.005 and ###P <0.005 compared with uptake at 0.1 and 0.5 mM phosphate, respectively using an unpaired t-test. $n = 6$.

Figure 3.9. Phosphate uptake in the distal colon, measured \textit{in vitro} using 0.1 mM, 0.5 mM and 2 mM phosphate with (shaded bars) or without (open bars) Na\textsuperscript{+} in uptake buffer. Results are mean ± SEM. ***P <0.005 and ###P <0.005 compared with values using 0.1 and 0.5 mM phosphate, respectively using an unpaired t-test. $n = 6$. 
Figure 3.10. A: Transepithelial absorption and B: Mucosal retention of phosphate across the proximal and distal colon, measured in vivo using 0.5 mM and 2 mM phosphate in the presence (shaded bars) and absence (open bars) of Na⁺ in uptake buffer. Results are mean ± SEM. ***P <0.005 compared with absorption at 0.5 mM phosphate and ###P <0.005 compared with Na⁺-dependent absorption in the distal colon using an unpaired t-test. n = 6.

A

Appearance of ³²P in blood

Proximal Colon

Distal Colon

B

Mucosal retention of ³²P

Proximal Colon

Distal Colon
Figure 3.11. Phosphate transport in the distal colon, measured *in vitro* using 2 mM phosphate with Na\(^+\)-containing buffer alone (shaded bar) and the addition of 10 mM PFA (hatched bar) to uptake and pre-incubation buffers. Results are mean ± SEM. *n* = 6.
Analysis of Na\textsuperscript{+} levels in nominally Na\textsuperscript{+}-free uptake buffer following uptake experiments revealed that Na\textsuperscript{+} was not present under \textit{in vitro} conditions, whereas low Na\textsuperscript{+} levels were evident in the \textit{in vivo} buffer (mean value 8.08 ± 5.98 mM, n=6), indicating little likelihood of Na\textsuperscript{+} influencing phosphate transport under these conditions.

3.3.8. Mannitol uptake in duodenum, jejunum and ileum \textit{in vitro}

The intestinal epithelium constitutes a barrier for the movement of ions in the mucosal to serosal direction. Mannitol is a marker commonly used for assessing intestinal permeability, facilitated by its small size and non-absorbability under normal conditions (Krugliak \textit{et al.}, 1994). Permeability of mannitol through the intestinal epithelium occurs by passive diffusion through tight junctions (Ma \textit{et al.}, 1993). Mannitol was used in the present study as an independent measure of differences in non-specific permeability and paracellular transport across the small intestine and colon. The jejunum, ileum and distal colon expressed minimal permeability to mannitol \textit{in vitro} (Fig. 3.12), making it unlikely that the high levels of Na\textsuperscript{+}-independent phosphate uptake observed in jejunum and distal colon were due to passive diffusion. At 2 mM luminal phosphate, Na\textsuperscript{+}-independent phosphate uptake was 8- and 12-fold higher than mannitol transport in the jejunum and colon, respectively. Interestingly, Na\textsuperscript{+}-independent phosphate uptake in the ileum at 2 mM phosphate was the same as that for mannitol transport, suggesting that the minimal Na\textsuperscript{+}-independent ileal phosphate transport observed represents phosphate diffusion and/or mucosal bound \textsuperscript{32}P (but not transcellular transport). Although there were variations in the rate of mannitol uptake between animals, this was not statistically significant.
Figure 3.12. Transport of $^3$H-mannitol and $^{32}$P measured in vitro. Transport was measured using uptake buffer containing 2 mM phosphate and 1 mM mannitol. Total and Na$^+$-independent transport refer to phosphate transported in the presence (red bars) and absence (white bars) of Na$^+$. Transport of mannitol (blue bars) was measured in the absence of Na$^+$. ***P < 0.005 and *P < 0.05 compared with Na$^+$-independent phosphate absorption using an unpaired t-test. Results are mean ± SEM. n = 6.
3.3.9. Mannitol absorption in the duodenum, jejunum and ileum *in vivo*

The *in vivo* permeability of mannitol is reported to be similar in the jejunum and ileum, but much higher in the colon (Krugliak *et al.*, 1994). In agreement with this finding, present data shows that distal colon was significantly more permeable to mannitol than jejunum or ileum (Fig. 3.13A). In addition, Hu *et al* (1997) reported a correlation between the permeabilities of phosphate and mannitol through the proposed intercellular pathway in distal colon *in vitro*. In keeping with this report, significant levels of radiolabelled mannitol were detected in the blood when (³H)-mannitol was instilled into the distal colon, demonstrating that mannitol was transferred from lumen to blood. The mucosal retention data (Fig. 3.13B) show minimal mannitol retention in the jejunum, ileum and distal colon. The low mucosal retention and high plasma appearance of mannitol imply absorption of mannitol between the colonic epithelial cells (through the tight junctions), rather than across the cells. Mucosal phosphate retention in the distal colon was Na⁺-independent and significantly higher than mannitol retention, implying different routes used by these substrates. Variability of mannitol absorption did not statistically differ between animals.
Figure 3.13. Absorption of $^3$H-mannitol and $^{32}$P measured *in vivo*. A: plasma appearance and B: mucosal retention of $^{32}$P and $^3$H measured using 2 mM phosphate and 1 mM mannitol in the instillation buffer. Total and Na$^+$-independent transport refer to phosphate transported across that region in the presence and absence of Na$^+$. Transport of mannitol was measured in the absence of Na$^+$. Results are mean ± SEM. ***P <0.005 compared with Na$^+$-independent phosphate absorption and ###P <0.005 compared with $^3$H-mannitol transport in jejunum and ileum, using a one-way analysis of variance with *post hoc* comparisons performed using the Bonferroni multiple comparisons test. $n = 6$.

A

**Appearance of $^{32}$P and $^3$H in blood**

<table>
<thead>
<tr>
<th>Region</th>
<th>Permeability (mmoles per 1ml plasma/5cm/1mM Pi or Mannitol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jejunum</td>
<td>1.5</td>
</tr>
<tr>
<td>Ileum</td>
<td>0.5</td>
</tr>
<tr>
<td>Distal Colon</td>
<td>0.5</td>
</tr>
</tbody>
</table>

- Total phosphate uptake
- Na$^+$-independent phosphate uptake
- Mannitol uptake

### P <0.005 compared with Na$^+$-independent phosphate absorption.

*** P <0.005 compared with $^3$H-mannitol transport in jejunum and ileum.
Mucosal retention of $^{32}\text{P}$ and $^3\text{H}$

- Total phosphate uptake
- Na$^+$-independent phosphate uptake
- Mannitol uptake

Not significant
3.4. Discussion

The mechanisms and control of intestinal phosphate absorption remain ill-defined. This is partly due to recognised species differences and the variety of techniques and experimental conditions that have been used to assess uptake, particularly the wide range, and often idiosyncratic choice, of phosphate concentration added to the uptake buffer. The work described here has established the concentration of phosphate normally available for absorption, and it has used *in vivo* and *in vitro* methods to characterise the relative contributions of Na⁺-dependent and Na⁺-independent phosphate transport in different regions of the rat small and large intestine.

Early studies aimed at defining the transport kinetics of phosphate transport across the rat jejunal BBM revealed a Na⁺-dependent process with a $K_t$ of 0.1 mM; NaPi-IIb was later identified and held responsible for this transport process (Berner *et al*., 1976; Hilfiker *et al*., 1998; Lee *et al*., 1986b; Loghman-Adham *et al*., 1992). To date, subsequent studies have mostly used low phosphate concentrations (around 0.1 mM) to favour examination of the NaPi-IIb-mediated transport. However, there is very little information regarding reliable values for the luminal 'free' phosphate concentration available for absorption. In weaning rats, the phosphate level in upper small intestine has been reported to be approximately 5 mM (Kirchner *et al*., 2008). Data obtained in the present work reveal similar values to that of Kirchner *et al* (2008) in duodenum and jejunum of adult animals. Using phosphate levels of up to 10 mM to assess absorption, present data confirms the jejunum to be the primary region for phosphate absorption. However, a surprising finding was that significant phosphate uptake occurs in the ileum and distal colon at millimolar phosphate levels.

The precise extracellular free phosphate concentration available for absorption along the intestine has not been clearly defined. There are
limited data on luminal phosphate concentrations in adult rats. In keeping with the finding by Kirchner et al (2008), data presented in this chapter found luminal free phosphate concentrations to be in the millimolar range in the stomach and along the small and large intestine of adult rats. This is the first ever report of the ‘free’ unbound concentration of phosphate available for absorption along the gastrointestinal tract. At these millimolar levels, it would be expected that Na\(^+\)-dependent transport via NaPi-IIb will be saturated. The $K_t$ of the phosphate transport system under \textit{in vivo} conditions, however, was reported to be 2 mM or higher (Kirchner et al., 2008; McHardy, 1956; Williams & DeLuca, 2007).

Low levels of phosphate are secreted into gastric juice, which might explain the higher luminal phosphate concentration seen in the stomach. The total microbiota population makes up about 35-50% of the colonic content volume, contributing towards the production of nutrients such as fatty acids (Tappenden & Deutsch, 2007; Munakata et al., 1995). These fatty acids are readily taken up by colonic mucosa and also may release free phosphate from bound proteins, thus increasing total available phosphate within the colon. This might be a reason why a higher luminal phosphate concentration was seen in the large intestine.

Based on the finding that free phosphate concentrations in the intestinal lumen are in the millimolar range, the next step of this study was to determine the relative contribution of Na\(^+\)-dependent and Na\(^+\)-independent transport in specific intestinal regions at normal phosphate levels in adult rats, both \textit{in vitro} and \textit{in vivo}. Increasing the phosphate concentration up to 10 mM caused a corresponding rise in total phosphate absorption, which did not reach saturation. This observation, together with recent reports of Na\(^+\)-independent phosphate transport by Williams & Deluca (2007) and Douard et al (2010), imply that the Na\(^+\)-gradient is not the rate-limiting step in phosphate absorption across the proximal small intestine. Interestingly, present \textit{in vitro} data revealed that Na\(^+\)-independent transport in the duodenum is linearly related to phosphate concentration, while the rate of Na\(^+\)-dependent uptake is not
so dependent on luminal phosphate level. However, in the jejunum, both Na\(^+\)-dependent and -independent transport are equally affected by increasing phosphate concentrations. In contrast, results in vivo show that Na\(^+\)-dependent transport in both regions is unaffected by luminal phosphate concentration, and that the Na\(^+\)-independent component is responsible for increased overall phosphate absorption at higher luminal phosphate levels. The unequivocal absence of a Na\(^+\) requirement for phosphate absorption at high phosphate levels in vivo is in keeping with previous in vivo and ex vivo studies (Aloia & Yeh, 1985; Douard et al., 2010; McHardy, 1956; Williams & DeLuca, 2007). At physiological phosphate concentrations, the fact that NaPi-IIb contributes little towards total phosphate absorption in adult rats is not surprising considering the decrease in NaPi-IIb expression with aging (Kirchner et al., 2008; Xu et al., 2002a). Furthermore, recent reports have claimed that in mature rats, not only is Na\(^+\)-dependent phosphate transport insignificant, but also that this component is unlikely to be regulated, in contrast to the situation seen in weanling animals (Douard et al., 2010; Kirchner et al., 2008; Williams & DeLuca, 2007).

Since in vitro phosphate uptake does not show saturation at phosphate concentrations of up to 10 mM, the K\(_t\) for the transport process is significantly higher than that reported for BBM vesicles (of 0.1 mM) and may be more representative of the K\(_t\) in vivo. The phenomenon of different K\(_t\) in vivo and in vitro has been reported for Na\(^+\)-dependent glucose transport across the small intestine, where the reported K\(_t\) in vivo is approximately 20 mM, a value significantly higher than the K\(_t\) of 4 mM determined using everted sleeves, and of less than 0.5 mM using BBM vesicles and oocytes (Wright, 2001; Debnam et al., 1988; Wright, 2001).

The disparity in the overall contribution of Na\(^+\)-dependent transport observed in vivo and in vitro highlights the value and importance of using both approaches to study phosphate transport. The use of a single method in most previous studies may explain the discrepancies in the literature concerning the mechanisms involved in phosphate transport.
Using everted sacs from mature rats (13-week old), Douard et al (2010) showed that phosphate absorption at 1.2 mM luminal phosphate is Na⁺-independent. Since the everted sac technique can measure paracellular transport in addition to transepithelial transfer, Douard et al (2010) proposed that phosphate transport is solely mediated via the paracellular pathway, rather than the transcellular (NaPi-IIb) route. Present data from the in vivo in situ loop technique, which measures both transepithelial and paracellular phosphate transport, leads to similar conclusions to that of Douard et al (2010). Furthermore, the in vivo in situ loop technique was also sensitive enough to detect the low rates of Na⁺-dependent transport at luminal phosphate levels ≤ 2 mM.

Data from work presented in this chapter suggest that the everted sleeve technique is a valid and useful measure of phosphate transport across the BBM of intact epithelium, since Na⁺-dependent transport is readily detectable. However, examining phosphate absorption in vivo may be more physiologically relevant and reveal regulatory changes in the dominant Na⁺-independent transport pathway. In this context, it is notable that a facilitative pathway for intestinal glucose transport, now attributed to GLUT2, was overlooked in early in vitro studies, because of the rapid internalization of the transport protein away from the BBM during preparation of tissue for in vitro uptake studies (Kellett, 2001).

Na⁺-independent phosphate absorption is mediated by either paracellular transport or an unidentified transcellular pathway. The apparent contradictions between the in vivo and in vitro data concerning the contributions of Na⁺-dependent and Na⁺-independent transport may reflect differences in paracellular transport in these preparations. It is interesting to note that a facilitative component of intestinal glucose transport, now attributed to GLUT2, was overlooked in early in vitro studies due to rapid shuttling of this transport protein away from the BBM upon removal of the tissue from its blood supply (Kellett, 2001). It is important to characterise the pathway responsible for the higher Na⁺-independent phosphate transport detected in vivo.
A potential role of type III transporters in intestinal phosphate handling has recently emerged. PiT1 mRNA has been detected in all regions of the rat small intestine, with highest expression levels in the ileum (Giral et al., 2009). However, the mRNA expression profile does not correspond to that seen for the translated protein: PiT1 protein levels are highest in the jejunum and absent in the ileum (Giral et al., 2009). PiT2 mRNA has been found at low levels throughout the rat small intestine (Giral et al., 2009), but to date a profile for the protein in this species has not been established. The $K_t$ for PiT1 and PiT2 has been estimated to be $\sim 25 \mu M$ in oocytes (Tatsumi et al., 1998), but the function of these proteins in phosphate absorption is unclear. Previous studies have shown PFA to be a specific inhibitor of NaPi-IIb-mediated transport, whilst only weakly inhibiting PiT-mediated transport when used at concentrations 50-fold higher than the level of phosphate (Bai et al., 2000; Ravera et al., 2007).

Thus, at the higher (millimolar) phosphate concentrations used in the present study, 10 mM PFA would have selectively inhibited NaPi-IIb-mediated transport, whereas at 0.1 mM phosphate 10 mM PFA is likely to have blocked both NaPi-IIb and PiT-mediated transport.

Present study used PFA to differentiate between the contribution of type II and type III transporters to the Na$^+$-dependent component of phosphate absorption. Increasing concentrations of PFA have been reported to progressively decrease renal Na$^+$-dependent phosphate transport and the EC$_{50}$ for PFA has been calculated to be $\sim 3 \text{ mM}$ (Picard et al., 2010). The present study did not see a progressive decline in intestinal phosphate transport with PFA in vivo or in vitro, suggesting that NaPi-IIb is maximally inhibited. It is interesting that PFA inhibited all the Na$^+$-dependent transport measured in vivo, but not in vitro, which may reflect the relatively low contribution of Na$^+$-dependent transport under in vivo conditions, or the possibility that PiT transporters contribute to phosphate transport across the BBM but not transepithelial transport. Non-specific effects of PFA have been documented at concentrations >10 mM (Picard et al., 2010; Ravera et al., 2007), making it unwise to use very high doses
of PFA in an attempt to totally block Na\(^+\)-dependent transport. More specific PiT and NaPi-IIb transporter inhibitors are needed to confidently establish a role for PiT-mediated transport in intestinal phosphate transport and to distinguish between the relative contribution of NaPi-IIb and PiT proteins to Na\(^+\)-dependent phosphate absorption.

Small differences in the \textit{in vivo} results between transepithelial absorption and mucosal retention data are partly due to unavoidable errors in the calculation of mucosal retention; e.g. errors in measurement of the length of intestinal section used for \textit{in vivo} studies and measurement of wet mucosal tissue weight instead of dry tissue. Additionally, some \(^{32}\text{P}\) activity in the scraped mucosa was probably due to bound \(^{32}\text{P}\), which would not influence calculations of transepithelial phosphate transport.

The involvement of the ileum in overall intestinal phosphate uptake is unclear. While some studies have reported the rat ileum to have little or no capacity to absorb phosphate (Marks \textit{et al.}, 2006; Peters & Binswanger, 1988), others reported that phosphate is absorbed equally by duodenum, jejunum and ileum, and to a lesser extent by colon (Cramer, 1961; Kayne \textit{et al.}, 1993). Present results show minimal ileal phosphate transport at 0.1 mM luminal phosphate. In contrast, at high levels of phosphate there is readily detectable Na\(^+\)-dependent phosphate transport both \textit{in vivo} and \textit{in vitro}, which is PFA-sensitive. However, the transport rate is lower than that detected in the duodenum and jejunum at the same luminal phosphate concentrations. Since NaPi-IIb and PiT1 proteins are not expressed at the ileal BBM in the rat (Giral \textit{et al.}, 2009; Marks \textit{et al.}, 2006), an additional, and as yet uncharacterised, low affinity Na\(^+\)-dependent transporter seems likely to be present in this region. Present data also provided evidence that the distal, but not proximal, colon can absorb phosphate from the lumen and transfer it into blood. Relative to the jejunum, the rate of phosphate absorption by the distal colon at 2 mM luminal phosphate is about 40\% and, importantly, is mediated by a Na\(^+\)-independent, PFA-insensitive pathway. Phosphate transport in the distal, but not the proximal, colon was previously detected.
under *in vitro* conditions and attributed to a paracellular pathway (Hu *et al*., 1997; Lee *et al*., 1980).

Gene expression of NaPi-IIb, PiT1 and PiT2 mRNA was detected in the distal colon by Capuano *et al* (2009). However, whether this is translated into protein expression is unknown. NaPi-IIb transporters function maximally at pH ≥7, whereas PiT transporters express higher level of Na\(^+\)-dependent phosphate transport activity at pH <7. The acidity of colonic contents is likely to favour PiT-mediated transport (Evans *et al*., 1988; Cummings, 1981; Berner *et al*., 1976). However, Berner *et al* (1976) demonstrated that phosphate uptake by intestinal BBM vesicles at pH 6 does not involve cotransport with Na\(^+\), a finding that suggests a non-PiT-mediated process. Thus, the mechanisms involved in the colonic transport process have yet to be elucidated. The significance of phosphate transport in the colon to overall phosphate homeostasis is unclear, since increasing solidity of colonic contents, particularly in the distal colon, would make phosphate less accessible for absorption. However, the uptake capacity of the colon for phosphate might be relevant to the clinical observation, and complication, of hyperphosphataemia following the use of high phosphate-containing enemas (Carl & Mitchell, 2007; Hunter *et al*., 1993; Hu *et al*., 1997).

Hu *et al* (1997) directly compared intestinal permeability of phosphate and mannitol and proposed that absorption of phosphate occurred by the same paracellular pathway used by mannitol. However, mannitol is neutrally charged whereas phosphate is negatively charged; the two substrates cannot be considered as strictly equivalent in assessing their involvement in paracellular transport since transport through the tight junctions is both size and ionic charge selective. Mannitol was used to assess whether differences in its permeability were obvious between *in vivo* and *in vitro* techniques, especially the latter where one might expect greater passive permeability arising from tissue damage during its preparation for uptake studies. Mannitol was also used as an indicator of bound levels of radiolabelled substrate following uptake and tissue
washing procedures, and differences in variability in uptakes between the two techniques and between intestinal regions.

As established previously (Krugliak et al., 1994), the jejunum, ileum and distal colon exhibited low mucosal permeability to mannitol in vivo and in vitro. This low mannitol permeability represents low levels of extracellular bound radiolabelled substrate, and demonstrates that phosphate uptake measured using both of these techniques is slightly overestimated. However, this is an expected limitation of any solute uptake techniques. Interestingly, data presented in this chapter indicate that transepithelial in vivo permeability to mannitol was greater in the distal colon compared with small intestine, as established previously (Krugliak et al., 1994). Combined with minimal mucosal mannitol retention, this data shows that transport of mannitol is via the paracellular, and not transcellular, pathway. Although jejunum and ileum are more ‘leaky’ epithelia, the high rate of water absorption (solvent drag) allows for high rate of mannitol permeability across the distal colon.

Paracellular transport of phosphate is the passive movement of the phosphate ion through tight junctions between adjacent epithelial cells, down its electrochemical gradient. After ingestion of a phosphate-containing meal, the intestinal lumen is exposed to high concentrations of phosphate, thus it is possible that paracellular transport of phosphate is the predominate postprandial route. Paracellular transport of phosphate may occur via claudins. It may be that an anion selective claudin in surface epithelium mediates phosphate uptake. This is not surprising since cation-selective claudin-12 has been shown to transport Ca$^{2+}$ (Fujita et al., 2008). Although over 20 different claudins have been identified, their species-specific expression, distribution and their roles have not yet been clearly defined.

Interest in the Na$^+$-independent component of phosphate transport is revived by the results presented in this chapter. Further experiments are needed to characterise this pathway in the proximal small intestine and
distal colon, especially under \textit{in vivo} conditions. It also remains to be elucidated whether this transport route is regulated, as it is the case for Na\textsuperscript{+}-dependent phosphate uptake. There are reports claiming that Na\textsuperscript{+}-independent phosphate transport in the small intestine is regulated (Yeh & Aloia, 1987; Debiec & Lorenc, 1985). In the colon, the presence of the vitamin D receptor (Lee \textit{et al.}, 1980) and the genomic upregulation of NaPi-IIb, PiT1 and PiT2 by dietary phosphate restriction (Capuano \textit{et al.}, 2009) suggest that phosphate absorption in the colon may involve regulation by vitamin D and/or the level of phosphate in the diet. Further work is, however, needed to establish the importance of regulation of phosphate uptake in the colon to overall phosphate balance.
3.5. Conclusion

In conclusion, the present study has shown phosphate concentrations along the intestine to be in the millimolar range, and the Na\(^+\)-independent transport may be the dominant pathway \textit{in vivo}. To date, study of the regulation of intestinal phosphate transport has used mainly \textit{in vitro} techniques to assess changes in Na\(^+\)-dependent phosphate transport; the pathway involved being attributed to NaPi-IIb. However, it is now clear that the contribution of Na\(^+\)-dependent transport to overall transport is relatively small \textit{in vivo}. Additional studies are needed to gain insight into the mechanisms and regulation of the Na\(^+\)-independent component of phosphate absorption in the jejunum and colon, as well as the proteins responsible for Na\(^+\)-dependent phosphate uptake that operate at normal luminal phosphate concentrations in the ileal lumen.
4.0. Genomic regulation of Na\(^+\)-dependent phosphate cotransporters by low phosphate diet, age, chronic renal failure and matrix extracellular phosphoglycoprotein
4.1. Introduction

Recent evidence suggests that in addition to the well characterised type II transporters, type III Na\(^+\)-dependent phosphate transporters PiT1 and PiT2 may also play a critical role in the absorption of phosphate. PiT1 and PiT2 transporters have a wide membrane expression, including the brush-border membrane (BBM) of the small intestine and kidney.

Very little is known about the regulation of PiT transporters. In summary, the genomic expression of PiT1 and PiT2 is regulated by phosphate deprivation, potassium deficiency and metabolic acidosis (Villa-Bellosta et al., 2009b; Villa-Bellosta & Sorribas, 2010b; Collins et al., 2004; Breusegem et al., 2009). PiT1 and PiT2 protein expression is regulated by phosphate depletion, acute low phosphate diet, Fibroblast growth factor-23 (FGF-23), and by conditions that affect NaPi-IIa directly such as deletion of NaPi-IIa gene (Fernandes et al., 1999; Kavanaugh et al., 1994; Nowik et al., 2008; Tomoe et al., 2010; Villa-Bellosta & Sorribas, 2010b). These reports suggest a more significant role for PiT transporters in phosphate homeostasis, in contrast to the previous notion that PiT transporters play only a housekeeping role at the basolateral membrane (Bai et al., 2000; Collins et al., 2004).

Rat and mouse are the two most common species used in the study of phosphate homeostasis. Recently, Giral et al (2009) showed that in rats PiT1 and PiT2 proteins are expressed at the renal BBM and that renal PiT2 protein is regulated by low phosphate diet. Conversely, they failed to find PiT2 mRNA expression in any regions along the small intestine. Although PiT1 protein was detected in the proximal small intestine, it was not regulated by dietary phosphate level. Region-specific expression of PiT transporters in the mouse small intestine is however unknown. To examine whether PiT transporters are differentially expressed in rat and mouse small intestine, and in different intestinal regions as reported for
NaPi-IIb expression (Marks et al., 2006), PiT1 and PiT2 mRNA expression was analysed in all three distinct areas of the small intestine.

The regulation of PiT transporters by chronic low phosphate diet and age is not well characterised. The effect of one of the most common phosphate disorders, hyperphosphataemia, a consequence of chronic renal failure, on PiT transporter expression has also not been evaluated to date. The regulation of NaPi-IIa and NaPi-IIb mRNA expression by chronic renal failure in the small intestine and kidney is, however, established (Douard et al., 2010; Marks et al., 2007). There is also growing interest in the effect of the bone-derived phosphatonin matrix extracellular phosphoglycoprotein (MEPE), which has been shown to inhibit both intestinal and renal Na\(^+\)-dependent phosphate transport (Marks et al., 2008; Shirley et al., 2010).

The aims of the present study were to investigate whether increased BBM Na\(^+\)-dependent phosphate transport, observed during the feeding of a low phosphate diet or during weaning, is mediated by changes in intestinal and renal PiT1 and PiT2 gene expression, and to establish the effects of severe chronic renal failure, using the 5/6 nephrectomy rat model. The effects of MEPE on NaPi-II and PiT transporters in the small intestine and kidney during chronic renal failure has also been examined. Since there are currently no suitable commercial antibodies for PiT1 and PiT2 proteins, changes in mRNA levels in respect of these proteins were analysed in rats using quantitative real-time polymerase chain reaction (PCR).
4.2. Materials and Methods

4.2.1. Animals and diet

4.2.1.1. Rats
Male Sprague Dawley (SD) rats aged 3 weeks, 8 weeks and 16 weeks were obtained from Charles River Laboratories (UK) and used in accordance with the Animals (Scientific Procedures) Act 1986. Control rats were allowed *ad libitum* access to a standard rodent chow containing 0.52% phosphate (Diet RM1, SDS Ltd, Witham, Essex, UK) and water. Some rats on a control diet were switched to a low phosphate diet containing 0.02% phosphate (Harlan Tekland, Indianapolis, USA) for 7 days prior to experimentation.

4.2.1.2. Mice
Male C57BL/6 mice (25 g body weight) were obtained from the Comparative Biology Unit at the Royal Free campus of UCL and used in accordance with the Animals (Scientific Procedures) Act 1986. Control mice were allowed *ad libitum* access to a standard rodent chow containing 0.52% phosphate (Diet RM1, SDS Ltd, Witham, Essex, UK) and water.

4.2.1.3. Chronic renal failure (5/6 nephrectomy)
Surgically 5/6 nephrectomised SD rats, and sham-operated controls were supplied by Charles River Laboratories (MA, USA) and delivered to the laboratories of Acologix Inc (California, USA). Animals were fed a control diet (5L79, Purina Charles River) prior to and following 5/6 nephrectomy surgery. To induce severe hyperphosphataemia and hyperparathyroidism, secondary characteristics of chronic renal failure, animals were switched to a high phosphate (1.2%), but low calcium (0.4%) diet (Diet TD.07363, Harlan Teklad, Indianapolis, USA) post-nephrectomy. MEPE treatment was also carried out at Acologix Inc. For this, sham and 5/6 nephrectomised animals were treated daily with an intravenous injection of HEPES-buffered saline (vehicle-treatment), or...
HEPES-containing 3 mg/kg *E.coli* expressed MEPE (an optimal dose of the phosphatonin previously established by Acologix and by Marks *et al* (2009)) for 2 weeks. During this time, animals were switched to a control phosphate, but low calcium (0.4%) diet. At the end of treatment, blood was collected from animals via a tail vein bleed. Acologix Inc measured parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D₃ levels in plasma using a BioActive ELISA kit (Immunotopics), and plasma and urine phosphate levels using a Quantichrom phosphate assay kit (BioAssay Systems, Hayward, CA), according to the manufacturer's instructions. Animals were killed by anaesthetic overdose and tissue was removed and snap-frozen with RNA*Later* solution (Ambion, Texas, USA) in liquid nitrogen and stored -70°C before shipment on dry ice to the UK. All experimentation carried out by Acologix were performed according to national and local ethical guidelines.

4.2.2. RNA extraction from rat and mouse tissue

Kidneys or intestinal mucosal scrapes from rats and mice (excluding those from 4.2.1.3) were snap-frozen with RNA*Later* solution (Ambion, Texas, USA) in liquid nitrogen and stored at -70°C until use. This was determined to be the best method for preserving RNA in stored tissue (Fig. 4.1). Tissue samples were ground to fine powder using liquid nitrogen, and pestle and mortar. RNA was then extracted from tissue using Trizol (Invitrogen, Paisley, UK), according to the manufacturer's instructions. Extracted total RNA samples (1.5 µl) combined with loading buffer (Sigma, Dorset, UK), were run on 1.2% agarose gel containing 0.1% ethidium bromide at 120 V to ensure that good quality RNA was extracted, evident by two bright ribosomal bands (28S and 18S, 2:1 ratio), and from the reading from Nanodrop (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK), where the 260/280 ratio was above 1.75. RNA concentration was also determined using the Nanodrop, and RNA was diluted to a concentration of 1 µg/µl.
4.2.3. mRNA quantification using real-time PCR

RNA was reverse transcribed with 0.5 µg of oligo-dT 12-18 primer and a First Strand cDNA synthesis kit (Superscript II RNase H⁻ reverse transcriptase; Life Technologies, Paisley, UK). NaPi-IIa and NaPi-IIb transporter mRNA expression levels were analysed using Real-Time PCR Chromo4™ Real-Time Detector (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK), using QuantiTech SYBR® Green PCR kit (Qiagen, West Sussex, UK), following the manufacturer’s instructions for calibrator normalised protocols. Specific primers against rat and mouse were designed and the primer most suitable at detecting the gene of interest was chosen on the basis of lower cycle number for amplification and steepest fluorescence emission peak (Fig. 4.2). β-actin transcript was chosen as the housekeeping gene and run in parallel cycles. β-actin has been shown to have less variance in expression across a variety of treatment conditions and it is a commonly used as a control/standard in real-time PCR. Primers were tested for efficiency and checked for formation of primer dimers by running the PCR product in a 2% agarose gel with ethidium bromide for 30 min in an electrophoresis tank at 120 V.

Primers were designed from PRIMER3 tool at: (http://www.ncbi.nlm.nih.gov/tools/primer-last/index.cgi?LINK_LOC=Blast Home), based on the published sequence of the specific gene of interest. Primer3 tool is useful for designing primers that are specific, in terms of the region of interest, minimum GC content and product size. The sequences of final primers chosen are listed in Table 4.1.

All primers were run on the same cycling conditions as follows: 95°C for 10 min followed by 45 cycles of 95°C for 15 s, 60°C for 20 s and 72°C for 40 s with transition rates of 20°C s⁻¹ and a single fluorescence acquisition at 81°C.
Table 4.1. Details of primers chosen for detecting type II and type III Na\(^+\)-dependent phosphate transporters in rats.

<table>
<thead>
<tr>
<th>Gene and GenBank AN(^1)</th>
<th>Forward</th>
<th>Reverse</th>
<th>Target species</th>
<th>Target size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaPi-IIa (NM_013030)</td>
<td>951–969</td>
<td>1315-1300</td>
<td>Rat</td>
<td>371</td>
</tr>
<tr>
<td>NaPi-IIb (AF_081499)</td>
<td>1402-1418</td>
<td>1631-1616</td>
<td>Rat and mouse</td>
<td>230</td>
</tr>
<tr>
<td>NaPi-IIc (NM_139338)</td>
<td>672-691</td>
<td>899-880</td>
<td>Rat</td>
<td>228</td>
</tr>
<tr>
<td>PiT1 (NM_031148)</td>
<td>768-787</td>
<td>1007-988</td>
<td>Rat and mouse</td>
<td>243</td>
</tr>
<tr>
<td>PiT2 (NM_017223)</td>
<td>118-1137</td>
<td>1370-1351</td>
<td>Rat</td>
<td>253</td>
</tr>
<tr>
<td>PiT2 (NM_011394)</td>
<td>1502-1521</td>
<td>1759-1740</td>
<td>Mice</td>
<td>258</td>
</tr>
<tr>
<td>β-Actin (NM_031144)</td>
<td>937-955</td>
<td>1223-1208</td>
<td>Rat and mouse</td>
<td>286</td>
</tr>
</tbody>
</table>

\(^1\)AN, accession number; bp, base pair
4.2.4. Calculation of expression fold change

A calibrator (control) for NaPi-IIa, NaPi-IIc or NaPi-IIb and β-actin was run for every PCR cycle. The fold change in expression was calculated using the threshold cycle (C_t) value for the actin, and for the gene of interest (GOI) of each sample. The fold-change in GOI was equal to $2^{(-\Delta\Delta C_t)}$, where $\Delta\Delta C_t = [C_t (\text{GOI}) - C_t (\text{actin})]_{\text{sample}} - [C_t (\text{GOI}) - C_t (\text{actin})]_{\text{calibrator}}$ (Livak & Schmittgen, 2001). The difference in threshold cycle values for each reaction, the $\Delta C_t$ value (control and experimental), were dependent on the amplification efficiency, which was used to compensate for efficiency differences between samples and the calibrator.

4.2.5. Statistical analysis

Data are presented as means ± SEM. Unpaired t-test or a one-way ANOVA, as appropriate, was used and group differences were considered statistically significant if $P < 0.05$; n represents the number of samples per group. Post hoc analysis was carried out within groups by using the Bonferroni correction.
4.3. Results

4.3.1. Extraction of high integrity RNA
RNA from animal tissues is prone to degradation. Intestinal tissue in particular is known to have the highest rate of RNA degradation (Lee et al., 2005). The initial choice of homogenising samples using an electrical homogeniser was not a sufficiently rapid and robust method, in comparison with grinding samples in liquid nitrogen using a pestle and mortar to prevent RNA degradation (Fig. 4.1A). On the basis of this finding, for all future experiments grinding with the liquid nitrogen method was used for extracting RNA. Tissues also need to be suitably stored if RNA extraction is needed at a later date. RNA later solution allows tissues to be stored for months without sacrificing the integrity of RNA, since it stabilises the RNA. In contrast to strong RNA bands (28S and 18S) seen after tissues were stored in RNA later, Fig. 4.1B demonstrates RNA from intestinal tissue was fully degraded (represented by smear on gel) following storage for months without treatment with reagents to preserve RNA although the tissues were extracted for RNA by grinding with liquid nitrogen.

4.3.2. Primer design for real-time PCR
Following extraction of good quality RNA from intestinal and kidney samples, primers were designed from the published sequence of the Na\(^+\)-dependent phosphate transporters NaPi-IIa, NaPi-IIb, NaPi-IIc, PiT1 and PiT2 for rat and mouse. A number of potential primers, between 200-300 base pairs, were tested for each gene of interest using SYBR green kit and a LightCycler Real-Time PCR instrument. The final choice of a primer for the gene of interest was made on the basis of the following: the steepest peak in fluorescence emission at the melting temperature of the PCR product (Fig. 4.2A), a lower cycle number, and a more linear response than the alternative primers tested for that gene (Fig. 4.2B).
Figure 4.1. Comparison of RNA extraction methods and storage conditions. A: extraction of a range of tissues using electrical homogeniser or liquid nitrogen (N₂) grinding process. Samples were stored in RNAlater at -70°C. Good quality of RNA is represented by strong 28S and 18S ribosomal RNA bands. B: represents complete RNA degradation in duodenum and jejunum samples stored at -20°C without stabilising solution (RNAlater), following tissue removal from animal.
Figure 4.2. Primers were tested for their use in real-time quantitative PCR, where A: the melting curve and B: the cycle number at which the cDNA begins to get amplified were measured for each set of primers tested for the reference gene. [1] Represents the final primer chosen for a particular gene.

A

B
Cycle number represents how many melting/annealment/extension cycles have occurred. The same cDNA sample was used for all primers tested and the lower the $C_t$ (cycle number above threshold) at which the curve starts appearing, the more efficient are the primers.

4.3.3. Comparison of phosphate transporter expression in rats and mice

Once the conditions for quantifying mRNA were successfully optimised, known Na$^+$-dependent phosphate transporters were quantified in three regions (duodenum, jejunum and ileum) of the small intestine of adult rats (Fig. 4.3) and mouse (Fig. 4.4). Although the jejunum plays the predominant role in Na$^+$-dependent phosphate absorption across the small intestine (see Chapter 2 for further details), NaPi-IIb mRNA levels detected showed no significant differences between the duodenum and jejunum of rats (Fig. 4.3A), as previously noted (Marks et al., 2006). In the ileum, the region not involved in Na$^+$-dependent phosphate absorption in the rat (see Chapter 2 for further details), NaPi-IIb mRNA expression was undetectable. In contrast, NaPi-IIb expression in the mouse ileum was significantly higher than that seen in the duodenum and jejunum (Fig. 4.4A), again as noted previously (Marks et al., 2006). This parallels previous NaPi-IIb protein expression and uptake data recorded in the mouse (Giral et al., 2009; Marks et al., 2006; Radanovic et al., 2005), which demonstrated this region to be largely responsible for overall intestinal phosphate absorption. Substantial levels of PiT1 mRNA (Figs. 4.3B and 4.4B) and PiT2 (Figs. 4.3C and 4.4C) were found in the duodenum and jejunum of rat and mouse. However, unlike NaPi-IIb, there was no region-specific expression pattern for PiT1 and PiT2 in either species.
Figure 4.3. Expression of A: NaPi-IIb, B: PiT1 and C: PiT2 mRNA in the mucosa of duodenum (duo), jejunum (jej) and ileum of adult rats fed a control diet, quantified using real-time PCR. Fold change was calculated using the comparative $\Delta\Delta C_t$ method using actin as a housekeeping gene. Results are means ± SEM. *P <0.05 and **P <0.01 compared with ileum using a one-way ANOVA with Bonferroni multiple comparisons post hoc test. n = 6.

A

![Graph showing NaPi-IIb expression in duodenum (Duo), jejunum (Jej), and ileum (Ileum).]

B

![Graph showing PiT1 expression in duodenum (Duo), jejunum (Jej), and ileum (Ileum).]
Fold change

Duo
Jej
Ileum

PIT2
Figure 4.4. Expression of A: NaPi-IIb, B: PiT1 and C: PiT2 mRNA in the mucosa of duodenum (duo), jejunum (jej) and ileum (ile) of adult mice fed a control diet, quantified using real-time PCR. Results are means ± SEM. *P <0.05 compared with mouse ileum using a one-way ANOVA with Kruskal–Wallis post hoc test. n = 6.

A

![Graph A: NaPi-IIb](image)

B

![Graph B: PiT1](image)
4.3.4. Response to chronic low phosphate diet

The effect of chronic low phosphate diet on mRNA expression of intestinal and renal Na\(^{+}\)-dependent phosphate transporters was investigated. Kidneys of animals that were chronically fed a low phosphate diet for 7 days showed higher levels of PiT2 and NaPi-IIa expression (upregulation by 3 and 6-fold, respectively, compared with animals fed a control diet) (Fig. 4.5A). This is in keeping with the previously established stimulatory effect of a chronic low phosphate diet on renal Na\(^{+}\)-dependent phosphate transport and NaPi-IIa, and PiT2 protein expression (Capuano et al., 2005; Giral et al., 2009; Madjdpour et al., 2004; Villa-Bellosta et al., 2009b). Therefore, the increase in renal Na\(^{+}\)-dependent phosphate transport following a chronic low phosphate diet is partly mediated by genomic changes in the level of NaPi-IIa and PiT2 transporters. No changes were observed with respect to renal PiT1 and NaPi-IIc mRNA expression.

There were no changes in the genomic level of any of the Na\(^{+}\)-dependent phosphate transporters, NaPi-IIb, PiT1 or PiT2, expressed at the jejunal BBM in rats fed a low phosphate diet (Fig. 4.5B). Hence, a chronic low phosphate diet causes genomic upregulation of Na\(^{+}\)-dependent phosphate transporters expressed in the kidney only. Present data imply that the increased jejunal Na\(^{+}\)-dependent phosphate transport observed with rats fed a low phosphate diet (see Chapter 2.3.3 for details) is not due to altered gene expression of NaPi-IIb, PiT1 or PiT2.

4.3.5. Transporter responses to aging

To evaluate whether intestinal and renal expression of PiT transporters are age-dependent at the genomic level, gene expression was analysed in 3-week and 8- or 16-week old rats. The level of NaPi-IIa mRNA was similar in both age groups (Fig. 4.6A). Renal NaPi-IIc and intestinal NaPi-IIb expression is reported to decline with age (Silverstein et al., 1997; Segawa et al., 2002; Xu et al., 2001).
Figure 4.5. Changes in the mRNA expression of type II and type III transporters induced by a low phosphate diet. A: kidney cortex and B: jejunal mucosa was taken from rats maintained on a control or low phosphate diet for 7 days. Fold change was calculated using the comparative ΔΔC_t method using actin as a house keeping gene. Results are mean ± SEM. *P <0.05 and **P <0.01 compared with control diet, using unpaired t-test. n = 6.
Figure 4.6. Age-related changes in the mRNA expression of type II and type III transporters in the A: kidney cortex from young (3 weeks) and adult (8 weeks) rats, and B: duodenal and C: jejunal mucosa from young (3 weeks) and old (16 weeks) rats. Fold change was calculated using the comparative ΔΔCt method using actin as a housekeeping gene. Results are means ± SEM. *P <0.05, ***P <0.005 compared with younger animals, using unpaired t-test. n = 6.
C

**Jejunum**

<table>
<thead>
<tr>
<th></th>
<th>3 weeks</th>
<th>16 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaPi-IIb</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
</tr>
<tr>
<td>PiT1</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="" /></td>
</tr>
<tr>
<td>PiT2</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
</tr>
</tbody>
</table>
Although NaPi-IIc mRNA expression appeared higher in 3-week old rats, this did not reach significance (P=0.23). Renal PiT1 and PiT2 mRNA levels were also similar in young and adult rats.

This study shows the expected age-dependent decline in NaPi-IIb expression in both the duodenum (Fig. 4.6B) and jejunum (Fig. 4.6C). Interestingly, jejunal, but not duodenal, expression of PiT2 mRNA was regulated by age. PiT2 mRNA was 9-fold higher in young animals. These results parallel previous findings that Na\(^+\)-dependent phosphate transport across the intestinal BBM is significantly greater in young rats (see Chapter 2.3.4 for further details). Intestinal PiT1 mRNA expression was similar in both age groups (P=0.09).

4.3.6. Transporter responses to chronic renal failure
To define the genomic effects of the 5/6 nephrectomy model of chronic renal failure on NaPi-II and PiT transporters, mRNA levels were quantified in the kidney and jejunum. For small intestine, only the jejunal region was analysed since it is responsible for the majority of Na\(^+\)-dependent phosphate transport in the rat (see Chapter 2.3.1). In the residual kidney of chronic renal failure rats, NaPi-IIa mRNA levels were significantly reduced (Fig. 4.7A). Interestingly, mRNA expression of PiT1 (Fig. 4.7C) and PiT2 (Fig. 4.7D) were also markedly reduced compared with sham-operated animals. The mean value for NaPi-IIc mRNA expression (Fig. 4.7B) was reduced by chronic renal failure, but this was not statistically significant (P=0.09). In contrast to previous findings (Douard et al., 2010; Marks et al., 2007), NaPi-IIb levels were significantly reduced in the jejunum (P=0.05) compared with sham-operated rats (Fig. 4.8A). Jejunal PiT1 (Fig. 4.8B) and PiT2 (Fig. 4.8C) mRNA levels were also downregulated significantly by chronic renal failure (P <0.05). Chronic renal failure reduced plasma 1,25-dihydroxyvitamin D\(_3\) levels (Table 4.2) and increased plasma levels of PTH and phosphate, as established previously (Marks et al, 2007).
Figure 4.7. mRNA expression of renal A: NaPi-IIa, B: NaPi-IIc, C: PiT1 and D: PiT2 in rats maintained on a control phosphate diet for 8 weeks and subjected to sham nephrectomy (Sham), 5/6 nephrectomy (CRF), or 5/6 nephrectomy and treatment with 3 mg/kg/day MEPE for 2 weeks (MEPE). Fold change was calculated using the comparative $\Delta\Delta C_t$ method using actin as a house keeping gene. Results are mean ± SEM. *P <0.05 and **P <0.01, compared with sham nephrectomy. $n = 3$ for sham and $n = 6$ for CRF and treatment with MEPE, respectively.
C

**PiT1**

Fold change

Sham CRF CRF + MEPE

D

**PiT2**

Fold change

Sham CRF CRF + MEPE
Figure 4.8. Changes in the mRNA expression of jejunal A: NaPi-IIb, B: PiT1 and C: PiT2 in rats maintained on a control phosphate diet for 8 weeks and subjected to sham nephrectomy (Sham), 5/6 nephrectomy (CRF), or 5/6 nephrectomy and treatment with 3 mg/kg/day MEPE for 2 weeks (MEPE). Fold change was calculated using the comparative ∆∆Ct method using actin as a housekeeping gene. Results are mean ± SEM. *P <0.05 and #P <0.05, compared with sham nephrectomy and 5/6 nephrectomy, respectively. n = 3 for sham and n = 6 for CRF and treatment with MEPE, respectively.

**A**

![Graph showing mRNA expression of NaPi-IIb](image)

**B**

![Graph showing mRNA expression of PiT1](image)
C

**PIT2**

![Bar chart showing fold change for Sham, CRF, and CRF + MEPE conditions.](chart.png)

- **Fold change**
  - **Sham**
  - **CRF**
  - **CRF + MEPE**

**Sham CRF CRF + MEPE**

* #
Table 4.2. Parameters confirming the induction of chronic renal failure (CRF) and the influence of chronic MEPE treatment. 5/6 nephrectomised model of CRF rats were injected daily with 3 mg/kg *E.coli*-derived MEPE for 2 weeks. Results are means ± SEM. *n* = 6 per experimental group.

<table>
<thead>
<tr>
<th></th>
<th>Plasma phosphate (mg/dl)</th>
<th>Plasma 1,25-dihydroxyvitamin D₃ (pg/ml)</th>
<th>Plasma PTH (pg/ml)</th>
<th>FEx phosphorus¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>6.63 ± 0.32</td>
<td>189.56 ± 32.46</td>
<td>191.5 ± 88.2</td>
<td>5.79 ± 1.07%</td>
</tr>
<tr>
<td>CRF</td>
<td>8.35 ± 0.55</td>
<td>93.50 ± 12.59</td>
<td>2378.85 ± 1248.4</td>
<td>26.4 ± 2.76%</td>
</tr>
<tr>
<td>CRF + MEPE</td>
<td>8.03 ± 0.50</td>
<td>115.35 ± 28.03</td>
<td>1640.75 ± 689.33</td>
<td>19.76 ± 3.65%</td>
</tr>
</tbody>
</table>

¹ Fractional phosphate excretion in urine
4.3.7. MEPE treatment of chronic renal failure rats

5/6 nephrectomised rats were injected daily with 3 mg/kg \textit{E.coli}-derived MEPE. Following 2 weeks of MEPE treatment, animals displayed significantly lower plasma PTH levels and increased urinary excretion of phosphate compared with vehicle-treated chronic renal failure rats (Table 4.2). Acute infusion of MEPE for 3 hours is known to decrease intestinal and renal phosphate absorption in healthy rats (Marks \textit{et al.}, 2008).

In the present work, the effect of chronic MEPE treatment of chronic renal failure rats on the levels of NaPi-II and PiT mRNA expression was measured. MEPE treatment did not affect NaPi-IIa, PiT1 or PiT2 mRNA expression in the residual kidney of chronic renal failure rats (Fig. 4.7A). Gene expression of NaPi-IIc (Fig. 4.7B), PiT1 (Fig. 4.7C) and PiT2 (Fig. 4.7D) appeared augmented at the end of the MEPE treatment period when compared with vehicle-treated chronic renal failure animals, although values did not reach statistical significance (P=0.15, 0.14 and 0.17, respectively). When compared with vehicle-treated chronic renal failure animals, MEPE did reduce further jejunal expression of NaPi-IIb (Fig. 4.8A) and PiT2 (Fig. 4.8C). It is, therefore, possible that the effect of MEPE in reducing jejunal phosphate absorption (Marks \textit{et al.}, 2008) is mediated via regulation of NaPi-IIb and/or PiT2. MEPE treatment of chronic renal failure animals had reduced PiT1 mRNA levels in the jejunum (Fig. 4.8A), but this was not statistically significant. Thus, PiT transporters in the small intestine and kidney seem to be differentially affected by chronic renal failure and MEPE.
4.4. Discussion

In contrast to NaPi-II transporters, which have been identified and studied in detail, the newly emerging type III transporters, the PiT transporters, were only recently revealed as high affinity BBM Na\(^{+}\)-dependent phosphate transporters. Although it is possible that PiT proteins may act as key transporters alongside type II transporters, in vivo evidence has not yet been shown to support this idea. The present study was designed to increase our understanding of the genomic expression and regulation of the type II and type III Na\(^{+}\)-dependent phosphate transporters in kidney and small intestine. Na\(^{+}\)-dependent phosphate transporters are differentially expressed in the small intestine and kidney, and there is thought to be functional heterogeneity between the type II and type III transporters (Marks et al., 2010; Villa-Bellosta et al., 2009b; Villa-Bellosta & Sorribas, 2010b; Virkki et al., 2007). A better understanding of the specific properties of renal and intestinal PiT transporters would be important in therapeutic intervention to treat hyperphosphataemia and help prevent vascular calcification in chronic renal failure patients.

Renal and intestinal phosphate absorption has been shown to be modulated by dietary phosphate intake, age, 1,25-dihydroxyvitamin D\(_3\), PTH and phosphatoninns such as MEPE. In the present study, the relative abundance of mRNA expression for PiT transporters in the small intestine and kidney was measured under conditions of chronic low phosphate diet, aging, and chronic renal failure, and compared with NaPi-II mRNA levels to study adaptation at the genomic level. In rats, renal PiT2 mRNA expression was augmented by chronic low phosphate diet. Results of the present study also provide compelling evidence that intestinal, but not renal, PiT2 gene expression is higher in young animals. PiT1 mRNA expression in both kidney and small intestine was not altered by chronic low phosphate diet or aging, highlighting the differential regulation of PiT1 and PiT2. Animals with chronic renal failure
displayed reduced intestinal and renal PiT1 and PiT2 mRNA expression. Furthermore, the data show that chronic treatment with the phosphaturic protein MEPE to chronic renal failure rats downregulates NaPi-IIb and PiT2 mRNA in the jejunum, but does not affect NaPi-IIa and PiT2 mRNA expression in the kidney. This is the first report of the effect of chronic renal failure on expression of PiT transporters and the effect of chronic treatment with MEPE on expression of both PiT and NaPi-II transporters.

Rat and mice exhibit significant differences in intestinal phosphate handling (Marks et al., 2006; Radanovic et al., 2005). In agreement with previous work, data in the present study show that NaPi-IIb mRNA transcript is highly expressed in the mouse ileum but undetectable in the rat ileum. Similar levels of NaPi-IIb are present in the rat duodenum and jejunum. PiT1 and PiT2 are equally expressed and at significant levels in all three regions of rat and mouse small intestine. This contradicts a recent report which demonstrated minimal PiT2 mRNA expression along the entire length of the rat small intestine (Giral et al., 2009). Giral et al (2009) also found that PiT1 mRNA was highly expressed in the ileum, but PiT1 protein was undetectable in the ileum. The present study shows no segmental or species differences in the genomic expression of PiT1 and PiT2 transporters in rat and mouse small intestine.

The rat ileum has very little ability to absorb phosphate and further evidence for this comes from previous studies showing negligible expression of NaPi-IIb and PiT1 at the ileal BBM (Giral et al., 2009; Marks et al., 2006). In contrast to the rat, all regions of the mouse small intestine have the capacity to absorb phosphate (Marks et al., 2006; Radanovic et al., 2005). So the fact that significant levels of PiT1 and PiT2 mRNA are detectable in the mouse small intestine could mean that PiT1 and PiT2 mediate Na\(^+\)-dependent phosphate absorption, particularly in the duodenum and jejunum, where there is minimal expression of both NaPi-IIb mRNA and protein (Marks et al., 2006; Radanovic et al., 2005). It has been suggested that the rat ileum is a site
of paracellular phosphate secretion (Peters & Binswanger, 1988), but this statement has yet to be confirmed.

The role of PiT transporters in phosphate homeostasis is only just emerging and their role under conditions that stimulate phosphate absorption, such as feeding a low phosphate diet, has yet to be defined. Restriction of dietary phosphate intake is used in patients with chronic renal failure to lower plasma phosphate levels. What is known so far is that PiT2 is a highly regulated protein (Breusegem et al., 2009; Giral et al., 2009; Villa-Bellosta et al., 2009b; Villa-Bellosta & Sorribas, 2010b). Villa-Bellosta et al (2009b) demonstrated that switching from a low to high phosphate diet inhibited PiT2-mediated transport to a greater degree than NaPi-IIa- and NaPi-IIc-mediated transport. Therefore, although both NaPi-IIb and PiT proteins transport the same substrate, they are differentially regulated. This is also evident from the finding that rat PiT2 is intermediate between NaPi-IIa and NaPi-IIc in the time taken for the half-maximal renal response to dietary phosphate loading (Villa-Bellosta et al., 2009b). Present results show that animals fed a chronic low phosphate diet had not only a significantly increased mRNA expression of renal NaPi-IIa, but also increased PiT2. NaPi-IIc and PiT1 mRNA levels were however unaffected. This parallels recent work that found increased protein expression of NaPi-IIa and PiT2 in phosphate-deprived rats (Giral et al., 2009; Villa-Bellosta et al., 2009b). Therefore, increased renal Na\(^{+}\)-dependent phosphate transport seen following a chronic low phosphate diet is mediated by genomic changes in NaPi-IIa, and probably PiT2. In adult rats, the relative contribution of non-NaPi-II-mediated transport (phosphonoformic acid (PFA)-insensitive) in kidney has been estimated to be between 25-50%, depending on the dietary conditions, and PiT2 is thought to be a strong candidate for this component (Villa-Bellosta & Sorribas, 2010b; Picard et al., 2010). In double knockout mice, where both NaPi-IIa and NaPi-IIc genes were deleted, substantial Na\(^{+}\)-dependent phosphate reabsorption remained (Segawa et al., 2009a); PiT transporters are thought to be responsible for this component. In mouse however, renal expression of PiT1 and
PiT2 mRNA is not regulated by a low phosphate diet (Hoag et al., 1999; Tenenhouse et al., 1998).

The mechanism by which low phosphate diet upregulates intestinal phosphate absorption does not involve changes to NaPi-IIb or PiT1 mRNA expression (Capuano et al., 2005; Giral et al., 2009; Hattenhauer et al., 1999; Katai et al., 1999). The response is, therefore, thought to involve post-translational mechanisms, where NaPi-IIb protein expression at the intestinal BBM is controlled according to dietary phosphate intake (Capuano et al., 2005; Hattenhauer et al., 1999; Katai et al., 1999; Giral et al., 2009). In the present study, NaPi-IIb and PiT1 mRNA levels were unchanged in rats fed a low phosphate diet. For the first time, the present study indicates that intestinal PiT2 mRNA expression is not regulated by a low phosphate diet.

Taken together with the uptake data presented in Chapter 2, the increased intestinal Na\(^+\)-dependent and Na\(^+\)-independent phosphate transport seen following a chronic low phosphate diet was not due to changes in mRNA expression of NaPi-IIb, PiT1 or PiT2. At the protein level, Giral et al (2009) showed that although significant levels of PiT1 proteins were detectable in duodenum and jejunum, their expression was not altered by a low phosphate or a high phosphate diet. Intestinal PiT2 protein expression under conditions of a low phosphate diet has not yet been examined. Thus, although intestinal PiT2 mRNA expression is unchanged by a low phosphate diet, PiT2 protein level may be regulated. Therefore, it is critical that future studies examine intestinal PiT2 protein expression following the feeding of a low phosphate diet.

Phosphate absorption is regulated according to body demand for phosphate during development. Na\(^+\)-dependent phosphate uptake by intestinal and renal BBM vesicles decreases with age (Hoag et al., 1999; Silverstein et al., 1997; Xu et al., 2002a). This is complemented at the genomic level, evident from the proportional age-dependent decline in intestinal NaPi-IIb and renal NaPi-Ilc expression (Kirchner et al., 2008;
Segawa et al., 2002; Silverstein et al., 1997; Xu et al., 2002a). In contrast, expression of NaPi-IIa protein increases with age and the highest levels are found in adult kidney (Arar et al., 1999; Chau et al., 2003; Sorribas et al., 1996). In the present study, expression of PiT transporters was analysed in young and adult rats to assess their role in phosphate absorption during growth. NaPi-IIa mRNA expression was similar in both young animals and adults, but surprisingly NaPi-IIc mRNA levels were also similar. It is generally accepted that NaPi-IIc expression is age-dependent. However, there are only two reports confirming this (Segawa et al., 2002; Silverstein et al., 1997). Before NaPi-IIc was cloned, Silverstein et al. (1997) demonstrated that a specific mRNA in rat kidney, partially homologous to the NaPi-IIa isoform, mediated higher renal Na⁺-dependent phosphate transport in younger rats than in adults. Later, Segawa et al. (2002) focused on rats at three developmental stages: sucklings (not separated from their mother, and milk is their only source of food), weaning animals (separated from their mother and subjected to some solid food), and adults. They found NaPi-IIc protein and mRNA expression was lowest in sucklings and highest in weaning animals, whilst in adults NaPi-IIc was lower. Data obtained in the present study did not show the age-dependent decline in NaPi-IIc mRNA expression. This is likely to be because sucklings were used in the present study instead of weaning animals, which have similar levels of NaPi-IIc expression to adult rats (Segawa et al., 2002). Current knowledge of NaPi-IIc protein, which is historically thought to play a minor role in kidney phosphate reabsorption, is being challenged by newly emerging roles for NaPi-IIc in adults, especially under pathophysiological conditions associated with phosphate wasting disorders (Bergwitz et al., 2006; Jaureguiberry et al., 2008; Segawa et al., 2009a & 2009b).

PiT1 and PiT2 are proposed to play an insignificant role in renal phosphate reabsorption in mice. A study by Hoag et al. (1999) showed that PiT1 and PiT2 are not age-dependent in wild type mice, although their genomic expression was age-dependent in NaPi-IIa knockout mice.
The results of this present study show that, similar to the mouse, rat renal PiT1 and PiT2 gene expression is not age-dependent.

Present data show that gene expression of NaPi-IIb is elevated in the duodenum and jejunum of young rats, corresponding to the increased Na\(^{+}\)-dependent phosphate uptake seen in the duodenum and jejunum (see Chapter 2). Similar to the kidney, intestinal PiT1 mRNA expression was also not regulated by age. In contrast, PiT2 mRNA expression in the jejunum, but not the duodenum, declined with age. Therefore, the small intestine of young rats adapts to increased phosphate need during growth by expressing augmented levels of PiT2 in the jejunum, the intestinal region where the majority of the phosphate is absorbed (see Chapter 2), in addition to higher NaPi-IIb mRNA levels in both the duodenum and jejunum. These data provide evidence for the first time of region-specific adaptation of PiT transporters. It is tempting to propose that, combined with the uptake data from rats of the three different ages (see Chapter 2), the elevated phosphate transport activity in young animals is mediated by higher expression of both NaPi-IIb and PiT2.

Information is lacking as to whether changes in mRNA data are matched at the protein level. Results of the present study are at the genomic level, which means they may not mirror the abundance of transporter protein. It is clear that neither renal nor intestinal PiT1 mRNA is regulated by a low phosphate diet and age, the two most important physiological regulators of phosphate absorption in mammals.

In chronic renal failure, hyperphosphataemia increases vascular and soft tissue calcification. There is compelling evidence that PiT1 has a direct role in promoting calcification in vascular smooth muscle cells (Li et al., 2006; Villa-Bellosta et al., 2009a). However, little is known concerning the expression of PiT transporters in the small intestine and kidney in chronic renal failure. Studies have reported a reduction of renal expression of NaPi-IIa in chronic renal failure rats (Bacic et al., 2006; Marks et al., 2006; Traebert et al., 2000). In keeping with these reports,
present data show that declining renal function is associated with downregulation of NaPi-IIa mRNA. In agreement with earlier reports (Imanishi et al., 1996; Nagano et al., 2006), chronic renal failure animals used in the present work showed secondary hyperparathyroidism with elevated plasma PTH levels. PTH is a major regulator of renal phosphate handling and causes both acute and chronic reductions in renal tubular phosphate reabsorption by promoting the internalisation, and subsequent lysosomal degradation of NaPi-IIa from the BBM (Bacic et al., 2006; Murer et al., 2000; Traebert et al., 2000). In contrast, PTH has no direct effect on intestinal phosphate transport. The chronic renal failure-induced reduction in NaPi-IIa mRNA expression may be mediated by the increased circulating PTH levels. Although PTH levels are elevated in chronic renal failure, the involvement of PTH in renal responses to chronic renal failure is uncertain, due to possible tissue resistance to the hormone (Rodriguez et al., 1991; Massry et al., 1973).

Segawa et al (2007) provided compelling evidence that PTH is also a major regulator of NaPi-IIc. In their study, NaPi-IIc protein levels were augmented in thyroparathyroidectomised rats, and acute administration of PTH caused a decrease in NaPi-IIc proteins, an effect that involved retrieval of NaPi-IIc protein from the BBM. Furthermore, Picard et al (2010) showed that PTH completely inhibited PFA-sensitive Na\(^+\)-dependent phosphate transport in the kidney (Picard et al., 2010). The PFA-sensitive uptake component in the kidney represents both NaPi-IIa- and NaPi-IIc-mediated transport. Although results presented in this chapter show that the mean value for renal NaPi-IIc mRNA expression is reduced by chronic renal failure, it was not statistically significant (P=0.09). However, the trend implies that chronic renal failure affects NaPi-IIc expression at the genomic level.

Hyp is a mouse mutation characterised by phosphate wasting, due to a defect in Na\(^+\)-dependent phosphate transport at the renal BBM. In the Hyp mouse, the serum phosphate level is lower than normal and NaPi-IIa mRNA is reduced by some 50% (Roy et al., 1997; Tenenhouse et al.,
1998). Interestingly, the study of Tenenhouse et al (1998) showed that renal mRNA expression of PiT1 but not PiT2 is higher in the Hyp mouse, suggesting that PiT1 plays a compensatory role for NaPi-IIa under phosphate wasting conditions in mouse. Therefore, the data of Tenenhouse et al (1998) suggests that the adaptive changes in PiT1 mRNA levels contribute to the effects of the Hyp mutation on the renal handling of phosphate. What happens to Na\(^+\)-dependent phosphate transport across the small intestine under these conditions is however unknown. Therefore, it was of interest to compare the regulation of PiT1 and PiT2 during hyperphosphataemia. Results presented in this chapter provide evidence, for the first time, that in chronic renal failure rats levels of both PiT1 and PiT2 mRNA are reduced in the kidney. Picard et al (2010) recently demonstrated that in the kidney of control rats, PTH not only reduced PFA-sensitive NaPi-IIa- and NaPi-IIc-mediated transport, but also PFA-resistant Na\(^+\)-dependent phosphate uptake, suggesting that PiT transporters are responsible for this PFA-resistant component, and that they are inhibited by PTH (Picard et al., 2010). Similarly, PTH inhibited PiT1-mediated Na\(^+\)-dependent phosphate uptake in human embryonic kidney cells in vitro (Fernandes et al., 1999). The reduced levels of renal PiT1 and PiT2 mRNA observed in the present study, therefore, may be mediated by the inhibitory action of PTH.

The intestinal handling of phosphate and the genomic and protein expression of NaPi-IIb are reported to be unaffected in chronic renal failure rats (Douard et al., 2010; Marks et al., 2007). Data presented in the present study however show that NaPi-IIb mRNA expression is reduced in chronic renal failure rats. The apparent discrepancy may be explained by the fact that the present study used chronic renal failure model that displayed both hyperphosphataemia and secondary hyperparathyroidism, which was induced by feeding chronic renal failure animals with a high phosphate/low calcium diet to accentuate the features normally seen in chronic renal failure animals that have not been given any dietary treatment. Thus, the severity of hyperphosphataemia may have differential effects on the intestinal
expression of Na\textsuperscript{+}-dependent phosphate transporters. Evidently, in patients with end-stage renal disease (severe chronic renal failure requiring haemodialysis), jejunal phosphate absorption is significantly reduced (Davis \textit{et al.}, 1983). A similar phenomenon has also been reported for NaPi-IIa, where NaPi-IIa protein and mRNA expression are reduced only in severe chronic renal failure animals (Laouari \textit{et al.}, 1997). Their study demonstrated that vitamin D\textsubscript{3} deficiency of chronic renal failure reduced active, but not passive, phosphate absorption by 50\%, an effect likely to have been mediated by reduced expression of Na\textsuperscript{+}-dependent phosphate transporters.

Data presented in this chapter provide evidence that intestinal expression of PiT1 and PiT2 mRNA is reduced in chronic renal failure rats. The mechanism through which this downregulation is achieved is not clear at the present time. As discussed previously, intestinal and renal handling of phosphate is regulated by different mechanisms and PTH is not thought to play a direct role in the regulation of intestinal phosphate absorption (Borle \textit{et al.}, 1963; Murer \textit{et al.}, 2001; Nemere & Larsson, 2002). Decreased secretion of 1,25-dihyrdroxyvitamin D\textsubscript{3} may be responsible for causing the reduced intestinal PiT1 and PiT2 and/or NaPi-IIb mRNA expression, since plasma 1,25-dihydroxyvitamin D\textsubscript{3} levels were reduced in the chronic renal failure animals used in the present work. Although it is clear that NaPi-IIb expression is increased by 1,25-dihydroxyvitamin D\textsubscript{3}, there is only one published report of 1,25-dihydroxyvitamin D\textsubscript{3} regulation of PiT transporters: Katai \textit{et al} (1999) demonstrated a greater rate of PiT2-mediated transport and increased expression of PiT2 mRNA in enterocytes from rats treated with 1,25-dihydroxyvitamin D\textsubscript{3}.

The phosphaturic protein MEPE inhibits both intestinal and renal Na\textsuperscript{+}-dependent phosphate transport in healthy animals (David \textit{et al.}, 2009; Marks \textit{et al.}, 2008; Rowe \textit{et al.}, 2004). Acute inhibition of renal phosphate reabsorption by MEPE is mediated by decreased NaPi-IIa protein expression at the BBM (David \textit{et al.}, 2009; Marks \textit{et al.}, 2008).
However, whether MEPE has a similar effect on phosphate transporter expression in chronic renal failure is unknown. The present work, therefore, focused on whether chronic MEPE treatment can further downregulate the expression of intestinal and renal phosphate transporters in chronic renal failure. To study this, the genomic expression of renal and jejunal NaPi-II and PiT transporters were analysed. Since there are currently no suitable antibodies available for detection of NaPi-Ilb and PiT transporters in rats, expression of these proteins could not be assessed. Data show that renal expression of NaPi-IIa, NaPi-IId, PiT1 and PiT2 mRNA is not significantly affected by chronic treatment with MEPE in chronic renal failure rats. Although mean values for expression of these transporters were increased by MEPE, the changes did not reach significance.

In the small intestine, acute infusion of MEPE is known to decrease jejunal, but not duodenal, phosphate absorption in a dose-dependent manner (Marks et al., 2008). Although the study of Marks et al (2008) did not involve the analysis of expression levels of Na\(^+\)-dependent phosphate transporters at the intestinal BBM, it is unlikely that the acute 3-hour infusion caused a change at the genomic level. Data presented in this chapter show that chronic treatment with MEPE reduced NaPi-Ilb and PiT2 mRNA expression in the jejunum of chronic renal failure animals. The mechanism of this action is unknown and may be direct or indirect. Intestinal PiT1 mRNA levels were not altered by MEPE. Taken together, these findings show that jejunal PiT2 is as highly regulated as NaPi-Ilb, since both transporters are regulated at the genomic level by aging, chronic renal failure, and MEPE.

Low dietary intake of phosphate and the use of oral phosphate binders are currently used to control hyperphosphataemia in man. However, these approaches have several disadvantages that make them not ideal for reducing plasma levels of phosphate. An alternative method would be to directly target renal and intestinal PiT transporters, in addition to NaPi-IIa and NaPi-Ilb transporters. The small intestine is arguably a better
target for agents designed to control hypephosphataemia, since chronic renal failure progressively reduces renal function. Thus, MEPE is a promising candidate for the suppression of intestinal phosphate uptake to reduce plasma levels of phosphate. Modulation of PiT expression and PiT-mediated transport in small intestine might also be used for prevention and treatment of hyperphosphataemia.

4.5. Conclusion

There are no clear regional differences between the rat and mouse regarding the intestinal expression of PiT1 and PiT2. These proteins are equally expressed at the genomic level in all three regions of the small intestine. Changes in intestinal Na\(^+-\)dependent phosphate transport mediated by a chronic low phosphate diet does not require de novo RNA synthesis of NaPi-IIb, PiT1 and PiT2. For the kidney, adaptation to a chronic low phosphate diet is mediated by changes to both NaPi-IIa and PiT2 expression. Young rats express higher levels of both NaPi-IIb and PiT2 mRNA in the small intestine in contrast to the kidney, which shows no obvious age-dependent adaptation of NaPi-IIa, NaPi-IIc, PiT1 and PiT2 mRNA levels. Chronic renal failure reduces PiT1 and PiT2 mRNA expression in both rat small intestine and kidney. This effect might be mediated by decreased 1,25-dihydroxyvitamin D\(_3\) and/or increased plasma PTH levels. However, the mechanism of 1,25-dihydroxyvitamin D\(_3\) and PTH on PiT expression remains to be elucidated. MEPE successfully downregulates intestinal expression of NaPi-IIb and PiT2 mRNA in chronic renal failure, but does not significantly affect expression of renal phosphate transporters, although there was a trend towards stimulation. PiT transporters have been strongly implicated in various conditions of altered phosphate balance; therefore, it is important to characterise further the role of PiT transporters in both normal and disease conditions. This study extends our previously limited knowledge of the regulation of PiT transporters and may help to define the ways in which these proteins are regulated.
5.0. The effect of duodenal phosphate instillation on intestinal phosphate uptake and expression of renal phosphate transporters
5.1. Introduction

The small intestine and kidney are highly specialised organs that have the capacity to communicate with each other to co-ordinate regulation of certain plasma constituents. An example of such regulation is seen after oral sodium loading. Sodium homeostasis involves an enteric-renal communication axis; the intestine produces guanylin and uroguanylin that are important for natriuresis and these peptides influence intestinal and renal epithelial cells to increase excretion of sodium and water (Currie et al., 1992; Hamra et al., 1993; Kita et al., 1999; Sindic & Schlatter, 2007). An enteric-renal axis has also been reported for calcium and potassium (Conigrave & Brown, 2006; Lee et al., 2007). Calcium-sensing receptors are widely expressed in epithelial cells of the gastrointestinal tract and kidney, and the receptor plays a key role in calcium homeostasis (Chattopadhyay et al., 1998; Conigrave & Brown, 2006; Hebert et al., 2004; Lee et al., 2007).

Berndt et al (2007) demonstrated that instillation of a high concentration of sodium-phosphate (1.3 M) into the proximal small intestine increased renal phosphate excretion; an acute effect achieved within 20 min. When an equimolar concentration of sodium chloride was instilled into the duodenum no effect on phosphate excretion was observed, and instillation of 1.3 M phosphate into the stomach was also without effect on phosphate excretion, indicating a specific effect of phosphate in the upper small intestine. Furthermore, the response was not due to changes in serum levels of phosphate, PTH, Fibroblast growth factor-23 (FGF-23) or secreted frizzled-related protein-4 (sFRP-4), or changes in glomerular filtration rate or the involvement of a neural reflex. Additionally, Berndt et al (2007) showed that duodenal infusion of an extract prepared from the duodenal mucosa had a phosphaturic effect. Thus, the duodenum is proposed to sense phosphate and secrete a phosphaturic humoral factor that is able to communicate rapidly with the kidney to regulate renal phosphate reabsorption. This factor is so far
unidentified and whether it affects phosphate absorption locally, in other regions of the small intestine, is also undetermined.

PTH is a major regulator of renal phosphate reabsorption and it is capable of changing renal phosphate transport within minutes. Martin et al (2005) showed that the introduction of phosphate (60 µmol) into the upper intestinal tract rapidly (within 10 minutes) increased plasma levels of phosphate and PTH, which throws some doubt on the mechanism causing the increased phosphate excretion in the experiments of Berndt et al (2007). Martin et al (2005) also showed that duodenal infusion of phosphonoformic acid (PFA - NaPi-IIb inhibitor) increased PTH levels rapidly (within 5 minutes), but this effect was independent of changes in plasma phosphate levels.

Recently, matrix extracellular phosphoglycoprotein (MEPE) mRNA was found to be highly expressed in the duodenum, almost 17-fold higher than in the jejunum and ileum (Marks et al., 2010). The phosphatonin MEPE, found also in the kidney (Ogbureke & Fisher, 2005), inhibits phosphate absorption in both the small intestine and the kidney, and its actions in the small intestine are independent of changes in levels of 1,25-dihydroxyvitamin D₃, PTH and FGF-23 (David et al., 2009; Marks et al., 2008; Rowe et al., 2000), as observed for the duodenal phosphaturic factor proposed by Berndt et al (2007). Thus, MEPE is a potential candidate for the ‘intestinal phosphatonin’. However, the pathway through which MEPE inhibits phosphate absorption has not yet been characterised. The inhibitory effect of MEPE on intestinal phosphate absorption is confined to the jejunum and does not affect the duodenum. It is therefore of interest to examine whether the putative intestinal phosphatonin affects phosphate uptake in the jejunum following exposure of the intestinal mucosa to high phosphate concentrations.

The 1.3 M phosphate concentration used by Berndt et al (2007) for instillation experiments is a non-physiological choice of phosphate
concentration, and the study needs to be repeated using phosphate levels normally seen after ingestion of a phosphate-containing meal. Furthermore, although the study by Berndt *et al.* (2007) showed an increase in fractional excretion of phosphate, renal expression of phosphate transporters NaPi-IIa and NaPi-IIc was not determined. Such information would be a key indicator of changes in renal Na\(^+\)-dependent phosphate transport.

Due to declining kidney function in chronic renal failure, the kidney cannot be targeted for treatment of hyperphosphataemia. The small intestine on the other hand is suitable, since intestinal phosphate absorption is not significantly altered in chronic renal failure (Douard *et al.*, 2010; Marks *et al.*, 2007). It is, therefore, important to investigate whether a proposed intestinal mucosal factor affects intestinal phosphate transport since the factor might be a potential candidate for the control of serum phosphate levels in hyperphosphataemia.

The aim of the present study was to establish whether acute duodenal exposure to high millimolar (physiological) and molar (non-physiological) concentrations of phosphate causes local adaptation of phosphate absorption in the upper small intestine, and whether the claimed effect on renal phosphate excretion, by Berndt *et al.* (2007), is mediated through changes in expression of NaPi-IIa or NaPi-IIc proteins. This was investigated by instilling phosphate into *in vivo* duodenal loops for 30 minutes, and subsequently measuring phosphate uptake using the everted sleeve technique. The expression of phosphate transporters in BBM prepared from kidneys taken from these animals was also quantified.
5.2. Materials and Methods

5.2.1. Animals
Male Sprague Dawley (SD) rats aged 8 weeks were obtained from Charles River Laboratories (UK) and used in accordance with the Animals (Scientific Procedures) Act 1986. Control rats were allowed *ad libitum* access to a standard rodent chow containing 0.52% phosphate (Diet RM1, SDS Ltd, Witham, Essex, UK), and to water. During anaesthesia, Animals were checked for pedal withdrawal to a pinch and eye-blink reflex before starting surgery. Additional anaesthetic was given if animals showed any of these two reflex responses.

5.2.2. Phosphate instillation into the duodenum

5.2.2.1. 15 mM phosphate
The procedures and buffers used were identical to those given by Berndt *et al* (2007) except that the instilled solution in the duodenum was prevented from entering the jejunal lumen by setting a ligature at the duodenal-jejunal junction. This allowed investigation of the local effect of duodenal instillation of phosphate on jejunal phosphate transport. Rats were anaesthetised with an intraperitoneal injection of 45 mg/kg pentobarbitone sodium and their body temperature was maintained at 37°C throughout the instillation period using a thermostatically controlled heating blanket. The duodenum was cannulated with a small incision ~1 cm from the pylorus. A needle was inserted and tied in place by a ligature. About 5 cm distal to the needle, a ligature was placed to create a closed duodenal loop. 1 ml of 15 mM NaH$_2$PO$_4$, dissolved in distilled water (pH 5), was then infused via the duodenal needle over a period of 15 sec. For control experiments, 1 ml of 15 mM NaCl was similarly instilled. An instillation period of 30 min was used.

5.2.2.2. 1.3 M phosphate
This was carried out as described above, except 1 ml of 1.3 M NaH$_2$PO$_4$ (test buffer), or 1.3 M NaCl (control buffer) dissolved in distilled water (pH
5) was used. Berndt et al (2007) do not mention the solution in which sodium chloride and sodium phosphate were dissolved; the present study opted to use distilled water because of the high solution osmolarity.

5.2.3. Phosphate uptake by everted intestinal sleeves
Phosphate uptake by duodenum (the region used for instillation) and jejunum (2-3 cm from the ligament of Treitz) was measured after 30 min exposure of the duodenum to the instillation solutions. The everted sleeve technique was used and the procedure was as described in section 2.2.2.1. The incubation buffer contained 0.1 mM \(^{32}\)P.

5.2.4. Preparation of kidney BBM
Kidney BBM were prepared using a double Mg\(^+\) chelation procedure at 4°C, as described previously by Biber et al (1981). Both kidneys were excised and transferred to a beaker containing ice cold 154 mM NaCl. Each kidney was placed on an ice-cold glass surface and the fat and capsule was removed and the kidney was sliced into 2 mm sections. The outer layer of cortex was removed and the remainder of the kidney was discarded. The weight of the cortex (representing proximal tubules) was recorded and in some cases kidney sections were pooled. A starting cortex weight of about 1 g was homogenised using an Ultra Turrax homogeniser for 2 min at setting 5 in 30 ml R1 buffer (pH 7.4) containing, in mM: 300 mannitol, 12 Tris-HCl, 5 EGTA and protease inhibitors (Sigma, UK). Deionised water (42 ml) and MgCl\(_2\) was added to the homogenate to a final Mg concentration of 12 mM, and stirred continuously for 15 min. This mixture was centrifuged at 3,000 g for 15 min and the supernatant further centrifuged at 27,000 g for 30 min. The resulting pellet was resuspended in 20 ml R2 buffer (pH 7.4) containing, in mM: 150 mannitol, 6 Tris-HCl, 2.5 EGTA and protease inhibitors, using a hand-held Teflon homogeniser. This mixture was stirred with 12 mM MgCl\(_2\) for 15 min and centrifuged twice as described above. The resulting pellet was resuspended in 20 ml R3 buffer (pH 7.4) containing, in mM: 300 mannitol, 12 Tris-HCl, 2.5 EGTA and protease inhibitors, using the hand-held homogeniser, and centrifuged at 27,000 g for 30
min. The final pellet was resuspended in ~500 µl R3 buffer, using a 21-gauge needle fitted to a syringe, to a final protein concentration of ~5-9 mg/ml. Aliquots of BBM suspension were immediately frozen in liquid nitrogen and stored at -20°C.

5.2.5. Determination of protein concentration and vesicle purity
Protein concentration was determined using the Bradford assay, as described in section 2.2.4.3.

BBM purity was assessed using alkaline phosphatase assay, as described in section 2.2.4.4. BBM showed enrichment of 6.12 ± 0.43 fold (n=24) with respect to the starting homogenate.

5.2.6. Western Blotting
The NaPi-IIa and NaPi-IIc antibodies listed in Table 5.1 were used to quantify changes in levels of these proteins at the BBM, as described in section 2.2.6.1. β-Actin was used as a loading control.

5.2.7. Statistical analysis.
Data are presented as means ± SEM. The unpaired t-test was used and group differences were considered statistically significant if P <0.05; n, represents the number of samples per group.
<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Source</th>
<th>Secondary Antibody</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaPi-IIa</td>
<td>1:2000</td>
<td>Amersham Pharmacia Biotech UK Limited, Bucks, UK</td>
<td>Donkey anti-rabbit</td>
<td>1:2000</td>
<td>Gift from Biber J (Institute of Physiology, University Zürich, Switzerland)</td>
</tr>
<tr>
<td>NaPi-IIc</td>
<td>1:2000</td>
<td>Amersham Pharmacia Biotech UK Limited, Bucks, UK</td>
<td>Donkey anti-rabbit</td>
<td>1:2000</td>
<td>Gift from Biber J (Institute of Physiology, University Zürich, Switzerland)</td>
</tr>
<tr>
<td>β-actin</td>
<td>1:5000</td>
<td>Sigma Ltd., Poole, UK</td>
<td>Mouse anti-mouse</td>
<td>1:5000</td>
<td>Acam, Cambridge, UK</td>
</tr>
</tbody>
</table>

**Table 5.1. Primary and secondary antibodies used in Western Blotting.**

- **Primary Antibody**: NaPi-IIa, NaPi-IIc, β-actin.
- **Dilution**: 1:2000, 1:5000.
- **Source**: Amersham Pharmacia Biotech UK Limited, Bucks, UK; Sigma Ltd., Poole, UK; Acam, Cambridge, UK.
- **Secondary Antibody**: Donkey anti-rabbit, Mouse anti-mouse.
- **Dilution**: 1:2000, 1:5000.
- **Source**: Gift from Biber J (Institute of Physiology, University Zürich, Switzerland).

**Species-specified epitope**
- NaPi-IIa: Rabbit C terminus, 12-mer C-terminal, peptide contained an extra cystein residue (Custer et al., 1994).
- NaPi-IIc: Rabbit Not known.
- β-actin: Goat First 14 residues, Xenopus laevis.
5.3. Results

5.3.1. Effect of 15 mM phosphate instillation on intestinal phosphate uptake

A 15 mM phosphate concentration was chosen to represent that normally present in the intestinal lumen following ingestion of a high phosphate-containing diet. Following ingestion of a normal diet, the average ‘free’ (unbound) concentration of phosphate noted in the lumen of stomach and duodenum of rats is ~15 and 7 mM, respectively (see Chapter 2, Table 2.1). In contrast to the protocol of Berndt et al (2007), the test solution instilled into the duodenum was prevented from entering the jejunum.

The jejunum displayed a higher rate of phosphate uptake than the duodenum (Figs. 5.1), as observed in Chapter 2. Rats instilled with 15 mM phosphate expressed similar levels of phosphate transport in the duodenum (Fig. 5.1A) and jejunum (Fig. 5.1B), as those instilled with control solution of equimolar concentration of saline.

5.3.2. Effect of 15 mM phosphate instillation on expression of Na\(^+\)-dependent phosphate cotransporters at the renal BBM

To determine whether the decreased renal phosphate reabsorption reported by Berndt et al (2007) is mediated through altered expression of Na\(^+\)-dependent phosphate transporters at the renal BBM, NaPi-IIa and NaPi-IIc proteins were quantified using western blotting. Results revealed no statistically significant difference in the expression of NaPi-IIa (P=0.36) or NaPi-IIc (P=0.15) between the two groups (Fig 5.2), although there was a trend for decrease in NaPi-IIa expression with 15 mM phosphate instillation. It was not possible to obtain urinary phosphate excretion data, since preliminary data showed that stress of the surgery caused instability in the glomerular filtration rate.
Figure 5.1. Phosphate transport measured *in vitro* using A: duodenum and B: jejunum, following instillation of 1ml 15 mM saline (NaCl) or 15 mM phosphate (KH$_2$PO$_4$) into the duodenum. Uptake was measured for 2 min using buffer containing 0.1 mM $^{32}$P. Data are means ± SEM. $n = 6$. 

A

**Duodenum**

![Bar chart showing phosphate uptake in duodenum](chart_a)

B

**Jejunum**

![Bar chart showing phosphate uptake in jejunum](chart_b)
Figure 5.2. Quantification of NaPi-IIa and NaPi-IIc proteins in renal BBM using western blotting. BBM was prepared from animals instilled with 1 ml of 15 mM saline (clear bars) or 15 mM phosphate (shaded bars) into the duodenum for 30 min. β-actin was used as a loading control to calculate expression of NaPi-IIa and NaPi-IIc proteins. Results are expressed as means ± SEM. \( n = 6 \).
5.3.3. Effect of 1.3 M phosphate instillation on intestinal phosphate uptake

To mimic the effects seen by Berndt et al (2007), 1.3 M phosphate was instilled into the duodenum for 30 minutes. An equimolar concentration of saline was used as a control. As observed with 15 mM instillation, jejunal phosphate transport was unaffected by instillation of 1.3 M phosphate into the duodenum (Fig 5.3B).

Interestingly, phosphate transport in the duodenum (Fig. 5.3A) was upregulated by 59% following instillation of 1.3 M phosphate, when compared with instillation of 1.3 M saline. A similar transport response was reported in BBM vesicles from duodenum, but not the jejunum, following acute feeding of a high phosphate diet (for 4 hours) to rats (Giral et al., 2009). Interestingly, duodenal, but not jejunal, phosphate transport activity was over 4-fold lower with 1.3 M phosphate instillation compared with 15 mM phosphate instillation (Fig. 5.1A vs Fig. 5.3A). This might be due to the much higher osmolarity of the 1.3 M phosphate solution.

5.3.4. Effect of 1.3 M phosphate instillation on expression of Na\(^+\)-dependent phosphate cotransporters at the renal BBM

As observed with instillation experiments using 15 mM phosphate, 1.3 M phosphate instillation did not cause a significant change in the BBM expression of NaPi-IIa or NaPi-IIc proteins when compared with control saline instillation (Fig. 5.4). This means that the increased urinary phosphate excretion observed by Berndt et al (2007) following instillation of 1.3 M sodium-phosphate into the duodenum is not due to reduced expression of transporter protein at the renal BBM.
Figure 5.3. Phosphate transport measured *in vitro* using A: duodenum and B: jejunum, following instillation of 1ml of 1.3 M saline (NaCl) or 1.3 M phosphate (KH$_2$PO$_4$) into the duodenum. Uptake was measured for 2 min using buffer containing 0.1 mM $^{32}$P. Data are means ± SEM. $n = 6$. ***P <0.005 compared with 1.3 M saline instillation.
Figure 5.4. Quantification of NaPi-IIa and NaPi-IIc proteins in renal BBM using western blotting. BBM was prepared from animals instilled with 1 ml of 1.3 M saline (clear bars) or 1.3 M phosphate (shaded bars) into the duodenum for 30 min. β-actin was used as a loading control to calculate expression of NaPi-IIa and NaPi-IIc proteins. Results are expressed as means ± SEM. n = 6.
5.4. Discussion

Despite our understanding of the mechanisms involved in phosphate homeostasis, little is known about the primary events involved in the intestinal detection of changes in dietary phosphate load and the subsequent downstream cascade of regulatory events. Past evidence demonstrates that the acute effect of dietary phosphate is independent of known regulators such as vitamin D$_3$, PTH and phosphatoninins (Berndt & Kumar, 2007; Berndt et al., 2005; Brautbar et al., 1979; Capuano et al., 2005; Segawa et al., 2004; Trohler et al., 1976). Furthermore, control of phosphate absorption by the major regulators generally occurs over a period of hours or days, rather than minutes (Bacic et al., 2006; Murer et al., 2000; Sommer et al., 2007). Berndt et al. (2007) recently demonstrated that instillation of phosphate into the duodenum caused an acute increase in the fractional excretion of phosphate within 20 minutes. Replication of their experiments in the present study proved to be difficult, since their original publication stated that the instillate contained 1.3 mM phosphate, and a subsequent erratum amended the phosphate concentration to 1.3 M (Berndt et al., 2007). The reason behind the use of a supra-physiological concentration of phosphate (which is also hypertonic) was not justified. An ‘intestinal phosphatonin’ was proposed to be responsible for the rapid reduction in renal phosphate reabsorption, since levels of PTH, the main hormone involved in phosphate balance, and FGF-23, were unaltered. Nishida et al. (2006) also observed in man an increased fractional excretion of phosphate that was independent of changes in FGF-23 levels within an hour of ingestion of a high phosphate diet (Nishida et al., 2006). The present study examined the local effect on intestinal phosphate absorption induced by duodenal instillation of physiological (15 mM) and non-physiological concentrations (1.3 M) of phosphate, using the everted sleeve technique. Findings in the present study demonstrated that the instillation of 15 mM phosphate into the duodenum did not affect either phosphate uptake by the duodenum and jejunum or NaPi-IIa and NaPi-IIc protein expression at the renal BBM. The only
significant effect observed was an increase in phosphate uptake in the duodenum, but not jejunum, following instillation of 1.3 M phosphate. Duodenal instillation of 1.3 M phosphate did not cause corresponding changes in expression of NaPi-IIa and NaPi-IIc proteins at the renal BBM.

Several reports suggest that phosphate regulation involves a bone-kidney axis, since phosphatoninns such as FGF-23 and MEPE are bone-derived and act on the kidney to regulate renal phosphate reabsorption (Quarles, 2003; Rowe et al., 2004; Shirley et al., 2010). This, in conjunction with a gut-renal cross talk proposed by Berndt et al (2007), may help maintain plasma phosphate concentrations within a narrow range. The phosphate-sensing effect, in gut-renal cross talk, is specific to the proximal small intestine, since the same response was not elicited by the stomach (Berndt et al., 2007). Certain regions of the gastrointestinal tract are able to sense different nutrients (Buchan, 1999; Flemstrom & Sjoblom, 2005; Furness et al., 1999); therefore, the idea that phosphate is also sensed by the small intestine has been proposed by several workers. However, the potential phosphate-sensing receptor and the mechanisms involved are so far uncharacterised.

An increased urinary phosphate excretion might be the result of a parallel change in plasma phosphate concentration (Fiske, 1921). However, the renal response to raised plasma phosphate concentration depends on maximum tubular reabsorption ($T_m$) of phosphate as a result of NaPi-IIa transporters at the renal brush-border membrane. The observations of Berndt et al (2007) could be explained by a reduction in $T_m$ leading to excess phosphate being excreted in the urine. PTH has been shown to lower $T_m$. The proposed phosphaturic factor might therefore have reduced $T_m$ under the experimental conditions used by Berndt et al (2007).

MEPE is a possible candidate for an ‘intestinal phosphatonin’, since it is expressed in the proximal small intestine and induces phosphaturia (Shirley et al., 2010; Marks et al., 2008 & 2010). NaPi-IIb, the primary
intestinal Na\textsuperscript+-dependent phosphate transporter that has been characterised, is another candidate for a phosphate sensor, since an acute intestinal phosphate load increases serum phosphate levels in wild type, but not NaPi-IIb-null, mice (Sabbagh et al., 2009).

Several reports have claimed that phosphaturic factors may be released from other organs, such as the liver. Phosphaturia and hypophosphatemia are frequently observed following liver injury and hepatectomy, an effect independent of changes in the major phosphate regulators FGF-23, FGF-7, sFRP-4, and PTH (George & Shiu, 1992; Pomposelli et al., 2001; Smyrniotis et al., 2003; Salem & Tray, 2005; Dawson et al., 1987; Nafidi et al., 2007). However, the phosphatonin MEPE, and ASARM (Acidic, Serine- and Aspartic acid-Rich Motif) peptides might be involved in this response, since the liver contains an abundant source of the protease cathepsin B that can convert MEPE into ASARM (Nafidi et al., 2009). This may be relevant to the communication axis between the small intestine and kidney in phosphate handling.

The observation of Berndt et al (2007) raises the question of whether the proposed duodenal factor causes a local effect in the small intestine. The present study used the everted sleeve method to investigate phosphate uptake in the duodenum and jejunum, the two main regions involved in phosphate transport, following instillation of phosphate into the duodenum for 30 minutes. A concentration of 15 mM phosphate was initially used, since this represents the ‘free’ (unbound) phosphate concentration in the upper gastrointestinal tract (see Chapter 3, Table 3.1). Data shows that duodenal and jejunal phosphate uptake was unaltered by duodenal exposure to 15 mM phosphate. This is not surprising, considering that dietary adaptation to phosphate levels in the small intestine involving Na\textsuperscript+-dependent phosphate cotransporters is a slow process compared to the kidney (Radanovic et al., 2005).

Additionally, solution containing 1.3 M phosphate was also instilled into the duodenum to assess whether this supra-physiological level of
phosphate would have a local effect on phosphate transport. Although the jejunum transported similar levels of phosphate when exposed to 1.3 M phosphate or equimolar concentration of saline, the duodenum interestingly exhibited an increase of 1.6 fold in phosphate transport with 1.3 M phosphate instillation. Acute regulation of phosphate uptake in duodenal, but not jejunal, BBM vesicles was reported previously following an acute feeding of a high phosphate diet (Giral et al., 2009). The study of Giral et al (2009) demonstrated that the increased duodenal Na\(^+\)-dependent phosphate uptake was associated with decreased NaPi-IIb protein expression in the duodenum. Renal expression of NaPi-IIa protein was also reduced. Although the present study noted a similar acute increase in duodenal but not jejunal phosphate uptake, studies of NaPi-IIb protein expression were not possible, since there are no reliable commercial antibodies for NaPi-IIb protein detection in rats (see Chapter 2, section 2.4), and access to the antibody used by Giral et al (2009) was not possible.

The study of Berndt et al (2007) showed that renal regulation imposed by duodenal instillation of a solution containing a high level of phosphate occurs independently of changes in PTH, FGF-23, sFRP-4 and plasma phosphate levels. The phosphaturic factors PTH, FGF-23 and sFRP-4 decrease renal phosphate reabsorption by causing the retrieval of renal NaPi-IIa transporters from the BBM (see Chapter 1 for further details). The phosphaturic factor released from the duodenum is, therefore, also likely to reduce renal phosphate reabsorption via the same cellular mechanism. Berndt et al (2007), however, did not quantify changes in renal NaPi-II proteins, despite the availability of antibodies for NaPi-IIa and NaPi-IIc. Thus, the present study examined changes in NaPi-IIa and NaPi-IIc proteins at the renal BBM following duodenal instillation of 15 mM or 1.3 M phosphate. The results showed no change in NaPi-IIa and NaPi-IIc expression following instillation of either concentration of phosphate.
The fact that the present study could not detect a significant decrease in NaPi-IIa protein expression may be due to the experimental protocol. A duodenal instillation period of 30 minutes was chosen, whereas Giral et al (2009) observed a decrease in NaPi-IIa protein 4 hours after the animals were switched from a low phosphate diet (of 7 days duration). Thus, switching from one extreme diet to another is more likely to induce adaptation compared with conditions used in the present study. Therefore, the findings of increased renal phosphate excretion reported by Berndt et al (2007) are unlikely to be due to reduced levels of NaPi-IIa or NaPi-IIc proteins at the BBM.

Interestingly, inhibition of renal phosphate reabsorption by PTH occurs via two distinct pathways one involving the retrieval of NaPi-IIa from the BBM, and the other involving no change in the membrane abundance of NaPi-IIa protein (see section 1.9.2.2 for further details). The latter pathway involves the ERK signal transduction pathway (Lederer et al., 2000). For example, potassium deficiency decreases renal Na\(^{+}\)-dependent phosphate transport but increases abundance of NaPi-IIa at the BBM (Inoue et al., 2004). This post-translational reduction of Na\(^{+}\)-dependent phosphate transport in potassium deficiency is facilitated by increased partitioning of NaPi-IIa into cholesterol-, sphingomyelin-, and glycosphingolipid-enriched BBM microdomains. Other studies have further supported the effect of alterations in membrane lipid composition on NaPi-IIa-mediated transport at the BBM (Breusegem et al., 2005; Levi et al., 1995; Zajicek et al., 2001). The study of Bielesz et al (2006) demonstrated that diurnal increases in renal phosphate excretion were linked only moderately to a decrease in renal Na\(^{+}\)-dependent phosphate transport, and that the abundance of NaPi-IIa was not altered. They proposed that diurnal changes in urinary phosphate excretion are dependent on changes in serum phosphate concentration and tubular reabsorptive threshold, and not NaPi-IIa expression at the BBM. Therefore it remains possible that duodenal instillation of 15 mM and/or 1.3 M phosphate could have inhibited renal BBM phosphate transport.
without affecting the BBM abundance of NaPi-IIa within the 30 minute
time period used.

Although regulation of proximal tubular phosphate reabsorption is largely
attributed to changes in the abundance of NaPi-IIa at the BBM, other
transporters are also likely to be involved in phosphate uptake. Evidence
is emerging that type III (PiT) transporters may be involved in phosphate
transport. PiT1 and PiT2 are Na\(^+\)-dependent phosphate transporters
expressed at the intestinal and renal BBM, respectively (Giral et al., 2009;
Villa-Bellosta et al., 2009b). Data presented in Chapter 4 established the
genomic upregulation of renal PiT2 by phosphate diet, a conclusion also
made by Giral et al (2009) who demonstrated upregulation of renal PiT2
protein following dietary restriction of phosphate. The present study also
revealed that significant levels of PiT2 mRNA is found in the rat small
intestine (see Chapter 4) and an in vitro study showed intestinal PiT2
regulation by 1,25-dihydroxyvitamin D\(_3\) (Katai et al., 1999). It was not
possible to detect PiT1 and PiT2 proteins in the present study due to lack
of suitable antibodies; access to the antibodies used by Giral et al (2009)
was not possible.

A long-standing hypothesis concerning phosphate homeostasis is that
phosphate-sensing may be an integral mechanism in certain organs to
communicate changes in local phosphate levels to other organs. Some
reports have hypothesised that PiT2 transporters may act as a
‘phosphate sensor’ (Rodrigues & Heard, 1999; Salaun et al., 2004),
although there is a lack of experimental evidence for this suggestion.
PiT1, the Na\(^+\)-dependent phosphate transporter present in the
parathyroid glands, was proposed to act as a ‘phosphate sensor’ in this
tissue, since changes in Na\(^+\)-dependent phosphate transport in
parathyroid cells correlated with extracellular phosphate concentrations
(Miyamoto et al., 1999; Tatsumi et al., 1998). Phosphate-sensing was
also described by Martin et al (2005) who demonstrated that duodenal
exposure to phosphate or PFA increased PTH levels acutely within
minutes, an effect that was independent of changes in serum phosphate
concentration. In agreement, Bevilacqua et al (2010) reported acute increases in PTH levels in response to high phosphate concentration in the jejunal lumen, and, therefore, proposed that a signal from the gastrointestinal tract to the parathyroids was responsible for this effect.
5.5. Conclusion

The study by Berndt et al (2007) postulated the release of a humoral factor(s) by the duodenum following sensing of increased phosphate levels in the intestinal lumen. This results in a rapid increase in renal phosphate excretion, whilst maintaining normal plasma phosphate concentration. The present study showed that instillation of physiological levels (15 mM) of phosphate into the duodenal lumen did not affect phosphate transport in either the duodenum or jejunum measured \textit{in vitro}. Duodenal instillation of a supra-physiological (1.3 M) concentration of phosphate was also without effect on jejunal phosphate transport, but increased duodenal phosphate uptake. However, instillation of these concentrations of phosphate into the duodenum did not alter renal BBM expression of NaPi-IIa and NaPi-IIc. The proposed gut-renal axis and the 'intestinal phosphatonin' requires further investigation, since elucidation of the phosphate-sensing mechanism may lead to development of therapy that could be used to control elevated plasma phosphate concentrations in renal failure.
6.0. General discussion and suggestions for future experiments
6.1. Short background

The small intestine has not previously been considered to be a major organ for maintenance of body phosphate, since postprandial increases in serum phosphate are rapidly handled by the kidney; thus past studies have focused on characterising the mechanism and control of phosphate transport in the kidney. Recent work, however, has shown that an enteric-renal axis can exert acute regulation of phosphate balance by releasing a phosphaturic factor into the circulation (Berndt et al., 2007; Martin et al., 2005). This suggests that the small intestine plays a more significant role in phosphate homeostasis than previously acknowledged. Phosphate absorption across the intestinal epithelium is mediated by transcellular and paracellular pathways. The former pathway is mediated by high affinity Na\(^+\)-dependent phosphate transporters NaPi-IIb and, potentially, PiT1 and PiT2, all three being expressed at the intestinal brush-border membrane (BBM) (Giral et al., 2009; Marks et al., 2010; Sabbagh et al., 2009). In comparison, little is known about the paracellular pathway for phosphate transport.

Studies involving animal knockout models, together with the preparation of purified BBM vesicles, revealed NaPi-IIb to be the main Na\(^+\)-dependent phosphate transporter for intestinal phosphate absorption (Giral et al., 2009; Loghman-Adham et al., 1987 & 1993; Sabbagh et al., 2009; Xu et al., 2002a). Furthermore, the majority of past studies in rats concluded that phosphate is absorbed predominantly across the proximal small intestine (Giral et al., 2009; Marks et al., 2006). In vivo studies however have suggested that the paracellular pathway is the predominant route for phosphate absorption (Aloia & Yeh, 1985; McHardy, 1956; Williams & DeLuca, 2007). The discrepancies between the contributions of Na\(^+\)-dependent (transcellular) and Na\(^+\)-independent pathways for total phosphate absorption are partly due to the wide variety of experimental techniques used for assessing uptake, as well as widely differing phosphate concentrations present in uptake buffers. The latter reflects
ignorance of normal postprandial phosphate levels present in the intestinal lumen.

6.2. Aims of experiments

An important objective of the present study was to validate suitable in vivo and in vitro techniques for the accurate robust measurement of phosphate uptake. The in vitro everted sleeve and in vivo loop technique, which measures respectively the appearance of luminal $^{32}$P in mucosa of specific regions of intestine and the appearance of $^{32}$P in the plasma, were used; uptake was measured in control, low phosphate diet and in rats of different ages. Both techniques have been previously adapted for use in solute uptake studies in several species (Balakrishnan et al., 2008, Marks et al., 2006 & 2007; Shultz et al., 1982; Starck et al., 2000; Stein & Williams, 2003). Far fewer studies have performed phosphate uptake studies in man due to ethical reasons. The triple lumen intestinal perfusion technique has been used to measure in vivo phosphate absorption in small intestine (Walton & Gray, 1979; Davis et al., 1983). With the use of the validated in vivo and in vitro methods in rats, the contribution of the Na$^+$-dependent and -independent components of total phosphate absorption at normal luminal phosphate concentrations were determined. In order to carry out these experiments under physiological conditions, a further aim of the present work was to determine the phosphate levels present in the intestinal lumen of rats following ingestion of a normal diet.

The ileum and colon are not thought to play a major role in phosphate absorption in rats (Cramer, 1961; Marks et al., 2006). However, a few studies found the ileum and colon to be capable of absorbing phosphate (Capuano et al., 2009; Cramer, 1961; Hu et al., 1997; Kayne et al., 1993). Thus, absorption of phosphate in the ileum and proximal and distal colon was investigated in vivo and in vitro at normal luminal phosphate concentrations.
The competitive inhibitor phosphonoformic acid (PFA) was used in order to distinguish between NaPi-IIb-mediated and non-NaPi-IIb-mediated transport to characterise a new pathway that may be involved in phosphate absorption, as proposed by several authors (Aloia & Yeh, 1985; Berner et al., 1976; Douard et al., 2010; Lee et al., 1986b; Williams & DeLuca, 2007).

In contrast to NaPi-II transporters, there is very little information on the newly emerging PiT family of Na\(^{+}\)-dependent phosphate transporters, therefore their genomic expression was investigated in those conditions known to affect phosphate absorption, i.e. low phosphate diet, age and hyperphosphataemia.

The effect of the proposed ‘intestinal phosphaturic’ factor on intestinal phosphate uptake, following duodenal instillation of millimolar and molar phosphate concentrations, was investigated in addition to the effect of this procedure on changes in expression of NaPi-II proteins at the renal BBM.

6.3. Measure of intestinal phosphate transport using in vitro and in vivo techniques

Using the everted sleeve technique, the study demonstrated that the feeding of a low phosphate diet for 7 days increased Na\(^{+}\)-dependent and Na\(^{+}\)-independent transport in the duodenum and jejunum; the increase seen in the Na\(^{+}\)-independent component was much smaller compared with that observed for the Na\(^{+}\)-dependent component. The enhanced Na\(^{+}\)-dependent transport was attributed to an increased expression of NaPi-IIb protein, as reported previously (Giral et al., 2009; Hattenhauer et al., 1999). However, the identity of the transporter responsible for the increased rate of Na\(^{+}\)-independent transport in the duodenum and jejunum following a low phosphate diet is not known and requires further investigation.
Studies carried out in vitro showed that the rate of Na\(^{+}\)-dependent transport was greater in the duodenum and jejunum of young animals compared with adults. Na\(^{+}\)-dependent phosphate transport did not decline further once animals reached adulthood. Ageing also did not affect Na\(^{+}\)-dependent phosphate transport in the ileum or the Na\(^{+}\)-independent transport in all regions of small intestine. The present study successfully used the in situ loop technique to distinguish between Na\(^{+}\)-dependent and Na\(^{+}\)-independent transport in three regions of small intestine. Past studies have failed to observe Na\(^{+}\)-dependent transport in vivo (Douard et al., 2010; McHardy, 1956; Williams & DeLuca, 2007). Furthermore, no previous study has investigated the changes in intestinal phosphate absorption, Na\(^{+}\)-dependent and Na\(^{+}\)-independent, caused by a low phosphate diet and ageing in vivo, and this technique may be employed for this purpose in future studies.

Under all conditions used, a higher rate of phosphate transport was observed in the jejunum compared with duodenum, as established previously (Loghman-Adham et al., 1987). Additionally, Marks et al (2006 & 2008) reported that regulation of intestinal phosphate absorption in the rat is largely confined to jejunum. Thus, future studies should focus on the jejunum when investigating the control of intestinal phosphate absorption.

In addition to low phosphate diet, increases in the level of 1,25-dihydroxyvitamin D\(_3\) (which may be caused by low phosphate diet per se) is another important regulator of intestinal phosphate transport (Hattenhauer et al., 1999; Katai et al., 1999; Lee et al., 1986b; Marks et al., 2006). In man, 1,25-dihydroxyvitamin D\(_3\) therapy does not increase jejunal phosphate absorption in normal subjects (Gray TK et al., 1977; Davis et al., 1983). Similarly, Williams & Deluca (2007) reported that in rats when molar concentrations of phosphate are used in in vivo uptake buffers, the stimulatory effect of 1,25-dihydroxyvitamin D\(_3\) on intestinal phosphate absorption is eliminated and transport at these high phosphate concentrations is completely Na\(^{+}\)-independent. Interestingly, the majority
of the early studies used 0.1 mM phosphate in uptake buffers whereas
more recent studies have used low millimolar phosphate concentrations
to measure uptake. The present study, using 0.1 mM phosphate in
uptake buffer, showed upregulation of Na\(^+\)-dependent transport in the
proximal small intestine of young animals and adults fed a low phosphate
diet. However, whether this would be the case at higher phosphate
concentrations need to be investigated. Furthermore, 0.1 mM phosphate
used for \textit{in vitro} uptake studies could have acted as a rate-limiting step
and masked a potentially bigger effect of low phosphate and age on the
Na\(^+\)-independent component than that observed in the present study.

6.4. Regional variations in Na\(^+\)-dependent and Na\(^+\)-independent
phosphate transport along the rat small intestine and colon

Analysis of intestinal luminal contents revealed, surprisingly, that
phosphate concentrations are in the low millimolar range following
ingestion of a normal phosphate diet. Uptake data presented in Chapter 3
showed that at these concentrations, transport in the jejunum was
predominantly Na\(^+\)-independent and this component was not inhibited by
the NaPi-IIb inhibitor PFA. The identity of this pathway, whether a Na\(^+\)-
independent transporter or passive diffusion, is not yet known. Phosphate
transport in the duodenum was Na\(^+\)-independent regardless of the
phosphate concentrations used for \textit{in vivo} and \textit{in vitro} uptake studies.
Thus, although NaPi-IIb is expressed in the duodenum, it is unlikely to
play a significant role in phosphate absorption in this region under normal
dietary conditions. This further highlights the differential regional
regulation of intestinal phosphate absorption. Although PFA is an ideal
inhibitor of NaPi-IIb-mediated transport, it is also a weak inhibitor of PiT-
mediated transport (Collins \textit{et al}., 2004; Ravera \textit{et al}., 2007; Virkkki \textit{et al}.,
2007). A suitable inhibitor is needed to determine the relative
contributions of NaPi-II-mediated and PiT-mediated transport in the
different regions of small intestine, and in the kidney.
Jejunal phosphate transport showed considerably more Na\textsuperscript{+}-dependency \textit{in vitro} than \textit{in vivo}. Rapid shuttling of a facilitative phosphate transporter to the BBM may explain the observed Na\textsuperscript{+}-independency \textit{in vivo}. A similar situation has been observed for glucose transport. The GLUT2 glucose transporter can be detected at the intestinal BBM during luminal exposure to high glucose concentrations (Kellett, 2001a). The existence of GLUT2 transporter at the BBM was overlooked due to rapid membrane turnover of the transporter in response to glucose level in the luminal fluid (Helliwell \textit{et al.}, 2000a & 2000b). Similarly, phosphate-sensing and uptake by NaPi-IIb may cause BBM expression of an alternative phosphate transport system following increased luminal phosphate levels. This theory could be explored by using techniques that were used for identifying shuttling of GLUT2 (Helliwell \textit{et al.}, 2000a & 2000b; Kellett, 2001). However, the factor responsible for the linear increase in \textit{in vitro} jejunal, but not duodenal, Na\textsuperscript{+}-dependent phosphate transport at higher luminal phosphate concentrations is not known, since NaPi-IIb has a K\textsubscript{t} of \(\sim 0.1\) mM (Hilfiker \textit{et al.}, 1998; Loghman-Adham \textit{et al.}, 1987). It is possible that there are additional molecular types of phosphate transporters at the intestinal BBM that mediate phosphate uptake.

Debiec & Lorenc (1985) showed an increase in, Na\textsuperscript{+}-independent phosphate uptake by duodenal and jejunal BBM vesicles when lactose was added to the pre-incubation medium. The mechanism behind this lactose-mediated increase in uptake is not known. Dietary lactose is also reported to stimulate calcium absorption across the small intestine (Bushnell & DeLuca, 1981; Lengemann & Comar, 1961; Sato \textit{et al.}, 1983). It is important to establish the route of passive permeability of phosphate. The human intestinal Caco-2 cell line is a potentially useful model to study the passive permeability of solutes and the contribution of paracellular transport, since the model has been successfully used to establish intestinal passive permeability of iron and copper (Ferruzza \textit{et al.}, 1999 & 2002).
Claudins are tight junction integral membrane proteins that act as the primary determinants of permeability of the tight junction (Kiuchi-Saishin et al., 2002; Teshima & Meddings, 2008). Claudins are, therefore, a potential target for modifying the absorption of solutes or drugs that are passively transported through the tight junctions (Kondoh et al., 2005). Claudins could be modulated at normal phosphate concentrations to determine their effects on phosphate absorption. Claudin-4 is preferentially expressed at the apices of intestinal villi and is associated with loosening of intercellular junctions, allowing access to the paracellular space; therefore, a substrate such as the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE), which directly interacts with claudin-4 in the jejunum (Kondoh et al., 2005; Tamagawa et al., 2003), could be used to determine if altered intestinal permeability, without damage to the intestinal mucosa, influences Na⁺-independent phosphate absorption in vivo.

Marks et al (2008) have reported that the phosphatonin matrix extracellular phosphoglycoprotein (MEPE), a potential treatment for hyperphosphataemia, dose-dependently inhibited jejunal phosphate absorption measured in vivo. The MEPE data were obtained using 0.1 mM phosphate in the uptake buffer. It would be interesting to see if the inhibitory effect of MEPE occurs at physiological (low millimolar) phosphate concentrations and whether MEPE affects Na⁺-independent phosphate uptake. Furthermore, detailed studies are needed to evaluate the effect of established regulators of phosphate uptake, i.e. glucocorticoids, growth hormone, thyroid hormone and phosphatonins such as FGF-23, on passive diffusion of phosphate across the small intestine.

At odds with previous reports, findings described in Chapter 3 demonstrated significant Na⁺-dependent phosphate transport in the ileum and distal colon in vivo and in vitro, at phosphate levels normally found in the intestinal lumen. The transport pathway responsible for the Na⁺-dependent, PFA-sensitive transport in the ileum is different from that
responsible for Na\textsuperscript{+}-independent PFA-insensitive transport in the distal colon. NaPi-IIb and PiT transporters are unlikely to be responsible for ileal phosphate transport, since levels of these proteins are not detectable in the ileum (Giral \textit{et al.}, 2009), and phosphate transport was minimal in the present study when measured using 0.1 mM phosphate in uptake buffer. In this context, a so far unidentified Na\textsuperscript{+}-dependent transporter, with a low affinity (high K\textsubscript{t}), is likely to be present at the ileal BBM to facilitate phosphate transport at millimolar phosphate concentrations. Further studies are needed for its characterisation.

Although the present study used mannitol to estimate passive intestinal permeability, it cannot be used as a probe for intestinal permeability specifically for phosphate due to charge and molecular size differences between mannitol (neutral molecule) and phosphate (an anion). Therefore, results presented in Chapter 3 cannot be used to establish whether colonic phosphate transport is mediated through a passive pathway, as proposed by Hu \textit{et al} (1997) or via a Na\textsuperscript{+}-independent phosphate transporter. Capuano \textit{et al} (2009) reported genomic expression of NaPi-IIb and PiT transporters in the distal colon, and their regulation by a chronic low phosphate diet, but it is not known whether translation to the transporter protein occurs. However, this is unlikely, based on the PFA-insensitive Na\textsuperscript{+}-independent colonic phosphate uptake data presented in Chapter 3. Further studies are needed for clarification. Interestingly, the present study detected significant transepithelial absorption of mannitol in the distal colon. The route is unknown at the present time and autoradiography (using tritiated mannitol) could be used to assess whether mannitol moves paracellularly or transcellularly across this epithelium. The involvement of claudins and the overall role of the paracellular pathway in mediating Na\textsuperscript{+}-independent phosphate uptake need to be characterised in detail. Changes in the electrical potential difference induced by the replacement of sodium with choline needs to be clearly studied in order to investigate whether a change in the potential difference was responsible for the apparent Na\textsuperscript{+}-independency seen in the experiments presented in this thesis, and if so to what extent.
The finding that the distal colon is capable of absorbing phosphate has implications for the clinical use of phosphate-containing enemas, which contain molar concentrations of phosphate and carries the risk of causing symptomatic hyperphosphataemia (Carl & Mitchell, 2007; Hu et al., 1997; Hunter et al., 1993;). Furthermore, the effectiveness of current treatments of hyperphosphataemia with oral phosphate binders is questionable, since the high levels of anions (chiefly short-chain fatty acids) in the colonic lumen might displace bound phosphate from these agents, thus making phosphate available for absorption by the distal colon. This could negate any upstream benefit of phosphate binders on reducing phosphate absorption.

6.5. Genomic regulation of Na\(^{+}\)-dependent phosphate transporters by low phosphate diet, age, chronic renal failure, and MEPE

PiT transporters were previously thought to serve housekeeping roles by absorbing phosphate from interstitial fluid for normal cellular functions (Tatsumi et al., 1998; Collins et al., 2004). However, recent evidence suggests that they have specific roles in intestinal and renal handling of phosphate (Giral et al., 2009; Villa-Bellosta et al., 2009b; Villa-Bellosta & Sorribas, 2010b). Genomic expression of NaPi-IIb in the small intestine is region-specific and differs between rat and mouse; in contrast, the data presented in Chapter 4 shows that gene expression of PiT1 and PiT2 are similar in the duodenum, jejunum and ileum of rat and mouse small intestine. However, this information needs to be supplemented with the protein expression of PiT1 and PiT2.

Data presented in Chapter 4 also show that intestinal, but not renal, expression of PiT2 in rats is age-dependent. PiT2 mRNA was significantly higher in the jejunum, but not duodenum, of young animals compared with adults. PiT2 was also upregulated in the kidneys of adult rats fed a chronic low phosphate diet. In contrast, PiT1 mRNA expression
in the small intestine and kidney was not regulated by age or low phosphate diet. This regulation of PiT2 highlights the differential regulation of phosphate transporters expressed at the BBM of small intestine and kidney. The study of Villa-Bellosta & Sorribas (2010b) reported that mRNA levels of PiT1 and PiT2 are low in rats. However, they have also provided immunohistochemical evidence of significant expression of PiT2 protein at the renal BBM and its upregulation by low phosphate diet (Villa-Bellosta et al., 2009b; Villa-Bellosta & Sorribas, 2010b). Immunohistochemical evidence of PiT1 protein expression and its regulation by low phosphate diet and age at the renal and intestinal BBM is needed. Whether PiT2 protein is expressed at the intestinal BBM is also not known.

The experiments described in Chapter 4 also provide evidence that intestinal NaPi-IIb, and intestinal and renal PiT1 and PiT2 mRNA are downregulated by a more severe model of chronic renal failure. This is surprising, since other reports claim that intestinal phosphate uptake and genomic expression of NaPi-IIb are unaffected by chronic renal failure (Douard et al., 2010; Marks et al., 2007). A factor to consider for this difference is the severity of chronic renal failure on phosphate homeostasis. Comparison of the present study to that reported by Douard et al. (2010) and Marks et al. (2007) suggests that intestinal Na⁺-dependent phosphate transporters are differentially regulated with progression of chronic renal failure. This changes the current view about intestinal phosphate uptake during chronic renal failure and has implications in the targeting of the process of intestinal phosphate uptake for therapeutic interventions during chronic renal failure.

In patients with chronic renal failure on haemodialysis, jejunal phosphate absorption in vivo was reduced compared with normal subjects and phosphate was absorbed by both passive and active pathways (Davis et al., 1983). The study of Davis et al. (1983) also demonstrated that phosphate absorption in chronic renal failure patients saturated at a luminal phosphate concentration above 2 mM. In chronic renal failure
patients the level of phosphate in the jejunal lumen was found to vary between 1 and 12 mM. Whether BBM Na\(^{+}\)-dependent and Na\(^{+}\)-independent transport is altered in chronic renal failure rats is not clearly established, especially considering that data presented in Chapter 4 show that Na\(^{+}\)-independent transport is the predominant pathway under normal (millimolar) luminal phosphate levels.

The present study found reduced renal expression of NaPi-IIa mRNA in chronic renal failure rats, as established previously (Brooks et al., 1997; Marks et al., 2007). Interestingly, mRNA levels of renal PiT1 and PiT2 were also downregulated. The mechanisms behind the reduction in the genomic levels of these phosphate transporters in chronic renal failure are not yet known. It remains to be established whether renal PiT1 and PiT2 transporters are regulated by major regulators of phosphate balance such as parathyroid hormone (PTH). PTH inhibits Na\(^{+}\)-dependent phosphate transport in the kidney by inducing the internalisation of NaPi-IIa. Picard et al (2010) recently showed that PTH inhibits PFA-resistant Na\(^{+}\)-dependent phosphate transport, suggesting an action of the hormone on PiT transporters. Thus, augmented PTH levels, as seen in chronic renal failure animals, may be responsible for reducing renal expression of NaPi-IIa, PiT1 and PiT2 mRNA in chronic renal failure rats. Due to a lack of suitable antibodies, it was not possible to analyse protein levels of PiT1 and PiT2 in renal BBM of chronic renal failure rats. Furthermore, the cellular signals leading to the internalisation of NaPi-IIb, NaPi-IIc, PiT1 and PiT2 are unknown.

The recently described genomic intestine expression of the phosphatonin MEPE (Marks et al., 2010) is relevant to the data presented in Chapter 4. Thus, chronic renal failure animals chronically treated with MEPE were analysed for expression of Na\(^{+}\)-dependent phosphate transporters. The fact that MEPE is expressed in both kidney and small intestine, and it inhibits phosphate absorption in both tissues (Marks et al., 2008 & 2010; Rowe et al., 2000 & Rowe et al., 2004) makes it a promising target for controlling hyperphosphataemia, especially, since data presented in
Chapter 4 show that intestinal NaPi-IIb and PiT2 mRNA were downregulated by MEPE in chronic renal failure rats. The reason why MEPE influences intestinal, but not renal, expression of Na\(^+\)-dependent phosphate transporters is not known. The effect of chronic renal failure on expression of PiT transporters in the small intestine and kidney before and after treatment with MEPE requires further study, particularly whether changes at the genomic levels are translated to the protein level. This could be carried out using BBM if suitable antibodies for NaPi-IIb and PiT1 and PiT2 become available.

There is no reliable assay for measurement of MEPE levels in blood. Knowledge of this is important to understand the link between normal and abnormal regulation of phosphate handling in small intestine and kidney. Although the cellular process by which MEPE exerts its inhibitory effect in the jejunum is unknown, the action of MEPE on phosphate transport occurs within 3 hours in rats and is independent of the main regulators of phosphate balance, PTH, 1,25-dihydroxyvitamin D\(_3\) and FGF-23. Thus, the inhibitory effect of MEPE on intestinal phosphate transport may be direct or indirect and this needs to be investigated in future studies. Renal PiT2, along with renal NaPi-II transporters, is reported to be involved in the phosphaturic effect of FGF-23, another phosphatonin. Further experiments are also needed to characterise the regulation of intestinal and renal expression of NaPi-II and PiT transporters by other phosphatonins such as FGF-23, FGF-7 and sFRP-4.

Intestinal phosphate absorption is thought to be primarily mediated by the Na\(^+\)-dependent transporter NaPi-IIb, which is pH sensitive and exhibits higher transport activities at alkaline pH (Hilfiker et al., 1998). In intestinal BBM vesicles, phosphate uptake was reduced at pH 7.4, but not at pH 6 under Na\(^+\)-free conditions (Berner et al., 1976). This raises the possibility that PiT transporters might be involved, since they function more effectively at acidic pH. If this is so, then it would be important to know whether PiT transporters strictly require Na\(^+\) for phosphate uptake. Although the present study and past reports show regulation of PiT
transporters at the genomic and protein level, there is no evidence that they are involved in phosphate absorption across the small intestine or kidney under normal conditions. However, evidence is emerging that PiT transporters are important in cellular processes in other tissues, such as vascular calcification and liver growth (Beck et al., 2010; Villa-Bellosta et al., 2007 & 2009a). The fact that PiT transporters were found to be affected by chronic renal failure in the present study, and by hypophosphataemia (in mice) (Tenenhouse et al., 1998), indicates that they may play a role in the pathophysiology of phosphate absorption.

6.6. The effect of duodenal phosphate instillation on intestinal phosphate uptake and expression of renal phosphate transporters

The importance of the small intestine in phosphate balance has led to suggestions of an ‘intestinal phosphaturic’ factor (Berndt et al., 2007; Martin et al., 2005). However, the identity of this enteric mucosal factor and the mechanisms involved in mediating the renal effect are unclear. Data presented in Chapter 5 show that instillation of molar (1.3 M), but not millimolar (15 mM), phosphate concentrations into the duodenum for 30 minutes increased duodenal phosphate transport. A similar acute effect was observed in duodenal BBM vesicles by Giral et al (2010), which was attributed to increased expression of NaPi-IIb protein at the duodenal BBM. The reason why the jejunum, which also expresses NaPi-IIb at similar levels to the duodenum (Giral et al., 2009; Marks et al., 2006), does not show this adaptation is not known.

Present data provide no evidence that changes in expression of NaPi-IIa and NaPi-IIc at the renal BBM following administration of 1.3 M phosphate into the duodenum were responsible for the increased urinary excretion of phosphate, as observed by Berndt et al (2007). However, Berndt et al (2007) used a supra-physiological concentration of 1.3 M phosphate for their experiments. Therefore, the significance of their finding is unlikely to apply at normal luminal phosphate concentrations in
man or experimental animals. Importantly, the present study establishes that duodenal exposure to such a high phosphate concentration does locally affect phosphate transport in the duodenum, but not in adjacent, unexposed regions of intestine.

Although duodenal phosphate instillation was without effect on expression of renal NaP-II transporters, PiT transporters may have been regulated during this procedure. However, expression of PiT1 and PiT2 proteins were not analysed owing to a lack of suitable antibodies. It is possible that changes in expression of NaPi-II proteins at the renal BBM might occur following a longer duodenal exposure to phosphate. Thus, it would be useful to repeat the present study, but using a longer duodenal phosphate instillation period. Also, the study of Berndt et al (2007), and the present study did not address whether exposure of the jejunum to phosphate is capable of inducing changes in urinary phosphate excretion.

The transport pathway responsible for the efflux of phosphate across the basolateral membrane in both small intestine and kidney epithelia has not been characterised. This is an important issue to resolve, since this pathway might also be targeted to decrease plasma phosphate levels in hyperphosphataemia.

A recent clinical report by Lynch et al (2011) caused controversy over the current understanding and application of therapy for hyperphosphataemia in chronic renal failure patients by demonstrating that restriction of phosphate in the diet was not associated with better survival rate among haemodialysis patients. They showed that increasing levels of phosphate restriction may cause greater mortality. The significance of this report and the future effect on patient treatment is not known. However, it is certain that this report is likely to play a role in the direction of future research on the control of intestinal and renal phosphate handling.
Figure 6.1 summarises our current understanding of the regulation of intestinal and renal phosphate absorption by the major factors considered in the present study: low phosphate diet, age and chronic renal failure.
6.7. Conclusion

Experiments described in this thesis have shown that millimolar phosphate concentrations are present in the intestinal lumen and phosphate absorption across all three regions of the rat small intestine and the distal colon occurs by mechanisms involving both Na$^{+}$-dependent and Na$^{+}$-independent pathways. In addition to NaPi-II, PiT transporters expressed in the small intestine and kidney are also regulated at the genomic level by chronic renal failure, low phosphate diet and age. Thus, PiT transporters may play an important role in the regulation of phosphate transport in these tissues. The proposed ‘phosphaturic factor’ that may be released from rat duodenum following exposure to high luminal phosphate concentrations does not affect jejunal phosphate transport nor the expression of NaPi-II proteins at the renal BBM.
Figure 6.1. Summary of changes in intestinal and renal phosphate transport in rats induced by low dietary phosphate, chronic renal failure (CRF) and in young animals. Findings from both previous studies and from the experiments conducted for this thesis are included. NaPi-II and PiT transporter expression mentioned refer at the genomic level only. ↑- increased, ↓-decreased and ↔ - no change.
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