Matrix extracellular phosphoglycoprotein (MEPE)

– a putative phosphatonin

A thesis submitted

by

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Abstract

The introduction to the thesis discusses the control of phosphate balance and considers recent developments in the field. The concept of novel circulating substances which influence, and perhaps regulate, plasma phosphate ('phosphatonins'), and which are important in tumour-induced hypophosphataemia, hereditary hypophosphataemias and, potentially, in normal phosphate physiology, is introduced. Finally, the isolation of the putative phosphatonin MEPE, and the current state of knowledge about this molecule, is described.

The initial practical studies deal with the development and validation of a novel micromethod for measurement of phosphate and, simultaneously, other anions in biological fluids, using capillary electrophoresis. The accuracy and reproducibility of the method are demonstrated, and the method is compared with an established micromethod for measurement of individual ions. Finally, capillary electrophoresis is applied to the measurement of phosphate and other anions in urine, plasma and tubular fluid samples.

In the next section, renal clearance experiments are performed in anaesthetized rats, in order to assess the effects of intravenously administered MEPE and to compare them with those of parathyroid hormone. MEPE is shown to be markedly phosphaturic. It does not alter glomerular filtration rate, blood pressure, plasma phosphate concentration or filtered phosphate load, but does cause large, dose-dependent increases in absolute and fractional phosphate excretion.

The final series of experiments uses micropuncture methods in anaesthetized rats, in which fluid is collected from individual proximal convoluted tubules. The phosphaturic effect of MEPE is confirmed, and is shown to result, at least in part, from a reduction in fractional phosphate reabsorption in the proximal convoluted tubule, without a change in filtered phosphate load.

The results obtained in these studies are discussed in relation to previous knowledge.

Disclaimer

This thesis has been prepared and written by me, supervised by Dr David Shirley and Prof Robert Unwin. The capillary electrophoresis experiments described in Chapter 2 were performed in collaboration with Dr Nuno Faria; sample collection was performed by me, work on the machine set-up was performed by him , and the actual analyses were done together. Some of these experiments are also described in his PhD thesis. The micropuncture experiments were performed with Dr Shirley, who performed some of the surgery and the tubular punctures. Micropipettes used for the micropuncture experiments were made by John Skinner. Otherwise, all experiments described in this thesis are entirely my own work.

Prior Publication

Many of the results presented in Chapter 2 were published in 2005.

Faria NJ, Dobbie H, Slater JM, Shirley DG, Stocking CJ, Unwin RJ. Simultaneous determination of anions in nanoliter volumes. *Kidney International* 2005;**67**(1):357-63

Those presented in Chapter 3 were published in 2008.

Dobbie H, Unwin RJ, Faria NJ, Shirley DG. Matrix extracellular phosphoglycoprotein causes phosphaturia in rats by inhibiting tubular phosphate reabsorption. *Nephrol Dial Transplant.* 2008;**23**:730-3.

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Chapter 1

Phosphate homeostasis

1 Introduction

Phosphate is the most abundant intracellular anion, and the human body contains about 600-900g of phosphorus. Of this, 80% is in the form of calcium salts in the bones, making the skeleton rigid. The remaining 20% is widely distributed in all the fluids and tissues of the body, with less than 0.1% of total body phosphate in the plasma.

Phosphate is vital to life. The major source of energy for cellular reactions is ATP; phosphate is a component of lipids, proteins, RNA and DNA; and protein phosphorylation and dephosphorylation is central to many control mechanisms. Therefore, control of phosphate balance is a key metabolic process, and phosphate regulatory pathways exist in organisms from yeast to man (Silver, 2003).

Whilst the key issue in the regulation of phosphate balance in mammals is usually how to get rid of excess phosphate, the situation in many lower animals is different. The phosphate concentration in many aquatic environments is extremely low - in the micromolar or even nanomolar range. In some systems, for example freshwater lakes in North America, phosphate is the limiting nutrient (Karl, 2000), and it has been suggested that low phosphate levels in Earth's early oceans acted as a brake on the evolution of multicellular organisms (Bjerrum & Canfield, 2002; Hayes, 2002). There is evidence that phosphorus availability provides the ultimate limit on the total amount of life in the oceans (Tyrrell, 1999). Thus, bacteria and yeasts have evolved extensive systems for the active uptake of phosphate from the surrounding environment (Wykoff & O'Shea, 2001). Many of these same mechanisms have been adapted to regulate phosphate balance in multicellular organisms (Werner & Kinne, 2001).

1.1 Phosphate balance in humans

The average British man consumed about 50mmol/day of phosphorus in the year 2000; the figure for women was 35mmol/day (Food Standards Agency, 2003). Slightly more recent figures from the USA are strikingly similar (US Department of Agriculture, 2004). Almost all of this is in the form of phosphate. Food items with high phosphate content include milk and dairy products, meat, fish and other proteins, and cereals. The modern

trend to eat more processed food may increase the average phosphate intake, as the phosphate content of these foods is especially high (Uribarri & Calvo, 2003). A crossover trial in graduate students found that they consumed twice as much phosphate when their diet was mainly processed food than when they consumed similar but unprocessed food (Raines Bell et al, 1977). Interestingly, the pre-meal serum phosphate levels of these students were the same, despite the two-fold difference in phosphate intake. Similarly, in another dietary manipulation study, a very low phosphate diet left early morning fasting values for serum phosphate similar to those of subjects on a normal phosphate diet, although there were differences in post-prandial levels (Portale et al, 1989). This is in contrast to the situation in experimental animals such as rats, where serum phosphate can be greatly reduced by restricting intake (for example, Slatopolski et al, 1996). This may simply reflect the fact that it is possible to control the diet of rats in a way which is unacceptable in human subjects.

The control of uptake of phosphate from the gut is currently not very well understood, but in health about 60-80% of ingested phosphate is absorbed from the lumen of the gastrointestinal tract (reviewed in Weisinger & Bellorin-Font, 1998). This uptake mainly takes place in the small intestine, and can occur by either the paracellular route (through tight junctions) or the transcellular route. The transcellular route, mediated by apical sodium-phosphate co-transporters, is thought to be the more important (Eto et al, 2006).

There is a circadian pattern to human serum phosphate concentrations, with plasma phosphate rising from a low in the mid-morning to a peak in the middle of the night. A study of healthy volunteers who gave blood every two hours and urine every four hours showed significant differences in serum phosphate depending on the time of day (from a minimum of 0.9mmol/L at 11am to maximum of 1.3mmol/L at 3am) (Kemp et al, 1992). Interestingly, and in contrast to the findings of Portale and colleagues discussed above, there was no obvious relationship to meals (unlike, for example, the circadian rhythm of serum glucose concentration). These findings imply that there is extensive tissue uptake of phosphate after meals followed by release during subsequent periods. Urinary phosphate excretion also varied in a circadian way, but less strikingly and there was more variation between individuals. Urinary excretion did not vary directly in proportion to serum concentration. A more recent study observed rises in serum phosphate that seemed to

occur after meals (Horwitz et al, 2003), but the number of subjects was too small to draw firm conclusions. A circadian rhythm to serum phosphate concentrations has also been reported in rats (Bielesz et al 2006), although the changes were relatively modest and phosphate concentrations were reported only for the normal working day.

Calcium and phosphate concentrations in human (and rodent) plasma are close to the saturation point for mineral precipitation; indeed, the solubility coefficient of calcium phosphate in water can be exceeded in some situations. Thus, moderate increases in serum phosphate might potentially lead to widespread vascular and soft tissue calcification. This could of course with equal accuracy be termed ‘phosphateification’, as the mineral is calcium phosphate, although this hardly trips off the tongue. As well as active control of serum phosphate levels, discussed more fully below, a number of proteins in the soft tissues and circulating in the serum seem to be able to inhibit mineralization (Schinke et al, 1999). The serum protein fetuin has been shown to act as an inhibitor of unwanted mineralization; mice deficient in this protein develop widespread and severe ectopic calcification (Schäfer et al, 2003), which is much more evident when they are fed a high-phosphate diet (Westenfeld et al, 2007). Low concentrations of this protein are associated with excess mortality in dialysis patients (Ketteler et al, 2003).

1.1.1 Hyperphosphataemia

Despite evidence of some regulation of phosphate uptake from the gastrointestinal tract, the main control of the concentration of phosphate in the serum, and of overall long-term phosphate balance, is exerted by the kidneys. Significant hyperphosphataemia is rare in people with normal kidney function. In contrast, in people with renal failure there is usually little change in phosphate intake whereas phosphate removal from the body is difficult; therefore many patients with end-stage renal failure have significant hyperphosphataemia.

1.1.1.1 Hyperphosphataemia in chronic renal failure

In a study of a large number of patients with significant renal impairment but not requiring dialysis (average estimated creatinine clearance 47ml/min), there was a strong positive relationship between serum phosphate and mortality, with each 0.33mmol/L increase in serum phosphate increasing mortality by 25%; mortality was increased once serum phosphate rose above 1.15mmol/L (*within* the normal range) (Kestenbaum et al, 2005). It must be said however that clinical information in this retrospective study may not be very accurate and not all confounders have been ruled out.

Registry data from the UK (UK renal registry report, 2006) and the USA (USRDS report, 2007) show that 40% or more of dialysis patients have hyperphosphataemia (even when defined by the generous criterion of plasma phosphate >1.8mmol/L; in fact the upper limit of the normal range in adults is usually considered to be 1.45mmol/L, so by this measure even more patients would be hyperphosphataemic). Conventional haemodialysis or peritoneal dialysis does not suffice to remove the average phosphate intake and it is necessary for almost all dialysis patients to take phosphate binding medication, aiming to reduce gastrointestinal phosphate uptake, and for them to follow a diet aimed at reducing phosphate intake.

Patients with end-stage renal failure undergoing dialysis have rates of death from cardiovascular disease which are at least ten times those of age-matched members of the general population, and in young patients this figure rises to up to 100 times (UK renal registry report 2006; Ansell et al 2007). Dialysis patients have multiple conventional risk factors for cardiovascular disease, including hypertension, dyslipidaemia and a high incidence of diabetes, but all these factors combined are not sufficient to account for the increased risk (Locatelli et al, 2003). It has been suggested that persistent hyperphosphataemia may provide part of the explanation for this additional risk (Block et al, 2002). Dialysis patients are prone to severe and widespread vascular calcification (Blacher et al, 2001; Oh et al 2002), and numerous studies have found a correlation between the degree of vascular calcification and serum phosphate levels or calcium/phosphate product (calculated simply by multiplying serum calcium by serum phosphate) (Shigematsu et al, 2003). Vascular calcification occurs at a younger age in dialysis patients than in the general population and is more widespread and severe. Vascular calcification and consequent increased vascular stiffness are strong predictors of

cardiovascular and all-cause mortality in dialysis patients (Blacher et al 2001). Studies of human vascular endothelial cells show that when grown in medium containing excess phosphate they lay down extracellular matrix proteins and mineralise these proteins (Jono et al, 2000) - in effect mimicking bone formation and providing a potential mechanism by which excess plasma phosphate might lead directly to mineralization and excess mortality. It is of particular note that the raised phosphate concentrations used in these experiments were only 2mmol/L - similar to those seen in many dialysis patients.

In a study that compared dialysis patients with higher and lower serum phosphate levels, hyperphosphataemic patients (with serum phosphates > 2mmol/L) were more hypertensive and had a higher cardiac output (Marchais et al, 1999). It is not clear which of these changes are primary and which secondary to the greater arterial stiffness seen in patients with vascular calcification. It has been suggested that vascular calcification in diabetic dialysis patients is more related to glycaemic control than to serum phosphate levels (Ishimura et al, 2002), although the number of diabetic patients in this study was small.

Longitudinal studies of phosphate control in patients with end-stage renal failure (that is, studies in which patients were grouped according to their serum phosphate levels at the beginning of the study and then followed up) have shown a relationship between serum phosphate levels and mortality, patients with the highest phosphate levels having the highest death rates (Block et al, 1998). Although highly consistent (the relationship has been seen in several US cohorts, in the UK, in Europe and in China), the magnitude of the increased mortality risk is relatively modest. In the original paper by Block et al, the highest quartile of serum phosphate was associated with a relative risk of 1.27, whilst in another study (Leggat et al, 1998) the relative risk associated with a grossly elevated serum phosphate, >2.5mmol/L, was only 1.13. A more recent study showed a relative risk of death of 1.82 in patients with a serum phosphate >2.26mmol/L compared to those with levels of <1.78mmol/L (Stevens et al, 2004). Supporting this is a longitudinal study from London's group (London et al, 2003) which showed that arterial medial calcification was strongly correlated with mean serum phosphate levels in long-term haemodialysis patients, and that medial calcification in turn was strongly correlated with the risk of death, mainly from cardiovascular causes. All these studies are based on stratifying patients into high or low phosphate groups on the basis of an initial phosphate value, which will lead to significant regression dilution bias (where the effect seen is reduced in

magnitude by the misclassification of patients initially- see MacMahon et al, 1990). A large long-term follow-up study with repeated phosphate determinations (at least monthly) would allow clearer connections between average or peak serum phosphate levels and clinical outcomes, but no such study has yet been performed. One study with repeated phosphate measurements, but of moderate size and with mean follow-up of only two years, confirmed an association of serum phosphate with mortality, with the hazard ratio for death in the highest quartile for serum phosphate of 1.6 (Melamed et al, 2006).

There is the additional complicating factor that serum phosphate is correlated with both nutritional status and patient compliance. Low phosphate may indicate inadequate nutrition, whilst elevated phosphate levels are often interpreted as indicating that the patient is not rigorous in following their diet or taking their medication, and thus may have other risk factors for mortality, for example poor blood pressure control (Leggat et al, 1998).

It has been suggested that vascular calcification in haemodialysis patients relates to the large amount of calcium many of them take by mouth, in the form of calcium carbonate (as gastrointestinal phosphate binder), aiming to reduce hyperphosphataemia. This leads to increased gastrointestinal uptake of calcium and thus (in the absence of appreciable renal calcium losses) a state of ongoing positive calcium balance. There is also the possibility that many dialysis patients are in positive calcium balance during dialysis itself (Gotch et al, 2007). Numerous comparisons have been made between calcium-containing phosphate binders and newer, non-calcium-containing binders, including sevelamer and lanthanum. It has been shown that vascular calcification progresses less rapidly in dialysis patients given non-calcium-based phosphate binders (Chertow et al, 2002 ; Kakuta et al, 2007), although others have found that progression of cardiac valve calcification is not different between calcium carbonate- and sevelamer-taking patients, provided their lipids are similar (Qunibi et al, 2007) . Some have (perhaps cynically) suggested that these analyses relate in part to the desire of pharmaceutical companies to sell phosphate binders which do not contain calcium (Nolan & Qunibi, 2003), these latter being under patent and expensive. A recent analysis of patients randomised to sevelamer or to calcium-based binders found no difference in all-cause mortality, but sub-group analyses suggested that mortality might be higher in older patients taking calcium-based binders (Suki et al, 2007). What is beyond dispute is that none of the currently available phosphate binding

regimes is particularly effective and most dialysis patients remain significantly hyperphosphataemic, with most studies suggesting that calcium acetate gives equivalent or better control of serum phosphate levels than sevelamer (reviewed in Qunibi et al, 2004).

As well as the possible direct harm caused by hyperphosphataemia, morbidity and mortality in dialysis patients have been shown to be increased by hyperparathyroidism (Melamed et al, 2006; Block et al, 2004). Although numerous factors contribute to hyperparathyroidism in renal impairment, persistently elevated plasma phosphate values are a major cause; most patients with renal impairment have both elevated PTH levels and hyperphosphataemia (see section 1.2.1. for a discussion of the inter-relationship between parathyroid hormone and plasma phosphate). In this context there is some interesting preliminary evidence that lowering serum parathyroid hormone *and* phosphate levels with the new therapeutic agent cinicalcet, (which acts as a calcimimetic at the calcium-sensing receptor on the parathyroid cell, and therefore reduces parathyroid hormone secretion directly and serum phosphate indirectly, by reducing bone breakdown) may reduce the risk of cardiovascular hospitalisation in dialysis patients (Cunningham et al, 2005); long-term clinical trials investigating the effects of this drug on the mortality of dialysis patients are in progress. There is experimental evidence (in rats which had been parathyroidectomised and then implanted with a minipump delivering controlled doses of PTH) that hyperphosphataemia causes myocardial hypertrophy and impaired renal function even when PTH is not elevated (Neves et al, 2004).

The risk of cardiac valvular calcification in dialysis patients has been found to correlate with their serum phosphate (Rubel & Milford, 2003); unfortunately this study is another in which patients are grouped according to a single value of phosphate at baseline and then followed up. The mechanism of valvular calcification is thought to be similar to that of arterial calcification, so this finding supports the view that raised serum phosphate is associated with an increased risk of abnormal mineral deposition at numerous sites.

Finally, there is evidence from a number of experimental models that hyperphosphataemia can promote the progression of chronic renal failure (Laouari et al 1997). There is also evidence that animals with chronic renal failure treated with phosphate-lowering medication have slower progression of renal disease (Nagano et al 2003). In accord with this, higher serum phosphate is associated with a more rapid decline in GFR in patients

with poor renal function approaching dialysis, as compared to patients with good control of serum phosphate (Voormolen et al, 2007).

Whether mortality within human populations with *normal* renal function is correlated with serum phosphate levels is not yet established. However, a recently published analysis of vascular risk factors in participants in the Framingham Offspring study found that those in the upper quartile for serum phosphate had a risk of vascular events of 1.55 as compared to those in the lowest quartile; this increased risk persisted even if analysis was limited to those with an estimated glomerular filtration rate of over 90ml/min and after adjustment for other recognised vascular risk factors (Dhingra et al, 2007). Very few patients had serum phosphate concentrations outside the normal range in this study, and the results were unchanged by their exclusion.

1.1.1.2 Hyperphosphataemia and calciphylaxis

Calciphylaxis, a syndrome of tissue necrosis and vascular calcification, was first described in detail by Gipstein, Coburn and Adams in the 1970s (Gipstein et al, 1976). They investigated patients with chronic renal failure and painful ischaemic ulcers of the skin or extremities, and found medial calcinosis of the arteries (that is, calcium deposits within the arterial wall). Unfortunately, parathyroid hormone assays were not available in 1976, but most of the patients were assumed to have secondary hyperparathyroidism; some improved dramatically when their parathyroid glands were removed. These authors also noted that all the patients had, at some point in their illness, greatly elevated serum phosphate concentrations. There are suggestions that the syndrome has become more common in recent years, although this may in part reflect longer average survival of dialysis patients (Don & Chin, 2003). Calciphylaxis has recently been more descriptively renamed calcific uraemic arteriopathy (Rogers et al, 2007). The condition has occasionally been reported in patients without renal failure and with a normal serum phosphate and calcium/phosphate product (Nigwekar et al, 2008), but this is most unusual, and hyperphosphataemia is regarded as playing a central role in the syndrome (Rogers et al, 2007); raised serum phosphate was the most significant risk factor for calciphylaxis in a multivariate analysis of patients in the largest single-centre study (Fine & Zacharias, 2002).

1.1.1.3. Acute hyperphosphataemia

Acute hyperphosphataemia may also lead to death; the most common situation in which this can occur is iatrogenic, when patients with poor ability to regulate phosphate (that is, with renal impairment or renal failure) are given phosphate-based medication as laxatives before colonoscopies or surgery (Tan et al, 2002). In a (probably small) proportion of cases a considerable amount of phosphate is taken up from the bowel and acute phosphate poisoning occurs. In one case in which such a patient was investigated by means of a renal biopsy (Desmeules et al, 2003), the biopsy was found to contain calcium-phosphate crystals blocking the renal tubules.

1.1.2 Hypophosphataemia

Since almost all foods contain phosphate, deficiency is rare in the developed world. When it does occur, severe hypophosphataemia can be life threatening; the subject has been thoroughly reviewed by Knochel (1977) and somewhat more recently by Subramanian and Khardori (2000). Severe hypophosphataemia usually occurs in patients with underlying phosphate depletion, for example poor nutrition, alcoholism or excessive use of phosphate-binding antacids. Hypophosphataemia can also, rarely, be caused by inherited conditions (see section 1.4.1), by renal phosphate wasting associated with kidney transplants or kidney stones (see section 1.4.2), or as a consequence of factors secreted by certain cancers (see section 1.4.3).

1.1.2.1 Hypophosphataemia in malnutrition

Studies in malnourished children have suggested that hypophosphataemia is common in this situation. Severely malnourished children with kwashiorkor were reported in one study to have a 12% incidence of severe hypophosphataemia (Manary et al, 1998); in another study over half the children had values below the normal range (Waterlow & Golden, 1994). Manary et al found that children with a very low serum phosphate (<0.32mmol/L) were at a much greater risk of death (mortality more than 60%) than children with serum phosphates nearer the normal range (mortality 22%). Some of the

deaths in these children were sudden and might have been due to heart rhythm disturbances or acute cardiac failure (which might have been caused directly by the low serum phosphate levels). In another study by the same group, also investigating severe malnutrition in young children, the overall mortality rate was 25% and about a quarter of all deaths were attributed in whole or in part to severe hypophosphataemia (Manary & Brewster, 2000). For a condition that is so common (admittedly usually far from academic medical centres), it is disappointing that there is so little literature in this area.

Hypophosphataemia is recognised as the key abnormality in the re-feeding syndrome. Total body phosphate is often low in malnourished patients. The resumption of eating (or administration of an intravenous glucose infusion) after a period of starvation causes plasma phosphate, often already low, to be reduced further as a result of increased phosphate uptake by cells, where it is required for increased protein and fatty acid synthesis. This precipitates severe hypophosphataemia and can cause respiratory and cardiac failure associated with rhabdomyolysis and leucocyte dysfunction; the syndrome can be fatal (Hearing, 2004). In a large retrospective study (Kagansky et al, 2005), hypophosphataemia was shown to be associated with the risk of death of elderly patients admitted to hospital, and was strongly associated with tube feeding and with intravenous glucose infusion, suggesting that many cases were caused by the re-feeding syndrome.

1.1.2.2 Hypophosphataemia in critical illness

In intensive care patients there is considerable evidence that hypophosphataemia is common, and that mortality is inversely correlated with serum phosphate; for example, in patients undergoing liver resection, mortality was four times higher in patients with a post-operative serum phosphate <0.5mmol/L than in those with a serum phosphate >0.8mmol/L (Giovannini et al, 2002). It is not clear, however, whether the relationship between severe hypophosphataemia and mortality is *causal* in critical illness: the authors of the study cited above suggest that hypophosphataemia might either be a cause of poor outcome or might be an early sign of complications. It has been shown that infusing the cytokines interleukin-6 and tumour necrosis factor-α into mice leads to hypophosphataemia (Barak et al, 1998), so adverse outcomes associated with low serum phosphate levels in critical illness might be consequences of high levels of these cytokines

rather than due to the low phosphate *per se*. In a study of intensive care patients, hypophosphataemia, defined as a serum phosphate <0.8mmol/L, was found in 29% of patients (Zazzo et al, 1995). These patients had a mortality of 30% compared to 15% in those patients with a normal serum phosphate, but the hypophosphataemic patients were more ill, in that they were more likely to have sepsis and require parenteral nutrition. In an extension of this study, glucose-1-phosphate was infused into eight patients with moderate or severe hypophosphataemia. In all eight, serum phosphate rose into the normal range (above it in one patient) and cardiac output (measured by thermodilution via a right heart catheter) improved significantly. This is the most convincing part of the study, and it suggests that control of serum phosphate is of considerable importance in this situation. In a similar (and equally small) study (Bollaert et al, 1995), critically ill patients on ventilators with serum phosphate <0.65mmol/L were infused rapidly with 20mmol of phosphate, which increased serum phosphate considerably. Mean blood pressure rose significantly after phosphate infusion, as did left ventricular stroke work - an indication of myocardial contractility. Despite these suggestive results, no clear consensus about the value or otherwise of routine phosphate supplementation in critically ill patients has been reached (Bugg & Jones, 1998).

In summary, normal control of serum phosphate depends on adequate renal function. Patients with renal failure often have significantly raised serum phosphate levels. There is considerable evidence that this raised serum phosphate is correlated with mortality, and there are plausible mechanisms by which hyperphosphataemia might contribute to premature death. It is not yet clear whether serum phosphate levels alter mortality in persons without renal impairment, but there is some evidence that they might. Hypophosphataemia is usually associated with undernutrition. It is also associated with poor outcome in several patient groups; however, it has not been conclusively demonstrated that it is hypophosphataemia that causes mortality in these groups.

1.2 Preservation of normal phosphate balance

Normal phosphate balance in man is maintained partly by regulation of uptake from the gastrointestinal tract, but principally by regulation of reabsorption of phosphate from the renal tubule. Phosphate is generally agreed to be freely filtered at the glomerulus

(Grimellec et al, 1975). Most is then actively reabsorbed in the proximal tubule; this process is under the control of a number of factors, in particular parathyroid hormone (Samiy et al, 1965; reviewed in Biber et al, 2000). The reabsorptive mechanism is specific and can be saturated. There is no evidence for tubular phosphate secretion, even under situations of phosphate loading (Handler, 1962).

Although much is known about how phosphate enters the renal tubular cell (by means of sodium-phosphate co-transporters on the apical brush-border membrane), little is known about how it leaves through the basolateral membrane into the circulation. Similarly, phosphate uptake from the gastrointestinal tract is mainly by means of sodium-phosphate co-transporters on the apical membrane of enterocytes, but little is known of how phosphate exits from these cells.

1.2.1 Parathyroid hormone and vitamin D

As indicated above, serum phosphate in human populations is regulated within the range 0.8 to 1.5 mmol/L, despite very variable phosphate intakes. It is generally agreed that the principal phosphate regulatory hormone is parathyroid hormone (PTH). There is a complicated inter-relationship between serum phosphate, PTH and vitamin D (see Fig. 1.1 and text below). PTH is synthesised as a preprohormone, processed, and secreted as a linear peptide by specialised cells in the parathyroid glands. PTH secretion is increased by a high phosphate diet and (as demonstrated in explanted parathyroid glands) by high serum phosphate (Almaden et al, 1998) and diminished by low serum phosphate (Felsenfeld et al, 2007); these effects are both genomic and post-transcriptional (Slatapolski et al, 1996). The release of PTH is markedly increased by low serum calcium and suppressed (though not completely halted) by high serum calcium. PTH synthesis and PTH secretion are both inhibited by the active form of vitamin D, calcitriol (see below).

PTH has widespread effects on phosphate. It increases the gastrointestinal uptake of phosphate, via raised calcitriol levels; it increases the production of calcitriol by up-regulating the enzyme 1α–hydroxylase in the kidney (see below); it increases the amount of phosphate lost in the urine by its actions on renal proximal tubular cells; and it stimulates bone breakdown, releasing phosphate into the plasma. It has significant, perhaps more striking, effects on serum calcium since it increases bone calcium loss and gastrointestinal calcium uptake (see Fig 1.1). PTH also has a number of less well

understood actions on the vascular system and elsewhere (Ogata et al, 2003); for example, prolonged exposure causes left ventricular hypertrophy which does not seem to be caused simply by hypertension (Piovesan et al, 1999).

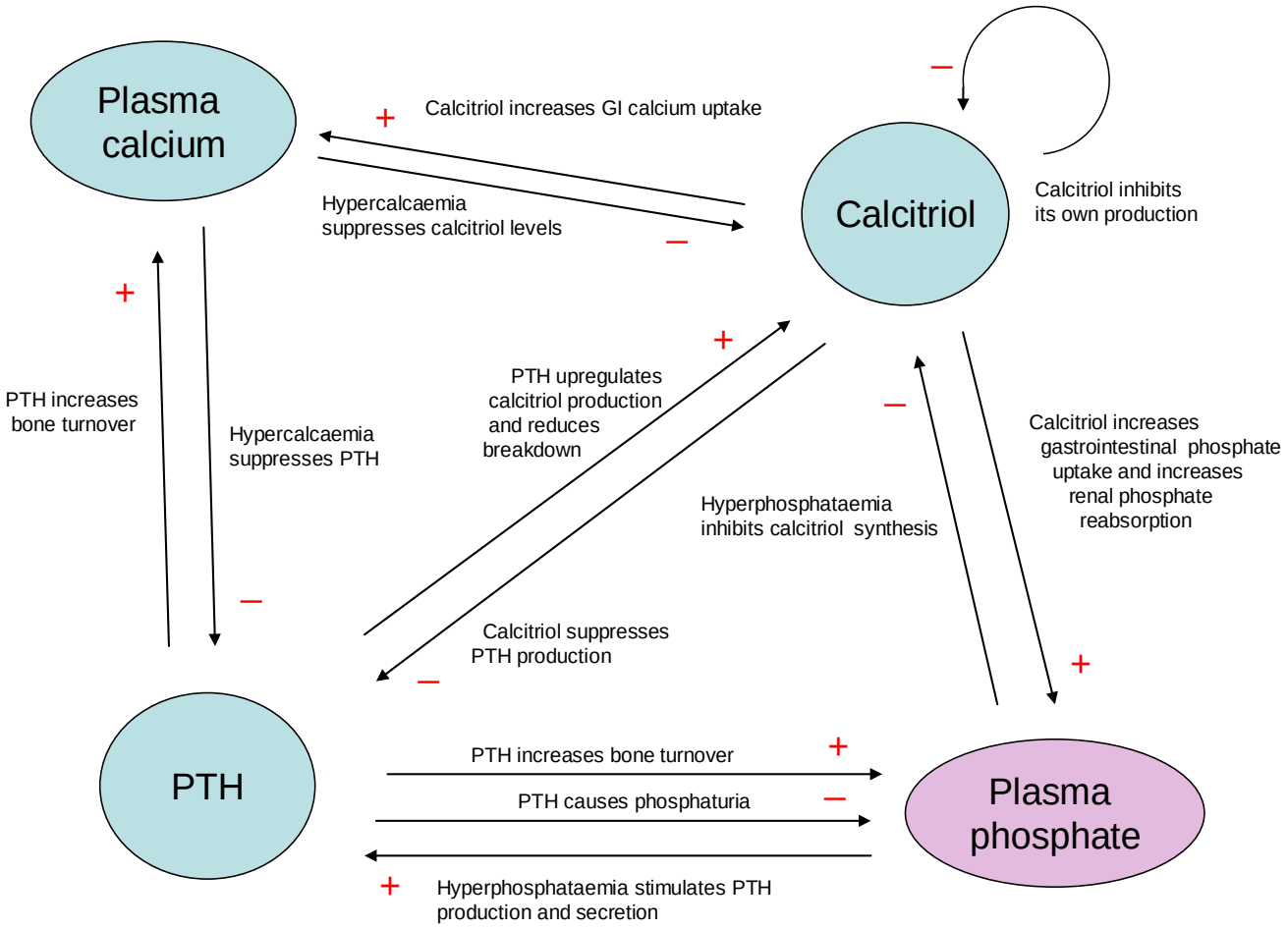


Fig. 1.1 Interactions between PTH, calcitriol, calcium and phosphate

Primary proliferative changes in the parathyroid glands leading to inappropriate excessive secretion of PTH (hyperparathyroidism) are not uncommon (Wermers et al, 1998). Interestingly, patients with mild hyperparathyroidism (PTH around twice the upper limit of normal) have modest hypercalcaemia but usually normal serum phosphate levels (Bilezikian & Silverberg, 2004), suggesting the existence of effective counter-regulatory systems able to compensate for the phosphaturic effects of modestly increased circulating PTH. (Note also in the section below on a clinical trial of subcutaneous PTH in osteoporosis, the absence of reported effects on serum phosphate.) It is clear that the main

clinical problems faced by patients with an excess of PTH are related to raised serum calcium rather than low serum phosphate (Peacock, 2002). In particular, patients with primary hyperparathyroidism do not often develop bone disease or myopathy; it is stated in Peacock's review that this is because they do not become phosphate depleted, although no reference is provided for this statement.

There is some evidence that PTH release may be stimulated by ingestion of oral phosphate via a humoral or nervous signal originating in the gastrointestinal tract, as well as by alterations in plasma phosphate; there are also data suggesting that the gastrointestinal tract may also produce other circulating phosphaturic substances. In uraemic rats fed a high-phosphate diet, duodenal infusion of the (non-absorbable) phosphate analogue phosphoformate induced a four-fold increase in PTH within 5min, without any change in plasma phosphate or calcium (Martin et al, 2005). It has recently been reported that instillation of phosphate into the duodenum in normal rats causes a rapid and large increase in renal phosphate excretion (Berndt et al, 2007). This effect occurs within 15 minutes and occurs without changes in serum phosphate; the effect is not altered by renal denervation and occurs in thyroparathyroidectomised animals. These workers assayed for changes in PTH and for alterations in the levels of the putative phosphate-regulating proteins fibroblast growth factor- 23 and frizzled-related protein-4 (see sections 1.4.3.3.1.and 1.4.3.3.3) and did not find any. These findings suggest a secreted substance originating in the small bowel and altering renal phosphate reabsorption; the nature of this substance (dubbed by the authors 'intestinal phosphatonin') and whether it has any actions on bone remain matters of conjecture.

Intravenous infusions of large doses of PTH in humans with normal renal function (Horwitz et al, 2003) and in rats (Brunette et al, 1973) lead (at least in the short term) to phosphaturia and reduced serum phosphate. PTH stimulates bone formation and resorption in humans; intermittent (daily) subcutaneous injection of the fragment PTH 1-34 have been shown to increase bone density and reduce the risk of further fractures in post-menopausal women with a history of fracture and osteoporosis (Neer et al, 2001). This finding is somewhat odd because continuous exposure to raised PTH levels, in primary hyperparathyroidism, causes reduced bone density and increases fracture risk; it is generally believed that short-term (less than two hours a day) exposure to elevated PTH is

anabolic for bone while long-term raised levels are catabolic (Potts, 2005). It is interesting that no mention of serum phosphate is made in this paper describing low-dose administration of PTH to over 1600 patients; it is probably reasonable to assume that there were no significant effects on serum phosphate levels.

The rate-limiting phosphate pathway in the renal tubule, and therefore in phosphate balance overall, is the type 2a sodium-phosphate co-transporter (NaPT2a) in the proximal tubular cell apical membrane (reviewed in Murer et al, 2001; see section 1.3.2 for a detailed discussion). It was demonstrated many years ago by means of micropuncture experiments that most renal phosphate reabsorption is in the early part of the proximal tubule (Brunette et al, 1973), and this is regulated by controlling the number and activity of these sodium-phosphate co-transporters. PTH regulates renal phosphate reabsorption by down-regulation of NaPT2a co-transporters; shortly after injection of PTH, the amount of NaPT2a present in the apical membrane decreases sharply, with the transporter protein being internalized and then degraded in the lysosomal compartment. There is currently no evidence for recycling of NaPT2a, although recycling back into the apical membrane has been demonstrated for other transporters. Thus, recovery of phosphate absorption depends on synthesis of new protein. PTH is able to act via both a protein kinase C- and a protein kinase A-dependent pathway (Hattenhauer et al, 1999), and has been shown to be active from both the luminal and basolateral sides of the mouse isolated proximal tubule (Traebert et al, 2000). It has been demonstrated that the luminal actions of PTH are mediated via the protein kinase C pathway, whilst the basolateral actions are mediated via both protein kinase C and protein kinase A pathways (Hattenhauer et al, 1999). No data exist to assess the relative importance of luminal and basolateral actions of PTH in the intact organism, but, as PTH is partially filtered at the glomerulus, luminal actions (especially in the more proximal tubule) may be of physiological significance. The interaction between PTH and the NaPT2a co-transporter is dealt with in more detail in section 1.3.2.

1.2.1.1 Vitamin D

Vitamin D is a steroid hormone with a complex metabolic and regulatory pathway (Dusso et al, 2005). When ultraviolet light strikes the skin, photons are absorbed by the steroid 7-dehydrocholesterol, splitting its beta-ring and forming vitamin D_3 (cholecalciferol). This

pro-hormone is then transported to the liver where a hydroxyl group is added by the liver enzyme, 25-hydroxylase. This leads to the formation of 25-hydroxyvitamin D_3 (calcidiol, sometimes written 25-(OH) D_3). Vitamin D can also enter the body via the gastrointestinal tract in food; the predominant form in food is vitamin D_2 (ergocalciferol) which is also converted to 25-hydroxyvitamin D_3 by 25-hydroxylase in the liver. The liver releases calcidiol into the circulation and it is acted on in the kidney (and in other tissues such as the skin and the prostate) by the enzyme 1α–hydroxylase to form 1α-,25-dihydroxyvitamin D_3 (calcitriol; sometimes written 1,25 $(OH)_2D_3$). Calcitriol interacts with a specific, high-affinity vitamin D receptor widely distributed in many tissues. Calcitriol formed in the kidneys circulates throughout the body, while that produced in other tissues seems mostly to have more local, paracrine functions. Calcitriol is by far the most active form of vitamin D; however, the greater chemical stability of calcidiol means that assays to measure vitamin D status in humans are usually aimed at measuring this compound. Figure 1.2 illustrates the relationships between the various forms of vitamin D.

The first step in the inactivation of vitamin D is 24-hydroxylation by the enzyme vitamin D 24-hydroxylase, present in the kidney and also in osteoblasts. This converts calcidiol to 24,25-$(OH)_2D_3$ and calcitriol to 1,24,25-$(OH)_3D_3$. Although there is evidence that 24,25-$(OH)_2D_3$ has some biological effects, it is much less active than calcitriol and no high-affinity receptor for it has yet been identified. The amount of calcitriol produced by the kidney reflects the activities of both the 1α-hydroxylase and 24-hydroxylase enzymes, as these are competing for the same substrate. Calcitriol administration (to mice) has been shown to increase 24-hydroxylase enzyme activity and mRNA abundance in the kidney (Akeno et al, 1997). 24-hydroxylase activity is also increased by high serum phosphate and reduced by PTH.

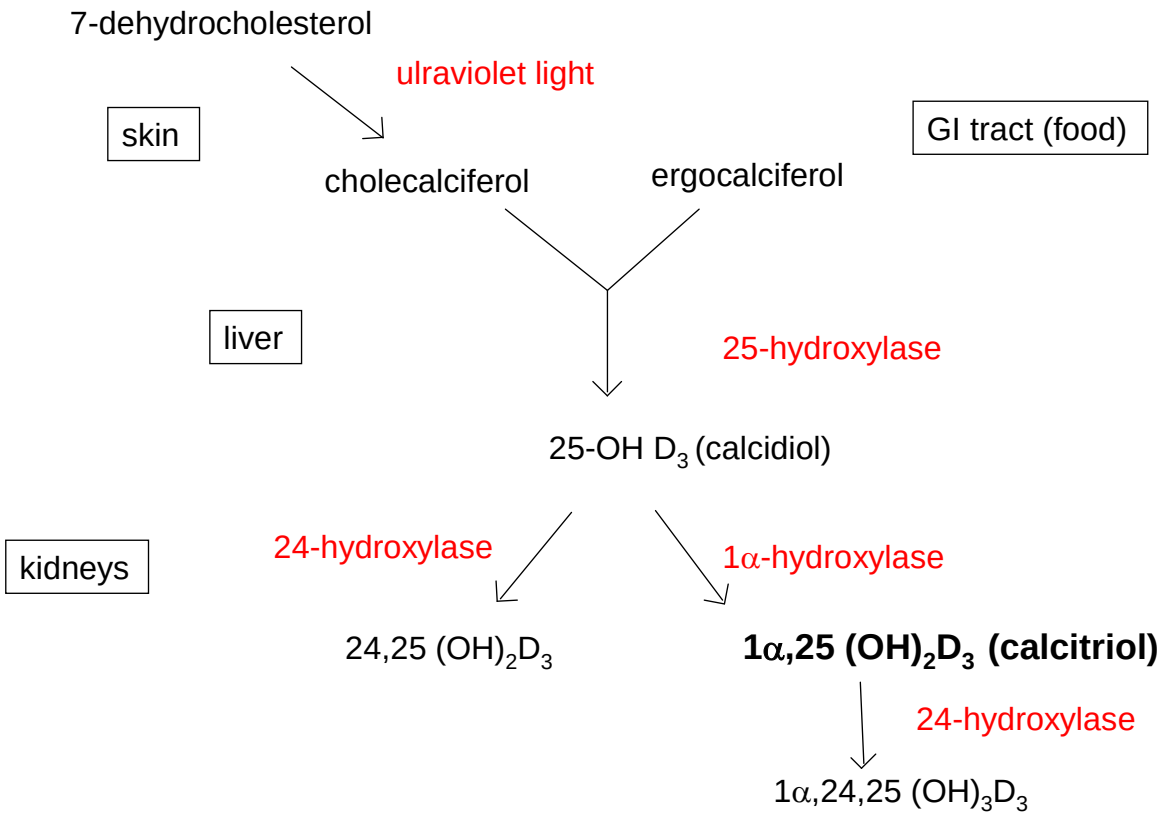


Fig. 1.2 Formation of calcitriol

High levels of active vitamin D (calcitriol) stimulate uptake of both calcium and phosphate from the gut. Calcitriol also stimulates sodium-phosphate co-transport in the renal tubular brush border in both intact rats and isolated renal tubule preparations (reviewed in Dusso et al, 2005); it directly suppresses PTH production by the parathyroid glands; and it also indirectly suppresses PTH by raising serum calcium, which then has a suppressive effect on the parathyroid glands. Low serum phosphate is a direct stimulant of renal 1α-hydroxylase activity, and thus leads to increased levels of calcitriol. Other regulators of renal 1α-hydroxylase activity are thought to be calcitriol itself, which inhibits enzyme activity, PTH and serum calcium (Moosgaard et al, 2007). Calcitriol levels are increased by PTH and by hypocalcaemia. In experiments using infusions of PTH and calcium chelators into dogs, in which PTH levels and calcium could be manipulated independently, it was shown that, when PTH is present to excess, alterations in plasma calcium determine whether calcitriol levels rise or fall (Hulter et al, 1985). This

suggests that the plasma calcium level can override PTH as a regulator of active vitamin D levels under certain circumstances.

As well as its role in the control of calcium and phosphate balance, vitamin D affects cell growth and is implicated in the development of some cancers and in modulation of the immune response (Dusso et al, 2005).

1.2.2 Other agents affecting renal phosphate handling

As well as PTH and vitamin D, a number of other factors have been shown to affect renal phosphate excretion. In most cases it seems unlikely that they control phosphate excretion, but they may nevertheless have an important influence under certain circumstances.

Glucagon

Glucagon is both phosphaturic and natriuretic; the phosphaturia has been shown to be mediated by cAMP, which is thought to be generated in the liver by glucagon and freely filtered at the glomerulus, passing into the renal tubule and acting from the luminal side of renal tubular cells (Bankir et al, 2002).

Thyroxine

Hypothyroidism is associated with a low serum phosphate concentration and thyroxine has been shown to increase the renal reabsorption of phosphate, by increasing the amount of NaPT2a protein in the proximal tubule brush-border membrane. Northern blot analysis confirmed that this effect was mediated by increasing the amount of mRNA for NaPT2a (Alcalde et al, 1999).

Growth hormone

Growth hormone increases serum phosphate and renal phosphate reabsorption, probably through an effect (at least in the rat) on NaPT2a numbers (Woda et al, 2004). Epidermal growth factor, the production of which is raised by growth hormone, has been reported to stimulate sodium-phosphate co-transport in isolated perfused renal tubules, but to inhibit sodium-phosphate co-transport in opossum kidney (OK) cells and in primary cultures of proximal tubule cells (Han et al, 2003).

Serotonin

It has been suggested that endogenous serotonin (5-hydroxytryptamine) may enhance phosphate reabsorption by stimulating proximal tubular cell phosphate transport (Gross et al, 2000).

Tubular calcium

Urinary phosphate excretion may also be related to (renal tubular) luminal calcium concentration. A calcium-sensing receptor is found in the apical membrane of the proximal tubule, and the expression of this receptor is reduced by a high phosphate diet and by PTH infusion. Exactly how this relates to the interlinked regulation of tubular calcium and phosphate handling is unclear (Rouse & Suki, 1985), but stimulation of these calcium receptors has been shown to inhibit the action of PTH on proximal tubular phosphate transport (Ba et al, 2003). Tubular calcium also appears to have effects on renal sodium handling, but this is mediated by actions in the loop of Henle and distal tubule (reviewed in Huang & Miller, 2007)

Glucocorticoids

Patients with Cushing's syndrome (glucocorticoid excess) are often hypophosphataemic. It has been show that dexamethasone (at least in adrenalectomised rats) causes phosphaturia, doing so by inhibition of sodium-dependent phosphate transport in the renal brush-border membrane (Webster et al, 1986).

Parathyroid hormone related protein (PTHrP)

The first 34 amino-acids of PTHrP are very similar to those of PTH, and PTHrP is expressed widely in many parts of the body (Strewler, 2000). It acts through the same receptor as PTH and, when infused in equal amounts, causes a similar degree of phosphaturia (Horwitz et al, 2003; Saito et al, 1993). PTHrP is often secreted in excess by lung tumours and can cause severe hypercalcaemia, but evidence suggests that it does not circulate in healthy people and that its main physiological roles are widespread local paracrine/autocrine actions on cell proliferation (Santos et al, 2001), without any significant action on renal phosphate handling.

Stanniocalcin

Stanniocalcin is a calcium-regulating hormone of bony fishes, known to stimulate phosphate reabsorption in the fish kidney. A similar sequence has been isolated from a

human lung cDNA library, named STC1, expressed in a bacterial system and injected into fish and rats (Olsen et al, 1996). In fish it caused reduced gill calcium transport, whilst in rats it caused a dose-dependent increase in renal phosphate reabsorption. Polyclonal antibodies raised against the human stanniocalcin protein bound to cells in the distal renal tubules of humans, suggesting this as a possible site of expression of the protein. Work on the stanniocalcins has disclosed a wide range of effects related to cell growth and differentiation, but it seems that the protein probably does not circulate in healthy humans, rather acting as a paracrine agent. Recent work has suggested a role for STC1 in endothelial gene expression and leukocyte migration in the kidney (Chakraborty et al, 2007).

1.2.2.1 Phosphatonins

The realisation that phosphate excretion depends not only on PTH and vitamin D but also on other factors, disturbance of which leads to the hereditary hypophosphataemic syndromes and also tumour-related hypophosphataemia (see section 1.4), led to the coining of the term ‘phosphatonin’ (reviewed in Kumar, 1997). ‘Phosphatonin’ is used to describe previously unrecognised substances which circulate in both normal and pathological states and which reduce serum phosphate by causing phosphaturia. Although at one time it was thought that one single new hormone might be found which would account for all phosphate-losing states and might additionally have a role in normal human physiology, it now seems more likely that there are a number of such substances with overlapping or interlocking functions. Current candidate phosphatonins have been identified from phosphate-wasting tumours (frizzled related protein-4, fibroblast growth factor-7, fibroblast growth factor-23 and matrix extracellular phosphoglycoprotein) and from the inherited phosphate-wasting disorders (fibroblast growth factor-23). These agents will be discussed in the context of human disorders of phosphate metabolism below.

1.3 Sodium-phosphate co-transporters

Phosphate is transported across cell membranes by a group of sodium-phosphate co-transporters, abbreviated to NaPTs. Currently, three main subtypes are recognised. NaPT1 is present on most cells of humans, rats and mice and seems to be responsible for

'housekeeping' phosphate transport, ensuring supplies of phosphate for cellular metabolism. The NaPT2 co-transporters seem to be responsible for high-capacity phosphate transport, NaPT2a and NaPT2c in the kidney and NaPT2b in the gut. The activity of these three subtypes controls serum phosphate, as phosphate enters the body from the bowel and leaves it in the urine. Although there is still much to be learned about these processes, it seems that most of the control is at the level of the renal proximal tubule NaPT2a co-transporter, perhaps with some contribution from renal NaPT2c. The NaPT3 group of proteins is less well understood currently, and their place in phosphate physiology remains a matter for speculation.

1.3.1 Sodium-phosphate co-transporter type 1 (NaPT1)

NaPT1 seems to be expressed in almost all cell types. Renal NaPT1 gene expression is not modulated by dietary phosphate intake (Tenenhouse et al, 1998) or by PTH (Zhao & Tenenhouse, 2000), in contrast to NaPT2a (see below). Levels of NaPT1 mRNA and protein were unchanged in the proximal tubules of NaPT2a knock-out mice, despite the grossly abnormal urine and serum phosphate levels (Zhao & Tenenhouse, 2000), which is perhaps an argument against NaPT1 having a role in phosphate regulation.

Hepatocyte nuclear factor 1α (HNF1α) is a protein found in many polarised epithelia, including the renal tubule; it transactivates a number of target genes including those coding for albumin, α1-antitrypsin and fibrinogens. Knock-out mice (HNF1α -/-) have a renal Fanconi syndrome, with aminoaciduria, glycosuria and phosphaturia. It has been shown that mRNA for NaPT2a in the kidneys of these mice is present in broadly normal amounts, but mRNA for NaPT1 is greatly reduced due to the absence of an activating effect of HNF1α (Cheret et al, 2002). This interesting finding appears to show that, in contrast to the generally held view, NaPT1 *is* a functionally important phosphate co-transporter in the kidney, although of course it does not necessarily mean that NaPT1 is a *regulated* phosphate transporter.

1.3.2 Sodium-phosphate co-transporter type 2a (NaPT2a)

The NaPT2a co-transporter has so far been found only in the proximal tubule and the osteoclast; it is absent from the bowel. The co-transporter is present largely in the convoluted part of the proximal tubule, decreasing toward the straight part. It is not found in the glomeruli, distal tubule or collecting ducts (Traebert et al, 1999b; Custer et al, 1994). NaPT2a localises to the apical (luminal) brush border of the proximal tubule and is not seen in the basolateral membrane. The transporter is a low affinity, high capacity system with closely related gene sequences in humans, rats, mice and other multicellular organisms (reviewed by Hernando et al, 2001). Homologues are generally not found in bacteria, although a similar protein has been found in the organism that causes cholera (Lebens et al, 2002); the suggestion has been made that it is an adaptation to survival in the human intestine where phosphate is plentiful.

A mouse knock-out of NaPT2a has been genetically engineered by Tenenhouse's group (Beck et al, 1998). This mouse has a moderately low serum phosphate, an increased fractional excretion of phosphate (FE_{Pi} of about 12% compared with about 4% in wild-type mice), and grows poorly with abnormal bones. Serum calcitriol is elevated (presumably to defend serum phosphate) and there is hypercalcaemia and hypercalciuria, which are assumed to result from increased bowel absorption of calcium under the influence of the raised calcitriol. Serum PTH is low, most likely suppressed by the hypercalcaemia and raised calcitriol. The animals do not develop rickets, and the abnormal bones become more normal as the animal ages. Renal brush-border membrane vesicles from NaPT2a null mice had about 30% of the sodium-dependent phosphate uptake of wild-type mice, which was interpreted by these workers as showing that NaPT2a is normally responsible for 70% of sodium-dependent phosphate uptake in the proximal tubule. Further experiments with this knock-out (Zhao & Tenenhouse, 2000) have shown that PTH has no effect on serum phosphate and only a minimal effect on fractional phosphate excretion and brush-border membrane vesicle sodium-phosphate co-transport, supporting the idea that the effects of PTH on renal phosphate excretion in mice are mediated via changes in NaPT2a. As indicated above, levels of NaPT1 mRNA and protein were unchanged in the knock-out mice; however, NaPT2c protein expression *was* increased in these animals - see section 1.3.4 (Tenenhouse et al, 2003).

Normal rats fed a low-phosphate diet have raised levels of NaPT2a protein, as assayed by Western blot of crude kidney brush-border membrane preparations. This effect occurs without a measurable change in serum PTH concentration (Laouari et al, 1997). Correspondingly, rats given a high-phosphate diet have a reduced amount of proximal tubular NaPT2a (Takahashi et al, 1998). Proximal tubule brush-border membrane vesicles from chronically thyroparathyroidectomised rats exhibit greater sodium-dependent phosphate uptake than is found in vesicles from sham-operated rats (Takahashi et al, 1998). Changing the diet of these thyroparathyroidectomised animals from low to high phosphate caused a rapid decrease in brush-border membrane vesicle phosphate uptake (over a period of only four hours). Immunohistochemical studies of the renal cortex confirmed that this effect was accompanied by a reduction in the amount of NaPT2a at the brush border. Clearly, the signal causing internalisation of the NaPT2a protein cannot have been carried by PTH in these animals, so this provides definite evidence that there is a non-PTH-dependent mechanism for the internalisation of NaPT2a in proximal tubular cells in response to dietary changes, as well as the recognised PTH-dependent mechanism. Other workers have shown that longer-term adaptation to alterations in dietary phosphate (over periods of days or weeks) are by means of changes in protein synthesis, but rapid changes can be made in a matter of hours by changes in NaPT2a transporter numbers in the brush-border membrane without new protein synthesis (Levi et al, 1994).

1.3.2.1 Trafficking of NaPT2a

NaPT2a is located only at the apical membrane of proximal tubular cells; phosphate can be effectively transported out of the renal tubular lumen only if this polarity is maintained (Hernando et al 2001 AP43). Work by Murer's group using green fluorescent protein-tagged NaPT2a has shown that the N-terminal region of the protein is not required for apical localisation, whilst the C-terminal end, which is required, contains a PDZ-binding motif (Bacic et al, 2004). Interactions between NaPT2a and the apical proteins NHERF1, NHERF2, PDZK1 and PDZK2 have been demonstrated and it is thought that these factors may help anchor the transporter to the brush-border membrane.

PTH leads to an increase in urinary phosphate excretion by decreasing the cell surface expression of NaPT2a. Work with biotin-labelled NaPT2a in OK cells demonstrated that the transport protein was internalised in response to a PTH signal (Jankowski et al, 1999) as the first step in the PTH-mediated regulation of tubular phosphate transport. NaPT2a

removal from the apical brush-border membrane is very rapid: within 60 minutes of exposure to PTH, most of the brush-border NaPT2a has disappeared, as assessed by immunofluorescence (Lotscher et al, 1999).

Considerable work has been done on the mechanism of retrieval of NaPT2a from the brush-border membrane. In mice with specific defects in the renal expression of megalin, a protein of importance in protein endocytosis in the proximal tubule, immunofluorescence studies found that steady-state levels of NaPT2a were raised (Bachmann et al, 2004). This correlated with reduced urinary phosphate excretion in the knock-out animals and suggests that there is active turnover and withdrawal of NaPT2a under basal conditions. Administration of PTH to these renal megalin knock-out animals was without effect - the absence of megalin prevented any response to exogenous PTH. However, there is no evidence of a direct interaction between megalin and NaPT2a, so the precise connection between them remains unclear.

A mouse knock-out for the ClC-5 intracellular H^+/Cl^- exchanger (an animal model for Dent's disease, which is caused by absence of the human ClC-5 protein) had significantly increased renal phosphate losses. Immunostaining demonstrated abnormal localization of the NaPT2a in the proximal tubule, with NaPT2a staining being mostly in subapical vesicles in the knock-out mice, rather than on the brush border as seen in wild-type animals. The abnormalities were not seen in the early S1 segment, but became more striking further down the proximal tubule (Piwon et al, 2000). The authors' hypothesis is that the phosphaturia seen in these animals is due to increased luminal activity of PTH, which is not undergoing the normal process of megalin-mediated endocytosis and instead is available to cause increased internalization of NaPT2a and consequent reduced tubular phosphate reabsorption.

Murer and co-workers (Karim-Jimenez et al, 2000) have investigated the molecular domains required to allow PTH to trigger internalisation of the NaPT2a protein in the model system of the OK cell. They showed by generating NaPT2a/NaPT2b chimeras that neither N nor C terminal region was required for internalisation, but that an area in the middle of the molecule containing a dibasic amino-acid motif, present in NaPT2a but absent from NaPT2b, was required

It has been shown, by use of inhibitors of the lysosomal and proteosomal pathways in OK cells, that NaPT2a is transported to, and degraded in, the late endosomes/lysosomes, and that this process is increased by exposing the cells to PTH (Pfister et al, 1998). The

process requires microtubule rearrangement and can be partly inhibited by the microtubule toxins colchicine and taxol (Lotscher et al, 1999). The actions of PTH on proximal tubular expression of the sodium-hydrogen exchanger 3 require the presence of the regulatory protein NHERF-1, but studies on OK cells expressing truncated forms of NHERF-1 showed no change in NaPT2a activity, suggesting that this regulatory protein is not involved in the trafficking of NaPT2a (Lederer et al, 2003). In contrast, mice with a null mutation for NHERF-1 have mislocalisation of the NaPT2a protein, with immunostaining showing that it is distributed within the cell rather than at the cell membrane. This corresponds with a large increase in the fractional excretion of phosphate (Shenolikar et al, 2002). These contrasting findings (OK cells with truncated NHERF-1 have normal NaPT2a activity, while NHERF-1 knock-out mice have reduced activity) may simply reflect species differences, or may point to the difficulty of generalising from model systems using immortalised mammalian cell lines.

Recent work (in mice) has suggested that PTH acts by inducing dissociation of a complex formed between NHERF-1 and NaPT2a; phosphorylation of a specific serine in a PDZ domain of NHERF-1 seems to cause dissociation of the NHERF-1/NaPT2a complex and thereby allow retrieval of NaPT2a from the brush-border membrane. This phosphorylation can be performed by protein kinase C (which is activated by luminal PTH, see section 1.2.1) and also by other phosphatases (Hernando et al, 2007).

Interestingly, high-affinity phosphate-transport proteins are found in the plasma membrane of yeast cells under low-phosphate conditions but rapidly trafficked from the membrane to vacuoles when phosphate is added to the environment (Lau et al, 2000), indicating that trafficking is an important regulated activity even in lower organisms.

1.3.3 Sodium-phosphate co-transporter type 2b (NaPT2b)

In the small bowel, another type 2 sodium-phosphate co-transporter has been identified, and localised to the enterocyte brush border (Hilfiker et al, 1998). This protein has been named the type 2b NaPT. Messenger RNA for this transporter has been identified in the lung, large bowel, liver and kidney, as well as in the small bowel.

NaPT2b protein has been localised by immunohistochemistry to the luminal epithelium of the alveolar cells in the lung (Traebert et al, 1999ii), where it was hypothesised that it might be involved in phosphate uptake for the synthesis of surfactant. Recently, mutations in the gene have been found in patients with the rare disease pulmonary alveolar microlithiasis (Huqun et al, 2007). One patient with this disease and a proven mutation in NaPT2b was found to have normal renal phosphate reabsorption (although the authors do not say how this was measured) and all patients in whom it was tested had normal serum phosphate levels (Corut et al, 2006); these findings suggest that renal control of phosphate is enough to maintain normal serum phosphate levels even if the main gastrointestinal regulatory system is absent; further studies on these patients would be of interest. The purpose of NaPT2b mRNA in the liver, kidney and large bowel remains unknown.

The important regulators of phosphate uptake in the small intestine that have so far been identified are a low-phosphate diet and calcitriol - each increasing intestinal phosphate absorption. It has been shown (in mice) that a low-phosphate diet or vitamin D administration causes increased expression of NaPT2b protein in the intestinal brush-border membrane (Hattenhauer et al, 1999). This effect is seen in animals fed a low-phosphate diet for a week; however, four hours of a low-phosphate diet are not enough to cause a measurable increase in NaPT2b transporter numbers. This observation led to the suggestion that the effects of a low-phosphate diet on the bowel may be mediated entirely via changes in vitamin D levels, in contrast to the rapid effects of PTH in the kidney mediated directly via PTH receptors. Interestingly, the amount of mRNA for NaPT2b was the same in animals on high or low phosphate diets and in the presence or absence of exogenous vitamin D, suggesting a post-genomic means of regulation of transporter numbers. Injection of animals with large doses of calcitriol increases subsequent small intestine phosphate uptake; this effect was associated with an increase in jejunal NaPT2b protein expression in both mice and rats (Marks et al, 2006).

1.3.4 Sodium-phosphate co-transporter type 2c (NaPT2c)

Children and young animals need a significant positive phosphate balance to provide the phosphate required for bone and other tissues (Spitzer & Barac-Nieto, 2001). Fractional

phosphate excretion in humans is only about 1% in newborns, about 5% in infants on human milk and about 20% in adults. During post-natal life no new nephrons are formed in humans, but GFR increases by increase of the GFR of each nephron. Absolute phosphate reabsorption by each nephron increases, but the maximum concentration of filtered phosphate which can be reabsorbed falls, during development (Kruse et al, 1982). Children have higher serum phosphate levels than adults, but there is an important difference between rodent and human physiology here, in that rodents grow throughout life and never fuse their epiphyses. The adult rodent may therefore have a positive phosphate balance more similar to a human child or adolescent than to an adult human. Nevertheless, it has been shown that even in rats there is twice as much NaPT2 (as assessed by Western blotting) in the proximal tubule of juvenile animals than in adults (Woda et al, 2001), initially leading to the suggestion that the greater phosphate reabsorption in young animals might be due to larger amounts of NaPT2a transporter.

Segawa and colleagues were the first to report the cloning of a growth-related sodium-phosphate co-transporter from both rat and human kidneys, which they called NaPT2c (Segawa et al, 2002). This protein was localised (in the rats) by immunohistochemistry to the apical membrane of the proximal tubule, and was found to be much more strongly expressed in weaning animals than in either newborns or adults (note that this does not correspond exactly with fractional phosphate reabsorption, which is highest in newborns, see above). The gene (at least as assessed by Northern blotting) was expressed only in the kidney.

The same group also cloned NaPT2c from mice. Sequence analysis showed 95% similarity to the rat sequence and 86% similarity to a human sequence assumed to be a homologue. Northern blot analysis of poly (A)-RNA from various tissues suggested that mRNA for NaPT2c was found only in the kidney, although RT-PCR suggested that transcripts were also to be found in the heart, placenta and spleen. No explanation or resolution of this difference between the two methods was offered (Ohkido et al, 2003). Western blotting of brush-border membrane vesicles showed that this transporter protein was greatly increased in animals fed a low-phosphate diet, and reduced to almost zero by a high-phosphate diet, suggesting that the transporter may be functional and regulated by changes in phosphate intake. Hybrid depletion experiments were performed in which antisense oligonucleotides were injected into *Xenopus* oocytes expressing poly(A)-RNA from mice on control and low-phosphate diets, and oocyte phosphate uptake was

measured. These showed no effect of NaPT2c antisense oligonucleotide in oocytes injected with RNA from control animals, but a significant fall in phosphate uptake in oocytes expressing RNA from phosphate-depleted animals (Ohkido et al, 2003). This might be taken to suggest that NaPT2c-mediated phosphate transport is only important when animals are phosphate depleted.

The NaPT2c protein has been shown to be internalised from the brush-border membrane of the renal proximal tubular cell in response to a high-phosphate diet in rats, just as NaPT2a is (Segawa et al, 2005). This response seems to be slower than the internalisation of NaPT2a; no change was demonstrated in the amount of brush-border membrane NaPT2c two hours after a high oral phosphate intake, whereas NaPT2a was already reduced by this time; at four and six hours the amounts of both were reduced. PTH has also been shown to affect internalisation of NaPT2c; NaPT2c protein and mRNA levels were increased in thyroparathyroidectomised rats, while injection of PTH into intact rats led to a reduction in NaPT2c in the tubular brush-border membrane, as assessed by immunoblots (Segawa et al, 2007). Again, this effect was not seen at two hours but was significant at 4, 8 and 12 hours.

Tenenhouse's group hypothesised that NaPT2c might be responsible for the remaining phosphate reabsorption in their NaPT2a knock-out mice (see section 1.3.2). They demonstrated that whilst overall brush-border membrane vesicle sodium-dependent phosphate uptake is greatly reduced in NaPT2a knock-outs, NaPT2c protein abundance (determined by Western blotting) is increased by more than two-fold (Tenenhouse et al, 2003). Interestingly, they also demonstrated that in *Hyp* mice (which carry a deletion of the *Phex* gene and are homologues of human X-linked hypophosphataemic rickets; see section 1.4.1.1) there was no increase in NaPT2c amounts despite hypophosphataemia. This suggests that adaptive up-regulation of NaPT2c requires *Phex* activity.

Mutations in the human NaPT2c gene have been shown to cause the syndrome hereditary hypophosphataemic rickets with hypercalciuria (HHRH; see section 1.4.1.3 below). The fact that humans without effective NaPT2c protein have moderate reductions in serum phosphate and quite significant bone disease has been interpreted as showing that the role of NaPT2c in overall phosphate balance may be more important than had previously been appreciated (Bergwitz et al, 2006). There is some preliminary evidence that phosphate metabolism in osteoblasts is abnormal in HHRH patients (Yamamoto et al, 2007); this is a

little hard to explain given that others have found that NaPT2c is absent from bone, but might represent secondary changes in osteoblasts due to either prolonged hypophosphataemia or exposure to abnormal levels of phosphate regulatory hormones.

1.3.5 Sodium-phosphate co-transporters type 3 (NaPT3s)

The receptors for two viruses, the gibbon ape leukaemia virus (named Glvr-1) and rat amphotophic virus (named Ram-1), turned out to be related. Both genes have been found to code for cell surface proteins which act as sodium-phosphate co-transporters. The proteins were renamed PiT-1 and PiT-2 (although this can cause confusion, as there is a nuclear factor important in pituitary function called pit-1) and were found to be 60% amino acid sequence identical, with only very weak homology to the type 1 and type 2 sodium-phosphate co-transporters discussed above. These proteins have now been named NaPT3s. The two proteins are widely distributed in rat tissues, including the liver, kidney, heart and muscles. In humans, PiT1 and PiT2 have been found in *all* tissues investigated and they are generally viewed as further 'housekeeping' transporters (Böttger et al, 2006). PiT1 and PiT2 are expressed in bone matrix vesicles and may have an important role in bone mineralization; mineralization in osteoblast cultures can be increased or reduced, respectively, by over- and under-expression of *PiT-1* (Yoshiko et al, 2007). It has also been shown that PiT-1 is important in the vascular calcification seen in hyperphosphataemia; such calcification can be reduced by transfecting vascular smooth muscle cells with PiT-1- specific small interfering RNA (Li and Giachelli, 2007).

1.4 Human disorders of phosphate metabolism

1.4.1 Hereditary hyperphosphaturias

1.4.1.1 X-linked hypophosphataemia (XLH)

Online Mendelian Inheritance in Man (OMIM) 307800

This is the commonest (that is to say, the least rare) of the inherited renal phosphate-wasting states in humans, and was first described by Winters and colleagues in the 1950s (Winters et al, 1958). This classic paper was reviewed, and discussed in the light of subsequent findings, by Scriver and colleagues (1991). The syndrome is characterized by growth retardation, rickets/osteomalacia, hypophosphataemia, abnormal renal phosphate reabsorption and failure of vitamin D[1] to rise in response to low serum phosphate. Serum calcium levels are normal. The mutated gene has been isolated from XLH patients and the gene product has been named 'protein with homology to endopeptidases on the X chromosome', or 'PHEX'. The PHEX gene has some expression in many tissues (parathyroids, lung, brain, muscle) but has its highest levels of expression in bone; it is not expressed in the kidneys (Beck, 1997). Exactly how abnormalities of this gene result in the XLH phenotype remains unclear, although the homology between the sequence of the PHEX gene and endopeptidase sequences has led to the hypothesis that the PHEX protein cleaves a circulating protein that affects renal phosphate handling. PHEX protein has been shown experimentally to cleave PTHrP but not PTH, endothelin or numerous other peptides (Boileau et al, 2001).

In a large retrospective study of patients with X-linked hypophosphataemia, no evidence was seen for genetic heterogeneity (Whyte et al, 1996). Male and female cases had no significant differences in phosphate concentrations or any other parameter of mineral metabolism, and female cases were (as expected in an X-linked dominant disease) roughly twice as frequent. This is a little difficult to reconcile with the subsequent discovery that

[1] Vitamin D is usually measured in humans by determination of 25-hydroxyvitamin D (calcidiol). The main active form is 1α-,25-dihydroxyvitamin D (calcitriol); calcidiol is converted to calcitriol by the enzyme 1α-hydroxylase, mainly in the kidney. Most experimental work in this area is performed using calcitriol if there is renal impairment; other forms of vitamin D are often used if renal function is normal. I have used 'calcitriol' in this thesis if this compound was used or measured, and 'vitamin D' if any form of vitamin D other than calcitriol was used or measured, or if the authors do not specify.

the mutation is in a proteolytic enzyme - because female cases still have a normal copy of the PHEX gene, while male cases do not; one would therefore expect females to be less affected or indeed not affected at all. This observation remains unexplained at present (although it is worth noting that the same phenomenon is also seen in the *Hyp* model discussed below).

A mouse model for this disease, known as the *Hyp* mouse, was discovered by a combination of chance and close observation at the Jackson Laboratory in the 1970s (Eicher et al, 1976). The *Hyp* mouse was found to carry an X-linked dominant mutation causing hypophosphataemia, rickets, reduced stature and renal phosphate wasting, and the almost exact resemblance to human X-linked hypophosphataemia was noted immediately. The presence of this mouse model helped to stimulate research interest in this area during the 1980s, when few other human diseases had as exact a model available. Nesbitt and co-workers (Nesbitt et al, 1992) carried out an elegant series of cross-transplantation experiments to investigate the basis of the metabolic abnormalities in these mice. They removed both native kidneys and transplanted one new kidney into each animal. In normal mice transplanted with a single normal kidney in this way, serum phosphate and tubular reabsorption of phosphate were no different from values in uninephrectomised controls. If a normal mouse received a transplant from a *Hyp* mouse, serum phosphate again was identical to that of control animals, as was the tubular reabsorption of phosphate. However, *Hyp* mice transplanted with a kidney from either other *Hyp* mice or normal animals had persistently low serum phosphates and reduced tubular reabsorption of phosphate compared with controls. These results show that the primary abnormality in the *Hyp* mouse does not lie in the kidney (although the phenotype is largely caused by renal phosphate wasting) but must be a circulating factor which can also cause phosphate wasting in normal kidneys. More recent work with this model has shown a reduction in NaPT2a mRNA and protein in the kidney, and also evidence for disordered regulation of vitamin D in these animals: in wild-type mice, low serum phosphate stimulates the activity of 1α–hydroxylase, whilst in *Hyp* mice it has exactly the opposite effect (Azam et al, 2003). Even though we now know the underlying genetic defect in *Hyp* mice, this observation is puzzling.

A database has been set up listing all known mutations in the PHEX gene, with the aim of allowing phenotype-genotype correlations and facilitating research on this gene (Sabbagh et al, 2000) (this is available online at phexdb.mcgill.ca). Almost all possible sorts of

mutation have been shown to cause disease: frameshifts, missense and nonsense mutations, abnormal splicing and deletions. These have been shown to result in abnormalities in the trafficking and/or conformation of the PHEX protein, or in its endopeptidase activity (Sabbagh et al, 2003). This range of types and location of mutations, with little evidence of variability in phenotype, suggests that the mutations are likely to cause non-function of the gene product rather than aberrant function.

1.4.1.2 Autosomal dominant hypophosphataemia (ADHR)

OMIM 193100

This disorder was first clearly distinguished from X-linked hypophosphataemia in the 1970s by Scriver and colleagues (Scriver et al, 1977) and was characterised in a large kindred as recently as 1997 (Econs & McEnery, 1997). The disease can present in childhood with rickets and skeletal deformity but can also present in adulthood with hypophosphataemia and weakness or bone pain. Some people with unequivocal hypophosphataemia were asymptomatic and were identified only as part of the family studies. Intriguingly, several male subjects had bone problems and low serum phosphate concentrations in childhood which improved in adulthood, with an absence of symptoms and low-normal serum phosphates. In all affected patients there is hypophosphataemia with (inappropriately) normal vitamin D levels, just as there is in XLH.

Genome-wide linkage analysis of a large kindred affected by ADHR allowed the abnormal gene to be localised to chromosome 12p13 (Econs et al, 1997). Subsequent positional cloning demonstrated that the syndrome is caused by mutations in a gene belonging to the fibroblast growth factor family, which has since been named FGF-23 (The ADHR consortium, 2000 and see below). Analysis of the mutations found in patients with ADHR has shown that they are mis-sense mutations, which render the protein less sensitive to protease cleavage than wild-type FGF-23 (White et al, 2001). The precise mechanism by which this leads to phosphaturia is not yet clear, although it has been shown that FGF-23 mutants suppress renal phosphate reabsorption by reducing sodium-dependent phosphate transport in the renal brush-border membrane, independently of PTH (Saito et al, 2003).

A recent paper has documented FGF-23 levels in people known to have FGF-23 mutations, and in control subjects (Imel et al, 2007). A large number of people with

definite FGF-23 mutations were not hypophosphataemic: only 16/42 subjects with ADHR mutations had low serum phosphates at the time of FGF-23 measurement. FGF-23 measurements in persons with ADHR mutations were widely scattered; some people with ADHR mutations had very high FGF-23 levels while many others had values in the normal range. In two patients in whom successive samples over a period of months were available, there were different patterns of FGF-23 concentration; one patient always had very high levels, but in the other the levels began high but subsequently normalised for no obvious reason. I think the conclusion must be that levels of FGF-23 are subject to some other forms of control which have not yet been discovered, and phosphate levels do not depend on FGF-23 levels in a straightforward way even in people with ADHR.

1.4.1.3 Hereditary hypophosphataemic rickets with hypercalciuria (HHRH)

OMIM 241530

This syndrome was first described in 1985 by Liberman's group in Israel (Tieder et al 1985). (The Middle East is a fertile ground for discovery of recessive genetic diseases, due to the presence there of many groups with a tradition of first cousin marriages.) The syndrome is characterised by rickets, poor growth and short stature, increased renal phosphate loss, hypercalciuria with normal serum calcium levels, raised vitamin D levels, increased gastrointestinal uptake of calcium and phosphate (probably owing to the raised vitamin D) and low or low/normal PTH levels despite very low serum phosphate. The clinical and biochemical abnormalities can be overcome by giving phosphate supplements, with the exception of the renal phosphate leak. This led to the hypothesis that the renal phosphate leak is the primary abnormality, with all the other changes being secondary. Note that in this syndrome, in contrast to XLH, ADHR and tumour-induced osteomalacia (see sections 1.4.1.1, 1.4.1.2 and 1.4.3), there is a significant (appropriate) increase in calcitriol levels. In one kindred (van den Heuvel et al, 2001), mutations in NaPT2a were specifically excluded as a cause of the syndrome; it was subsequently shown simultaneously by two groups, working independently but using very similar search strategies using genome-wide linkage scanning, that the syndrome was caused by mutations in the NaPT2c gene (Bergwitz et al, 2006; Lorenz-Depiereux et al, 2006).

1.4.1.4 Autosomal recessive hypophosphataemic rickets (ARHR)
OMIM 241520

This syndrome was described for the first time in 2006 by Feng and colleagues in two unrelated kindreds (Feng et al, 2006). In each kindred, affected individuals had renal phosphate wasting, low, but not very low, serum phosphate (0.7-0.9mmol/l) and rickets. PTH was normal, as were serum and urinary calcium levels. Serum calcitriol levels were inappropriately normal for the level of hypophosphataemia. Parents and siblings of affected individuals showed no abnormality of calcium, phosphate, PTH or calcitriol levels. This syndrome is differentiated from HHRH (above) by the normal vitamin D and urinary calcium levels. Based on the similarity between this syndrome and a mouse knock-out of the gene for dentin matrix protein-1 (*Dmp-1*; a protein of importance in bone mineralization), the authors hypothesised that a mutation in the human gene *DMP-1* might be the cause of these patients' abnormalities. They were able to demonstrate such mutations, a frame-shift in one kindred and nucleotide substitution in a presumed critical sequence in the other. DMP-1 is a member of the SIBLING group of proteins (see section 1.4.3.3.4) and is found mainly in mineralised tissues. The authors found greatly elevated serum FGF-23 levels in the *Dmp1*-null mouse but more modestly elevated values in the patients with ARHR (less than twice normal on average, and two of four patients within the normal range). How *DMP-1* mutations elevate FGF-23, and whether other phosphaturic substances are also elevated in this syndrome, are not yet known.

1.4.1.5 McCune-Albright syndrome and fibrous dysplasia of bone
OMIM 174800

Fibrous dysplasia of bone is characterised by replacement of normal bone by fibrous tissue with irregular trabeculae of woven bone irregularly distributed within it. It can affect a single bone or multiple bones and can occur as part of the McCune-Albright syndrome, a triad of café-au-lait spots, precocious puberty and fibrous dysplasia, often with other endocrine abnormalities (Ringel et al, 1996). The McCune-Albright syndrome is due to activating mutations in the α subunit of the G protein that stimulates cAMP formation via adenylate cyclase, known as Gs (Weinstein et al, 1991). These mutations lead to the constitutive activation of adenylate cyclase and consequent increased signalling through the intracellular cyclic AMP pathway. The mutations are also seen in fibrous dysplasia of bone, with the difference between the two syndromes thought to lie in the

time at which the mutation occurred. Mutation events occurring during the early stages of embryogenesis lead to an individual with mosaic populations of normal and mutated cells in many organs and consequently the many and various features of McCune-Albright syndrome. Persons in whom the mutation occurs in late development and in osteoid precursors have abnormalities limited to bone. Hypophosphataemia and renal phosphate wasting are often seen in patients with fibrous dysplasia of bone, and it is thought that a phosphaturic factor released from the abnormal bone circulates in these patients. The mechanisms linking increased intracellular cAMP and release of a phosphaturic factor are not understood, although it has been reported that there is increased expression of the c-*fos* proto-oncogene in the bone of patients with fibrous dysplasia (Candeliere et al, 1995). This gene interacts with nuclear transcription factors and leads to alterations in the expression of many other genes. It is quite possible that a number of different factors with activity in the renal proximal tubule might be circulating in patients with fibrous dysplasia of bone.

A study of 42 patients with McCune-Albright syndrome/fibrous dysplasia of bone found that 20 of them had some degree of renal phosphate wasting (Collins et al, 2001). Interestingly, even larger proportions of these patients had proteinuria (86%) and aminoaciduria (94%). The proteinuria was mild and reported to be tubular, although the data to support this were not given in the paper. The aminoaciduria was non-specific. These results strongly suggest that many patients with fibrous dysplasia of bone have a generalised dysfunction of proximal renal tubular function, perhaps caused by a circulating factor produced by bone. The possibility that there is a primary renal abnormality in these patients should also be considered: as the mutation is post-zygotic and sufferers are mosaics, there might be genetic abnormalities in the kidneys. Mutated protein has been detected at post mortem in the kidneys of at least some patients with McCune-Albright syndrome (Weinstein et al, 1991 as cited above), and it might exert a local effect, perhaps by means of raised levels of cAMP, which is known to cause phosphaturia (Bankir et al, 2002).

A paper by Riminucci and colleagues attempted to make the case that the renal phosphate wasting found in fibrous dysplasia of bone is due to FGF-23 (Riminucci et al, 2003). They elegantly showed that FGF-23 is expressed in fibroblasts and osteoblasts in dysplastic bone from fibrous dysplasia patients, and also in active remodelling bone (at a recent fracture site) from normal persons, whilst it is expressed at very low levels in normal

bone. The serum FGF-23 concentration in patients with fibrous dysplasia correlated both with the overall disease burden and with phosphaturia. The relationship was statistically robust but there were considerable variations; some patients with significantly elevated FGF-23 levels had normal renal phosphate handling. In a study of bone biopsies from patients with fibrous dysplasia, FGF-23 protein could be found by immunostaining in 4 out of 12 cases; these four cases had lower average serum phosphate values than patients without FGF-23 staining, but in only one case was serum phosphate outside the normal range (Kobayashi et al, 2006).

Although the evidence that FGF-23 is the sole implicated circulating factor in phosphaturic patients with fibrous dysplasia of bone is not conclusive, it is clear that mutations affecting intracellular cAMP in bone are able to trigger the release of a factor which can cause renal phosphate leak and, in at least some patients, other abnormalities of proximal tubular function.

1.4.1.6 Jansen's metaphyseal chondrodysplasia

OMIM 156400

This very rare autosomal dominant condition is characterised by dwarfism (due to abnormalities of the metaphyseal growth plate) and hypercalcaemia with a low serum phosphate, decreased tubular phosphate reabsorption and low PTH levels. The syndrome is caused by activating mutations of the gene encoding the PTH/PTHrP receptor (Schipani et al, 1999). It is thought that the bone growth abnormalities reflect activation of intracellular pathways normally triggered by PTHrP, while the renal and other metabolic abnormalities reflect over-activation of intracellular pathways normally affected by PTH. The sum of cases in the world literature is less than 20.

1.4.2 Other hyperphosphaturic syndromes

1.4.2.1 Post-transplant hypophosphataemia

Up to 50-80% of patients receiving renal transplants develop hypophosphataemia (Moorhead et al, 1974). This has been attributed to a combination of hyperparathyroidism (which is very common), calcitriol deficiency (not so common, at least if the graft works well, the (actually rather modest) phosphaturic effect of glucocorticoids and tubular toxicity of ciclosporin (reviewed by Massari, 1997). Some patients with marked, persistent hypophosphataemia do not have hyperparathyroidism and it has long been thought that there may be circulating phosphaturic factors other than PTH in these patients (Levi, 2001).

Falkiewicz and colleagues (Falkiewicz et al, 2003) observed that hypophosphataemia was common in patients treated with either of the immune suppressants tacrolimus or ciclosporin but was less severe in those treated with tacrolimus. This would suggest that post-transplant hypophosphataemia might be mostly an effect of the toxicity of these drugs on the renal tubules rather than any endogenous circulating factor. Unfortunately, however, the data were so confusingly presented that it is impossible to determine whether these conclusions, given in the text, could be substantiated; furthermore, the doses of corticosteroid given to the two groups of patients differed greatly. A second paper by the same workers, probably reporting further on the same group of patients, does not make their findings any clearer (Falkiewicz et al, 2006). Other workers have reported that the (relatively) new immune suppressive drug rapamycin is another cause of impaired renal phosphate handling after renal transplantation (Schwartz et al, 2001).

A carefully conducted study by Rosenbaum et al (Rosenbaum et al, 1981) investigated patients with stable kidney function more than six months after a kidney transplant. These patients were transplanted during the 1970s and so were taking steroids, but neither ciclosporin nor tacrolimus. About a third of patients had a low serum phosphate (<0.83mmol/L). Individual patient PTH levels were not given; however, the average serum PTH was no different from that in transplant recipients with normal serum phosphate levels, although both groups had higher serum PTH values than healthy controls. Tubular reabsorption of phosphate, determined during infusion of increasing doses of phosphate, was lower in the hypophosphataemic group than in patients with

normal serum phosphate levels. In some patients, renal phosphate handling could be restored to normal by infusions of calcium, which reduced PTH levels. However, in others, calcium infusion, despite lowering PTH, failed to reverse the renal phosphate transport defect. The results indicate a long-lasting abnormality in renal phosphate handling in many patients after renal transplantation, which in some cases is due to, and in others independent of, PTH.

Green and co-workers (Green et al, 2001) set out to look for a circulating phosphaturic factor in the serum of patients after a renal transplant. They studied a range of patients - some on dialysis, some with advanced renal failure but not yet on dialysis, and two groups of transplant patients: one early after transplant (within one month of a successful graft) and a second transplanted group 9-12 months after a graft. Mean serum phosphate in the early post-transplant group (0.3mmol/L) was markedly reduced compared to all the other groups, with a fractional excretion of phosphate of almost 70%. This led the authors to suggest that a phosphaturic factor might be circulating in this group of patients. They took serum from all patients and added it to opossum kidney (OK) cells, then measured sodium-dependent phosphate uptake. They found that serum samples from dialysis patients, pre-dialysis patients and early post-transplant patients were all able to inhibit sodium-phosphate co-transport. However, they found a rapid and significant *increase* in type 2 phosphate co-transporter abundance, as assessed by Western blotting. Over a longer time-course, an increase in co-transporter mRNA was also found. It is difficult to square the increase in co-transporter numbers and mRNA message with the decrease in its biological activity, especially as most other experiments seem to find that activity and co-transporter numbers are closely linked. Indeed, it was reported in the same paper that PTH added to OK cells causes a reduction both in sodium-dependent phosphate transport and in co-transporter protein. The authors suggested that the circulating factor found in post-transplant patients acts in a unique way on sodium-phosphate co-transporters, increasing transcription while reducing activity.

Elevated FGF-23 levels have been reported in renal transplant recipients (Evenepoel et al, 2007). These authors found that almost all transplant patients had transient hypophosphataemia after their graft; significant persistent hypophosphataemia was seen in patients who had raised levels of both FGF-23 and PTH. A second research group looked at FGF-23 levels on the day of transplant and regularly thereafter; in general, FGF-23

levels fell after a successful transplant and there was a significant inverse association between FGF-23 levels after the transplant and serum phosphate levels (Bhan et al, 2006).

1.4.2.2 Renal phosphate wasting and kidney stones

Prie's group has a long-standing interest in a possible connection between increased urinary phosphate concentrations and nephrolithiasis (kidney stones). Kidney stones are commonly made of calcium phosphate in combination with calcium oxalate. It is known that a major risk factor for calcium-containing stones is a raised urinary calcium excretion, and some of these patients also have a renal phosphate leak. It has been hypothesised that the renal phosphate leak is the primary problem in some patients, leading to low serum phosphate, consequent stimulation of vitamin D production and increased bowel uptake of calcium and phosphate. This increased uptake of calcium would then lead to hypercalciuria. Prie et al investigated 230 patients with calcium stones (Prie et al, 2001), comparing maximal phosphate reabsorptive capacity (Tm_{Pi}) and other renal parameters with corresponding values in 105 control subjects with no known kidney stones. They found that 10% of patients had hyperparathyroidism and a low Tm_{Pi}. However, another 20% of the patients had a low Tm_{Pi} yet normal serum PTH levels. This study shows that a low Tm_{Pi} is not an uncommon finding in patients with kidney stones. Interestingly there was a normal distribution of Tm_{Pi} in both normal subjects and stone formers, with no evidence of a bimodal distribution. The authors (reasonably) suggested that this finding indicates a complex series of influences on renal phosphate threshold, rather than just a few simple genetic traits. Syndromes characterised by renal phosphate wasting without changes in vitamin D (such as tumour-induced osteomalacia; see section 1.4.3) are rarely accompanied by kidney stones (Prie et al, 2004). In another large retrospective study, almost 10% of patients assessed in a renal stone clinic were found to have low serum phosphates and a renal phosphate leak but normal PTH levels (Levy et al, 1995), again suggesting a primary abnormality of the proximal tubule and/or unrecognised circulating phosphaturic factors in these patients.

Another publication by Prie and co-workers (Prie et al, 2002) reported that the primary sequence of the NaPT2a gene was abnormal in two patients with low serum phosphate and either kidney stones or osteoporosis. Prior to this, no clinical phenotype had been reported as due to an abnormality of the NaPT2a gene, despite the fact that it is regarded

as the most important regulated phosphate transporter in the kidney. Twenty patients with a renal phosphate leak but a normal PTH level had their NaPT2a genes sequenced. Eighteen of them were found to have no abnormality of the coding sequence of NaPT2a, but two different heterozygous abnormalities were found in two unrelated patients. One of these patients had kidney stones and a low serum phosphate but no other clinical abnormalities (he was of normal height and had no reported skeletal problems). The other patient had osteoporosis and a daughter with skeletal problems, but did not have kidney stones. Subsequent work by Murer's group (Virkki et al, 2003) has cast some doubt on the significance of the original findings. This second group was unable to reproduce the original findings that these sequence changes had functional consequences in terms of phosphate transport, particularly when expressed heterozygously. This raises the possibility that these sequence changes are simply neutral polymorphisms of little functional significance, and emphasizes that the mechanism of renal phosphate leak in stone-formers is not yet understood.

FGF-23 levels are raised in some patients with low serum phosphate levels and renal stones; however, in many others FGF-23 levels are within the range found in control subjects (Rendina et al, 2006).

1.4.3 Tumour-induced osteomalacia (TIO)

In this rare syndrome there is a combination of acquired renal phosphate wasting, low serum phosphate levels, low or low-normal calcitriol levels (calcitriol production would normally be stimulated by low serum phosphate, so normal levels are unphysiological in this situation) and a tumour, usually of mesenchymal origin. Surgical removal of the tumour results in rapid reversal of the renal abnormalities and normalisation of phosphate balance. The syndrome has obvious similarities to the genetic forms of hypophosphataemic rickets. It is unclear, and this should be borne in mind throughout the discussion below, whether there is one or several circulating phosphaturic factors and whether these factors are the same or different in different patients.

Although most cases of TIO are associated with mesenchymal tumours of relatively low malignant potential, there are case reports of hypophosphataemia and renal phosphate leak associated with small cell lung cancer (Robin et al, 1994), acute myeloid leukaemia (Liamis & Elisaf, 2000) and multiple myeloma (Dash et al, 1997). As these patients are

often terminally ill, little clinical information is available and the presumed circulating factors causing phosphaturia are entirely uncharacterised. It is of note, however, that both the myeloma patients had remission of their phosphaturia after chemotherapy treatment.

1.4.3.1 Pathogenesis of tumour-induced osteomalacia

Jonsson and co-workers tested extracts of tumours removed from four patients with oncogenic (tumour-induced) osteomalacia for their ability to inhibit phosphate uptake by opossum kidney (OK) cells (Jonsson et al, 2001). They found a range of phosphate uptake inhibition, with uptake inhibited by 55% by one active extract, by about 30% by two others and no significant inhibition by the remaining extract. Interestingly, although normal kidney extract produced no change in phosphate uptake in their assay, liver extracts produced 20% inhibition which reached statistical significance (no comment is made in the paper about this finding). Normal bone was not tried in this assay, although the tumours were of bone/soft tissue origin and so this would have been an appropriate control tissue. Further studies on the most active extract suggested that the active factor was of low molecular weight and resistant to trypsin and protease K. An earlier study (Cai et al, 1994), using medium obtained from tumour-cell cultures derived from a single patient, also demonstrated inhibition of phosphate uptake by OK cells.The active substance had a molecular weight between 8kD and 25kD and could be degraded by heating to boiling point. A study on conditioned medium from a culture of tumour-induced osteomalacia cells (Nelson et al, 2001) also suggested that the circulating factor was small (in this case ~5kD). These latter workers found no change in the amount of mRNA for the type 2 sodium-phosphate transporter in OK cells exposed to the conditioned medium, despite changes in phosphate transport. However, as control of activity of the type 2 sodium-phosphate transporter is usually primarily at the membrane insertion level rather than at the mRNA level, this finding does not disprove an involvement of this transporter in the actions of the phosphaturic factor.

1.4.3.2 Identification of circulating factors in tumour-induced osteomalacia

De Beur and co-workers used a technique called serial analysis of gene expression ('SAGE') to study over-expressed genes in tumour-induced osteomalacia (De Beur et al, 2002). This technique involves creating cDNA from the mRNA of tumour samples, then creating a 'SAGE tag' or small fragment by restriction enzyme digestion. These tags are then amplified, cloned and sequenced. Tags of 10 base pairs are short enough to be sequenced quickly but long enough to be unique. The frequency of tags is related to the frequency of the original mRNA message in the sample. These workers used other tumours, histologically similar to those involved in tumour-induced osteomalacia (TIO) but not associated with hypophosphataemia, as controls; they compared the tag frequency in osteomalacia-associated samples with that in these other tumours. Three osteomalacia-associated tumours were studied and a large number of over-expressed genes was found. The genes with the highest ratio of expression in TIO tumours compared with other tumours were those encoding Matrix Extracellular Phosphoglycoprotein (MEPE, a protein previously isolated from a patient with TIO and discussed in greater detail below in section 1.4.3.3.4), a novel protein called Frizzled-Related Protein-4 (FRP-4 , see section 1.4.3.3.3) and Dentin Matrix Protein-1 (DMP-1). Other genes so identified included those encoding the phosphate transporter pit1/glvr-1 (NaPT3), PHEX and FGF-23, along with a number of genes never previously identified. Many of these genes have subsequently emerged as important in the regulation of phosphate balance. These findings suggest that the phosphaturia of tumour-induced osteomalacia may involve the over-expression of a considerable number of genes, which may affect each other in complex ways. Interestingly, MEPE was the most over-expressed message in one tumour sample and one of the top five in both other tumour samples, while it was not seen at all in either of the control samples, thus being the most impressive positive result (though the authors chose to emphasise FRP-4).

A technically different but intellectually similar approach was followed by Shimada's group (Shimada et al, 2001). These workers constructed a cDNA library from frozen tumour tissue taken at operation from a patient with tumour-induced osteomalacia. The resection of the tumour had cured the patient, providing good evidence that this tumour was producing a phosphaturic factor. They also constructed a cDNA library from the

adjacent normal bone tissue. Probes were constructed against tumour and normal bone, and plaques generated from the tumour cDNA library were screened with both sets of probes. Any plaque hybridizing only with the tumour probes and not with the bone probes was considered as possibly significant and was therefore sequenced. In total, 456 clones were sequenced and the most frequent five sequences found were: *DMP1*, Heat Shock Protein 90 (*HSP-90*), osteopontin, *FGF-23* and *MEPE*, in that order.

1.4.3.3 Novel factors identified in tumour-induced osteomalacia

1.4.3.3.1 Fibroblast growth factor-23 (FGF-23)

FGF-23 was discovered by Yamashita and colleagues in 2000 (Yamashita et al, 2000). These workers were looking for genes similar to previously recognised fibroblast growth factors, and detected a sequence in the GenBank Nucleotide Sequence Database with marked similarities to other members of the family. They were able to find a cDNA for this novel protein in a library created from mouse skin poly(A) RNA, and they then amplified and sequenced this cDNA. As it was the 23rd member of the FGF family to be identified, they named the protein Fgf-23. They found *Fgf-23* mRNA expression in adult mouse brain, thymus and small intestine, but not in kidney, muscle or spleen (nor, interestingly, in skin, even though the sequence was first found in a skin cDNA library; bone was not tested). At that time any function of Fgf-23 was purely speculative.

After Shimada's group had suggested that FGF-23 was expressed in cells from a tumour causing TIO (see above), Bowe and colleagues found that FGF-23 inhibited phosphate transport in OK cells (Bowe et al, 2001). They used experimentally conditioned medium from COS-7 cells transiently transfected with cDNA for FGF-23, mutant FGF-23 from a patient with ADHR, human PTH, osteopontin or DMP-1. Forty-eight hours after transfection, the medium was used to bathe OK cells, and phosphate uptake over the subsequent 30 minutes was determined by accumulation of ^{32}P. Medium from cells transfected with osteopontin or DMP-1 had no effect on phosphate uptake. The medium

assumed to contain either form of FGF-23 caused a 60% reduction in phosphate uptake, slightly greater than the reduction seen with the presumed PTH-containing medium.

In contrast, Shimada et al were unable to demonstrate an effect of purified FGF-23 on phosphate transport in OK cells (Shimada et al, 2001). It has subsequently been shown that FGF-23 has an effect on these cells only when heparin-like compounds are also present (Yamashita et al, 2002). This later paper identified the receptor FGFr3c as being important in the interaction between FGF-23 and OK cells and found evidence that the effects (reduction in phosphate transport, presumably by internalisation of sodium-phosphate co-transporters) were mediated via the mitogen-activated protein kinase (MAPK) pathway and occurred without changes in sodium-phosphate co-transporter mRNA levels, at least in the short term.

Klotho

The Klotho gene was initially identified as a gene which might have importance in our understanding of ageing, since mice with abnormalities of this gene were found to have osteoporosis, vascular calcification and other diseases found in the old. They also had poor growth, short life-span and very abnormal calcium/phosphate metabolism (markedly elevated plasma phosphate, markedly elevated vitamin D, FGF-23 levels over 100 times normal, normal PTH levels). The very high levels of serum phosphate and vitamin D are similar to those found in knock-outs of Fgf-*23* (see below) and further investigation has now demonstrated that *Klotho* interacts with the FGF receptor 3c to convert this general FGF receptor into one specific for FGF-23 (Urakawa et al, 2006). In the absence of *Klotho,* FGF-23 does not seem to be able to interact with cells. Other functions of the *Klotho* gene remain speculative; it now seems possible that the 'premature ageing' phenotype caused by abnormal expression of the gene may be mainly a result of greatly increased levels of calcitriol in these animals (Lanske & Razzaque, 2007), although, interestingly, over-expression of *Klotho* in mice lengthens their lifespan.

Genetic manipulation in animals

FGF-23 has been shown to decrease serum phosphate, without altering serum calcium, when given by intravenous injection to mice; this effect took approximately 9 hours to become apparent (Shimada et al, 2004*a*). Calcitriol levels fell more rapidly (within 3

hours), probably due to an effect of FGF-23 on the enzymes 1α-hydroxylase and 24-hydroxylase. It was also found that injection of vitamin D into mice could increase serum FGF-23 levels, suggesting that FGF-23 might act as a feedback inhibitor of vitamin D.

Shimada's group has also reported on transgenic mice over-expressing human *FGF-23* (Shimida et al, 2004*b*). These animals grew as well as their litter-mates until weaning, but subsequently more slowly. They had small spleens and thymus glands and females were usually infertile, but the brain, liver, kidneys and skeletal muscle were apparently normal. The *FGF-23* construct was widely expressed, but interestingly not in the liver, the organ of highest mRNA expression in humans. The paper does not state whether these animals expressed *FGF-23* in bone. Serum phosphate was about half normal in transgenic animals, with a large increase in fractional excretion of phosphate (FE_{Pi}). Levels of calcitriol and PTH were both very low; serum calcium was slightly low. The low calcitriol levels can be explained by the previously demonstrated effect of FGF-23 on 1α-hydroxylase protein (Shimada et al, 2001), but the low PTH levels are puzzling, as one might expect them to rise in the presence of low calcium and vitamin D levels. (Patients with tumour-induced osteomalacia usually have normal PTH levels.) Immunohistochemical study of the kidneys showed a low level of NaPT2a in the proximal tubules of the transgenic animals, suggesting that the renal phosphate leak was due to a reduction in the amount of this protein.

In another transgenic mouse model, a different group of workers expressed human *FGF-23* in osteoblasts under the control of the α1(I) collagen promoter (Larsson et al, 2004). These animals had markedly elevated circulating FGF-23 levels and low serum phosphate concentrations. However, they had raised PTH and normal calcitriol levels. Bone density was greatly reduced in the transgenic animals and examination of the renal tubules disclosed reduced levels of NaPT2a, and also of NaPT2c and NaPT1. These differences between the two transgenic models are striking, unexplained and not commented on in the later report.

In a third transgenic mouse model, a mutant form of *FGF-23* derived from a patient with ADHR was over-expressed in the liver of transgenic animals (Bai et al, 2004). High levels of circulating FGF-23 were confirmed and animals developed hypophosphataemia, phosphaturia and low calcitriol levels. They had marked hyperparathyroidism and the activity of the 1α-hydroxylase enzyme was lower than in control animals.

Human *FGF-23* has been expressed temporarily in rats by intravenous injection of naked DNA plasmids (Segawa et al, 2003). Under these circumstances the DNA is taken up, mainly by the liver, transcribed and the FGF-23 protein expressed for 2-4 weeks. Segawa and colleagues injected both a normal *FGF-23* DNA sequence and one mutated at a consensus proteolytic site, similar to sequences seen in ADHR and known to prolong the half-life of FGF-23 in the circulation. Analysis of the animals' serum indicated that the mutated, but not the normal, DNA injection led to greatly elevated circulating FGF-23 levels. Animals on a normal diet had reduced serum phosphate after injection of the mutated DNA, and also reduced vitamin D levels. Animals fed a low-phosphate diet had reduced serum phosphate compared with those on a normal diet, and indeed a lower serum phosphate than was seen in the animals on a normal phosphate diet injected with the mutant DNA; there was no further change when they were injected with mutated DNA, although again they had much reduced vitamin D levels. These results confirm a connection between raised FGF-23 levels and low serum phosphate, but the absence of any effect in phosphate-deficient animals suggests that phosphaturic effects of FGF-23 can be overridden under these circumstances. Analysis of sodium-phosphate co-transporter numbers by Western blotting of renal extracts showed that animals injected with the mutant *FGF-23* had greatly reduced levels of NaPT2a and (even more strikingly) NaPT2c, with no change seen in NaPT1; this was seen both in animals on the normal diet and in those on the low-phosphate diet. (This does not seem consistent with the absence of an effect of the injection on phosphate excretion in mice on a low phosphate diet, but is reported without comment in the paper.)

A knock-out mouse strain lacking the *Fgf-23* gene has been generated by Shimada et al (Shimada et al, 2004*b*). Homozygote *Fgf-23 -/-* mice were born at the expected Mendelian frequency, indicating that the absence of the FGF-23 protein does not significantly reduce embryonic survival. After birth, wild-type and *Fgf-23+/-* mice were indistinguishable, but there was growth retardation and shortened survival in *Fgf-23 -/-* mice. Serum phosphate was normal in the *Fgf-23* null mice at birth and at 6 days, but was subsequently significantly elevated; serum calcium was more modestly elevated from two weeks. The most striking biochemical abnormality was a greatly elevated vitamin D level in the *Fgf-23* null mice. There were also abnormalities in lipid and glucose metabolism. These results might be interpreted as suggesting that *Fgf-23* does not play a central role in

phosphate physiology *in utero* or in early life but that its absence is significant in adults, at least in mice.

Transgenic animals have made a significant contribution to our understanding of the physiology of FGF-23, but it is striking that different transgenic models show rather different patterns of disturbance to phosphate physiology; this underlines the fact the interpretation of data from transgenic animals can sometimes be difficult.

Clinical studies

FGF-23 has been assayed in patients with TIO and XLH (Jonsson et al, 2003). In most (but not all) patients with TIO, FGF-23 levels were markedly elevated. In patients with XLH, FGF-23 concentrations were on average significantly elevated, but many patients had levels within the normal range; although the authors point out that all XLH patients were on treatment (with phosphate and/or vitamin D), it is unclear whether this would be expected to alter FGF-23 levels, particularly as almost all the XLH patients had very low serum phosphates.

It has been shown that dialysis patients have greatly elevated levels of circulating FGF-23 (Larsson et al, 2003), whilst the levels in patients with chronic renal failure nearing dialysis are raised, but not to the same extent. It is not clear whether these results simply represent accumulation of FGF-23 in renal failure or are in part due to increased production of FGF-23, perhaps in response to hyperphosphataemia. The magnitude of the concentration difference between healthy individuals and dialysis patients (more than 1000-fold) suggests that accumulation of FGF-23 in renal failure must be a large part of the explanation. A study of serum FGF-23 levels in men on dialysis (Imanishi et al, 2004) showed a significant correlation between serum inorganic phosphate and FGF-23 levels, but FGF-23 levels were grossly elevated even in those dialysis patients with normal or low serum phosphate concentrations and the scatter of the results is considerable. A small sub-study, not reported in detail, looked for changes in FGF-23 levels in healthy men in response to either dietary phosphate loading or phosphate deprivation (Larsson et al, 2003). Although significant changes in FE_{Pi} were induced by these dietary manipulations, there were no changes in FGF-23 levels (nor, in agreement with previous studies, in either PTH or serum phosphate levels).

Another small study reports levels of FGF-23 in patients with primary hyperparathyroidism (in which the mean level of FGF-23 was slightly elevated compared with controls) and in patients with humoral hypercalcaemia of malignancy (in which FGF-23 levels were more convincingly elevated) (Singh & Kumar, 2003); in neither patient group could a relationship between serum phosphate and FGF-23 levels be demonstrated. A second, larger, study of patients with primary hyperparathyroidism (Yamashita et al, 2004) also failed to show a correlation between serum phosphate and FGF-23 levels; the main predictor of FGF-23 levels in these patients was creatinine clearance. This study reported that serum FGF-23 concentrations were not different between normal controls and patients with primary hyperparathyroidism and normal renal function. Multiple regression analysis suggested that serum calcium levels and creatinine clearance, but not serum phosphate or PTH levels, were correlated with FGF-23 levels. Further, the levels of FGF-23 did not fall after parathyroidectomy despite normalisation of serum calcium. Many of the patients with low serum phosphate levels had FGF-23 levels within the normal range. This study does not support the case for FGF-23 as a regulator of serum phosphate levels in patients with hyperparathyroidism. In patients with hypoparathyroidism, there is hyperphosphataemia and hypocalcaemia. A study (again very small) of such patients found that they had raised levels of FGF-23 compared with controls (Gupta et al, 2004). The authors attributed this to hyperphosphataemia, but other possible explanations might include significant influences of vitamin D treatment, renal function, age or removal of a suppressive effect of PTH, none of which were assessed in the report.

Slightly surprisingly, in view of the wide acceptance of the hypothesis that PHEX mutations cause an excess of FGF-23, the data for raised FGF-23 levels in X-linked hypophosphataemic rickets (XLH) are not very conclusive. Initial studies (Yamazaki et al, 2002) in 6 patients with XLH showed average serum FGF-23 levels to be around 150 ng/L, with the normal range given as 8-53 ng/L. One of the six patients had an unequivocally normal value and two others had borderline elevation only. In addition, it is not clear whether any account was taken of the possibility of renal impairment in these patients. Given the strong relationship between FGF-23 and renal impairment noted above, this might have had a major influence on the results. A more recent paper, concerned mainly with assessing different methods of measuring FGF-23 levels in human

serum, found 5/29 patients with XLH had FGF-23 levels within the normal range (Ito et al, 2005, AP565).

1.4.3.3.2 Fibroblast growth factor-7 (FGF-7)

FGF-7 was identified as a potential cause of phosphaturia in tumour-induced osteomalacia by Carpenter et al (Carpenter et al, 2005). These workers established tissue cultures of cells grown from the tumours of two patients with TIO. Conditioned medium from these cultures was shown to inhibit phosphate transport in OK cells; some cultures subsequently lost this ability as the cells were repeatedly grown to confluence. Gene expression in cells that continued to elaborate a phosphate transport inhibiting substance was compared with gene expression in cells that had lost this effect (by RNA expression analysis using reverse transcription followed by endonuclease digestion, gel electrophoresis and fluorescence detection). By this means, FGF-7 was found to be greatly over-expressed in cells which inhibited phosphate transport. Subsequent renal clearance experiments have shown that FGF-7 increases renal phosphate clearance when given intravenously to rats (Shaikh et al, 2007). No information on levels of FGF-7 in human health or disease is yet available.

1.4.3.3.3 Frizzled-related protein-4 (FRP-4)

As indicated above, this protein was identified by Kumar's group when they examined four tumours by serial analysis of gene expression (SAGE) to identify genes up-regulated in tumour-induced osteomalacia (De Beur et al, 2002). It had not been identified as significant in the earlier studies of Rowe et al (2000) or Shimada et al (2001). Kumar's group also identified MEPE by this technique but were less interested in it. They subsequently cloned and expressed human FRP-4 in an insect expression system and performed experiments to determine whether it altered renal phosphate handling. Clearance experiments in rats demonstrated that acute FRP-4 infusions caused an increase in fractional phosphate excretion without significant changes in serum phosphate (Berndt et al, 2003). This was shown to be due to internalisation of the NaPT2a transporter in the renal tubular brush-border membrane, as assessed by immunoblotting of membrane

vesicles (Berndt et al, 2006). Longer-term infusions into rats (eight hours) caused a fall in serum phosphate and a marked increase in fractional phosphate excretion. One report, from the same group that originally isolated the protein, states that serum FRP-4 levels are not altered in patients with chronic kidney disease, or in those with renal transplants in whom phosphate balance is abnormal (Pande et al, 2006). All that can be said at present is that FRP-4 is a candidate phosphatonin about which little is known other than that it is expressed by tumours causing TIO and it is phosphaturic in rats.

1.4.3.3.4 Matrix extracellular phosphoglycoprotein (MEPE)

Matrix extracellular phosphoglycoprotein (MEPE) was first isolated in 2000 from tumour-derived tissue from a patient with tumour-induced osteomalacia (Rowe at al 2000). Messenger RNA was obtained from resected tumour and reverse transcribed to cDNA. This was then cloned into a bacteriophage vector after the addition of suitable primers, and the clones amplified by use of *E. coli*. The patient's preoperative serum was used to generate antisera by injection into rabbits, and the resulting rabbit antiserum was extensively pre-absorbed with normal human serum and with *E. coli* lysate. This antiserum was used to screen the bacteriophage library; the rationale was that proteins circulating pre-operatively in the patient with tumour-induced osteomalacia, but which were not present in ordinary human serum, would be recognised. Bacteriophage plaques that were recognised by the preabsorbed antiserum were transformed into plasmids, transfected into *E. coli* and sequenced. The entire procedure outlined above gave rise to nine clones. Three were human clusterin sequences and the remaining six were *MEPE* sequences of differing lengths, between 227 and 430 residues. The gene sequence suggests a 525 amino-acid protein, with a number of sequences which might be cleaved by proteases such as PHEX. The sequence contains no obvious transmembrane sections and suggests a protein which might be secreted.

MEPE was independently described by another group as the product of a bone-specific gene, which they called osteoblast/osteocyte factor 45 or OF45 (Petersen et al, 2000). These workers used the fact that primary bone marrow cultures from the rat can be induced to differentiate into osteoblasts by the addition of differentiation factors such as

dexamethasone whereas, without the addition of such factors, primary bone marrow cultures do not contain osteoblasts. By using subtractive hybridisation of cDNA from dexamethasone-treated cultures *vs.* cDNA from untreated cultures, it is possible to obtain a cDNA library greatly enriched for genes induced during osteoblast differentiation. This technique produced a large number of clones of which the most frequent was osteopontin, a gene already known to be induced during osteoblast differentiation. This provides evidence that the technique is able to identify genes of importance in osteoblast differentiation. The second most common clone found, in 17% of all sequences, was a previously novel protein now known as MEPE.

Mouse *Mepe* was subsequently isolated from a bone cDNA library (Argiro et al, 2001). Sequence analysis showed 56% overall homology between mouse and human cDNAs, but with large areas of close sequence homology. A section of 75 amino acids is present in the human sequence but absent from the mouse sequence. Northern blot analysis identified *Mepe* mRNA in bone, but not in brain, heart, liver, lung, muscle or kidney. MEPE expression was markedly increased in mineralising cultures of bone marrow cells, as opposed to non-mineralising cultures. Finally, *Mepe* expression in the bones and osteoblasts of *Hyp* mice (that is, the mouse homologue of X-linked hypophosphataemic rickets, known to be due to mutations in the mouse *Phex* gene; see section 1.4.1.1) was three times that seen in normal mice, suggesting the possibility of an interaction between MEPE and PHEX.

The genes responsible for a number of inherited dental syndromes in humans are known to be located on chromosome 4q21; MacDougall et al noted that the *MEPE* gene is also found in this area and therefore investigated its expression in teeth (MacDougall et al, 2002). They performed PCR analysis of a mouse tooth cDNA library and of cDNA generated by an odontoblast cell line and demonstrated *Mepe* expression in both, strongly suggesting that MEPE is expressed in developing teeth.

Researchers employed by Pfizer have succeeded in creating a *Mepe* knock-out mouse (Gowen et al, 2003). Unfortunately, these workers were mainly interested in bone morphology and reported only briefly about other aspects of these mice. They found that

homozygote and heterozygote knock-out animals appeared healthy, grew normally and were fertile. Homozygous knock-out mice had increased bone mass and increased osteoblast numbers, by a mechanism yet to be characterised; there was also some change in bone structure in heterozygotes. Homozygous animals were reported as having normal serum calcium and phosphate concentrations, although there were modest differences between *Mepe* knock-outs and wild-type controls and the variability between animals was large. Further, serum phosphate was determined at only one time point (one year of age) and there was no determination of serum PTH. These workers' conclusion that mineral metabolism in the knock-outs is normal therefore requires further assessment.

Fisher and Fedarko, in a mainly theoretical paper (Fisher and Fedarko, 2003), put forward the hypothesis that *MEPE* belongs to a gene family which they dubbed 'SIBLING' (Small Integrin Binding Ligand, N-linked Glycoprotein). Other described gene families are linked by sequence similarities and are usually assumed to have come about by gene duplication and subsequent divergent evolution, but this family is linked in terms of their chromosomal location and gene structure. The primary sequences of the six genes they place in the SIBLING family are unrelated, so a common origin with subsequent divergent evolution is ruled out. It is unclear what the authors feel is the implication of similarity of secondary features (see below) in the absence of primary sequence similarity; this might represent similar function of proteins with different evolutionary origins. I am unconvinced that the term 'gene family' is appropriately applied to this group of proteins and feel it is better reserved for genes with similar (and therefore presumably related, in an evolutionary sense) DNA sequences. The paper presents data comparing the exon structure of six genes coded on the long arm of chromosome four, including *MEPE*, pointing out that the number and structure of exons is similar and that there are areas of potentially important sequence similarity, including the integrin-binding tripeptide ArgGlyAsp, a proline-rich region and a consensus sequence for casein kinase II phosphorylation. The authors therefore suggested that MEPE - in common with the other proteins in the SIBLING family - would have a role as a soluble protein involved in extracellular matrix accumulation and calcification. In a more recent publication these authors have dropped one of the original six SIBLINGs (Ogbureke & Fisher, 2004). One of the genes placed in the SIBLING group is Dentin Matrix Protein-1 (*DMP-1*); mutations in this gene cause autosomal recessive hypophosphataemic rickets (ARHR; see section

1.4.1.4). Work on this protein suggests that it is cleaved by PHEX at multiple points, and that it is the cleaved fragments which have biological activity; PHEX might act on MEPE in a similar way, producing active fragments (Qin et al, 2003). *DMP-1* and three of the other genes placed in the SIBLING group have been shown to interact with specific matrix metalloproteinases (MMPs) (Ogbureke & Fisher, 2004). It is not known whether MEPE has a specific MMP partner. *MEPE* mRNA was found in human kidney homogenates by RT-PCR (note it was not found in rat kidney by Agiro's group, see above; this may represent a species difference or may be due to the different techniques used, PCR usually being more sensitive than Northern blotting), and MEPE protein was found by immunohistochemisty in the proximal tubule of the kidney in monkeys; staining was most marked on the luminal surface or luminal half of proximal tubular cells (Ogbureke and Fisher, 2005). The latter observations are consistent with binding of filtered MEPE to the luminal side of proximal tubular cells, particularly as the staining was strongest in the proximal convoluted tubule and less so in the straight tubule; there was no staining in the loop of Henle or the distal tubule. Ogbureke and Fisher hypothesise that the SIBLING proteins are involved in cell-cell interactions; since no MMP for MEPE has been identified, the role of MEPE in this remains speculative. These workers have also found *Mepe* mRNA (as well as RNA for other members of the SIBLING group) in mouse salivary glands; what it is doing there is unknown (Ogbureke and Fisher, 2004).

A report by Rowe and colleagues (Rowe et al, 2004) gives some information on the effects of MEPE on renal phosphate handling. These workers used full-length human MEPE and injected it intraperitoneally into mice. PTH was used as a positive control. Two different doses of MEPE were used. Serum phosphate was not altered after 7 hours in any group but after 30 hours was significantly lower in animals given PTH or the higher dose of MEPE than in saline-injected control animals. Urinary excretion of phosphate was measured, but only in pooled samples, and 'fractional excretion of phosphate' was determined on the basis of measurements of creatinine clearance as an assessment of GFR. As determined in this (less-than-satisfactory) way, fractional phosphate excretion was increased by both MEPE and PTH. Although these results are suggestive of an effect of MEPE on tubular phosphate handling, a more rigorous assessment is required.

Full-length MEPE has a molecular weight of about 44kD, and it is therefore possible that there are a number of active fragments, possibly with different activities. In this context, Liu and co-workers (Liu et al, 2004) have shown that a small (23 amino-acid) fragment from the centre of the full-length *MEPE* sequence can enhance dental pulp cell proliferation. They have called this fragment Dentonin. This report clearly leaves open the function of the remaining portions of the *MEPE* sequence. Another group (Hayashibara et al, 2004) studied the effects of the same peptide on bone. (Not being interested in teeth, they did not call it Dentonin.) They found that the peptide stimulated bone formation not only in organ culture but also *in vivo*, when injected directly next to the bone in mice. Subsequent work by this group has suggested that full-length MEPE decreases osteoclast formation in bone marrow cultures and might thus also have the effect of reducing bone breakdown or turnover (Hayashibara et al, 2007). Rowe and colleagues have speculated that a sequence at the C-terminal end of the MEPE molecule, called the ASARM motif ('acidic serine aspartate rich motif'), might be the fraction of the molecule which is active in the kidney and the bones; these workers demonstrated raised levels of proteins carrying this motif in both *Hyp* mice and humans with XLH (Bresler et al, 2004). In *Hyp* mice, the abnormal mineralization of the bones could be improved significantly by treating the animals with protease inhibitors; this improvement in bone histology was associated with a reduction (towards normal) of circulating ASARM peptides and of local bony accumulation of ASARM peptides (Rowe et al, 2006). It should be noted, however, that MEPE is not the only protein to carry this motif, so these data do not prove that the raised levels were MEPE-derived.

It has been shown that the *Hyp* phenotype is not rescued by transgenic expression of *Phex* restricted to osteoblasts; although bone mineralization is improved in these animals they remain hypophosphataemic (Liu et al, 2002; Bai et al, 2002). When this problem was approached from the other direction and two groups of mice were created, one with global *Phex* knock-out and the other with conditional (osteocalcin-promoted, therefore bone-limited) *Phex* inactivation, the two groups of mice had similar (low) levels of serum phosphate, equally reduced renal NaPT2a levels and osteomalacia (Yuan et al, 2008). FGF-23 levels were increased in both groups of mice, whereas *Mepe* mRNA (in bone samples) was greatly increased in animals with the global *Phex* mutation but not in those with the mutation limited to bone cells. The authors interpreted these findings as suggesting that FGF-23, but not MEPE, is involved in the hypophosphataemia in *Phex*-

mutated animals, since animals with *Phex* loss limited to bone cells had normal MEPE levels but low serum phosphates. In my view it is not certain that this is the correct conclusion, since it is hard to be sure that *Phex* inactivation in this model is limited to bones and alternative experimental approaches (as noted above) lead to contradictory results.

It has been shown that the PHEX protein is able to interact with MEPE (Rowe et al, 2005) and that the ASARM fragment of MEPE is the part of the molecule that mediates this interaction (Martin et al, 2008). Both MEPE protein and ASARM motifs (which can also come from other proteins, including DMP-1) are increased in *Hyp* mice, and this has led to the hypothesis that it is the ASARM peptides that lead to the bone mineralization defect in XLH (Martin et al, 2008); in view of the apparent phosphaturic effect of MEPE, either the full length molecule or the ASARM fraction might also be responsible in part for the phosphaturia found in this condition.

It has been demonstrated by Jain et al (2004) that MEPE circulates in normal humans. These workers had previous experience in the measurement of other members of the SIBLING family, including DMP-1, and had found that they circulate bound to complement factor H. They developed a protocol based on reducing and processing serum so as to separate these proteins, and found that it could be applied to detect MEPE using a competitive ELISA. They reported a very wide range of values for serum MEPE levels, which were approximately normally distributed in healthy adults, but declined with age. MEPE levels were negatively correlated with serum PTH and positively correlated with serum phosphate. The decline in MEPE levels with age might indicate a relationship to bone turnover, which also declines with age. It was also found that serum MEPE concentration was correlated with bone density in people over 60 (Jain et al, 2004). The positive correlation between MEPE levels and serum phosphate might be consistent with a physiological effect of MEPE on serum phosphate.

Preliminary work has suggested that MEPE fragments are elevated in the circulation of patients with XLH and also in those with tumour-induced osteomalacia (Jan de Beur et al, 2004). These authors developed ELISA assays for both full-length MEPE and a C-terminal fragment; they found that although full-length MEPE concentrations did not

differ between controls and patients with hypophosphataemic syndromes, the C-terminal fragment was markedly elevated in each group of patients. In two cases of TIO in which pre-and post-surgical samples were available, the C-terminal fragment concentration fell after surgery while serum phosphate normalised. These data are clearly consistent with MEPE being a circulating phosphaturic substance in these patients, although it should be noted that they have yet to appear in a full publication.

In summary, several lines of evidence lead towards the conclusion that MEPE might be a significant regulator of phosphate balance. The results from patients with tumour-induced osteomalacia suggest that it circulates in at least some of these patients and might have direct phosphaturic effects. Analysis of predicted molecular structure is consistent with an excreted, perhaps circulating, protein. The fact that MEPE can be found in normal human serum is supportive of a role in normal phosphate physiology, potentially linking the kidneys (the main controllers of overall phosphate balance) and the bones (the main source of accessible phosphate).

1.5 Summary paragraph

Phosphate homeostasis depends on inter-regulated control of the gastrointestinal tract (site of phosphate uptake), the kidney (site of phosphate excretion) and the bones (main store of inorganic phosphate). The PTH/vitamin D axis alone does not explain the complexity of normal phosphate homeostasis, nor does it account for many of the hereditary or acquired phosphaturic states. Phosphaturic syndromes have been shown to be caused by mutations in genes transcribed both in the kidney (*NaPT2c)* and the bones (*DMP-1*, *PHEX*, *FGF-23*). There is increasing interest in peptides (‘phosphatonins’) that might link bone biology and renal phosphate handling; MEPE is one such peptide.

1.6 Objectives of the present study

The present study aimed to establish and quantify the effect of MEPE on phosphate excretion in rats; to determine, using clearance experiments, whether the effect was tubular in origin; and to assess, using micropuncture experiments, which nephron segment is responsible. In order to measure phosphate concentrations in tubular fluid samples, it was necessary to develop a suitable microassay technique.

Chapter 2

Measurement of phosphate in biological samples using capillary electrophoresis

2.1 Introduction

2.1.1 Need for microanalysis

The study of renal tubular transport requires the ability to measure accurately ion concentrations in samples taken from single tubules as well as in plasma and urine. A wide variety of methods has been used over the past fifty years, but all have disadvantages. Sample collection and analysis are laborious, so methods allowing determination of several ion species in a single small volume sample would have advantages. My interest was primarily the measurement of phosphate in small volumes, but capillary electrophoresis also allows for analysis of other anions. This chapter describes the development of a method for the simultaneous analysis of anions at physiological concentrations in nanolitre volumes of tubular fluid or other biological samples.

Much micro analytical work is currently performed using radioisotopes. These have a number of advantages, particularly that they allow accurate determination in very small volumes. The health risks to workers using tritiated compounds (most commonly used for single-nephron GFR determination) are very low, but the bureaucratic burden is significant. The health risks of using ^{32}P are more significant and the bureaucratic and safety requirements correspondingly greater. Use of radioactivity is also expensive, so there is considerable interest in using non-radioactive means to perform microanalyses and to measure single-nephron GFR; in particular non-radioactive iothalamate has been proposed as an alternative to radioactive inulin (Capasso et al, 2002).

2.1.2 Methods used previously, their advantages and disadvantages

The routine analytical technique for the simultaneous determination of small anions is ion chromatography. This technique has been applied to samples with low ion concentrations but requires sample volumes of 30-600 μL (Meissner et al, 1999; Schminke & Suebert,

2000; Tanaka et al, 2000). These sample volumes are large compared with those collected during micropuncture experiments, in which tubular fluid volumes of significantly less than one microlitre are usually obtained.

For measurements in very small sample volumes, a range of different techniques has been employed. Bicarbonate concentration has been determined by calorimetry (Vurek et al, 1975) and fluorometry (Star, 1990) and by the use of microelectrodes (Yoshitomi & Fromter, 1984). Chloride concentration has been determined by spectroscopic (Levinson, 1976; Illsley & Verkman, 1987; Zhelyaskov et al, 2000), electrochemical (Yoshitomi & Fromter, 1984; Fujimoto & Kokota, 1976) and radio-isotopic methods (Isozaki et al, 1989; Ando et al, 1989). Phosphate has been measured in micropuncture samples using microcolorimetric techniques (Vurek, 1981; Woda et al, 2001), which were first developed by Chen and colleagues (Chen et al, 1956), and by the use of the radioisotope ^{32}P. Although all these techniques are capable of quantifying individual anion species in small volumes at physiological concentrations, they require specialized equipment and do not have the advantage of allowing simultaneous analysis of several anions.

2.2 Capillary electrophoresis

Capillary electrophoresis (CE) is a technique based on passing samples in solution along a narrow capillary and using the different mobilities of charged species in solution to provide separation of species. It can provide a method of separating and quantifying numerous anions quickly and with high efficiency (Jandic & Jones, 1991); moreover, it can be applied to a wide range of samples and analytes (Monnig & Kennedy, 1994). Because the method uses very small-bore capillaries, the sample volumes required are intrinsically small. For applications in which sample volume is a limiting factor, such as the analysis of tubular fluid, this is potentially a significant advantage. Dilution of the original sample is required to prevent overloading of the capillary, but the dilution factor can be altered to optimize detection limits.

Capillary electrophoresis has previously been used for the analysis of small ions in non-biological aqueous samples (reviewed in Doble & Haddad, 1999) and has been used to study airway surface fluid, a biological fluid available only in nanolitre volumes (Cowley et al, 1997). Furthermore, Wildman et al have previously published a method for the detection and quantification of multiple anions in urine using capillary electrophoresis

(Wildman et al, 1991). However, they did not publish on linearity of response, detection limits or repeatability. Moreover, their method required large sample volumes, such as are available in the analysis of human urine but not in that of laboratory rodents. Only one previous study has used capillary electrophoresis to analyse tubular fluid: colleagues in the renal laboratories at UCL have reported simultaneous analysis of cations in tubular fluid using conductivity detection (Stocking et al, 1999), but this is now a somewhat dated technique which has been largely supplanted by the more flexible and sensitive UV detection.

2.3 Methods

Capillary electrophoresis machines are available off the shelf from a variety of manufacturers; innovation in the use of these machines lies in the modification of the experimental conditions to optimise detection limits, separation of analytes and reproducibility. A large number of variables can be altered to improve analytical results.

A pictorial representation of a standard capillary electrophoresis apparatus is shown in Figure 2.1. Samples are place in vials on a rotating tray. The samples can be loaded into the capillary by a potential difference, by pressure injection or under the influence of gravity. They are then moved in the liquid phase along the hollow capillary, under the influence of a potential difference between the two ends of the capillary. Anions are surrounded by hydration spheres of water molecules and movement of these spheres towards the cathode creates bulk flow of fluid along the capillary. Different anions move at different speeds according to their charge and the size of their hydration spheres and are therefore separated. Peaks corresponding to different anion species are generated in this system by indirect UV detection, which allows a considerable number of different species to be seen at the expense of a lower sensitivity than can be found in direct detection systems. A high background signal is generated by including chromium ions in the run buffer; these provide a constant and high level of UV absorption. When other ions are present in the capillary they displace chromium, and lead to a reduced level of absorption and more UV light reaching the detector.

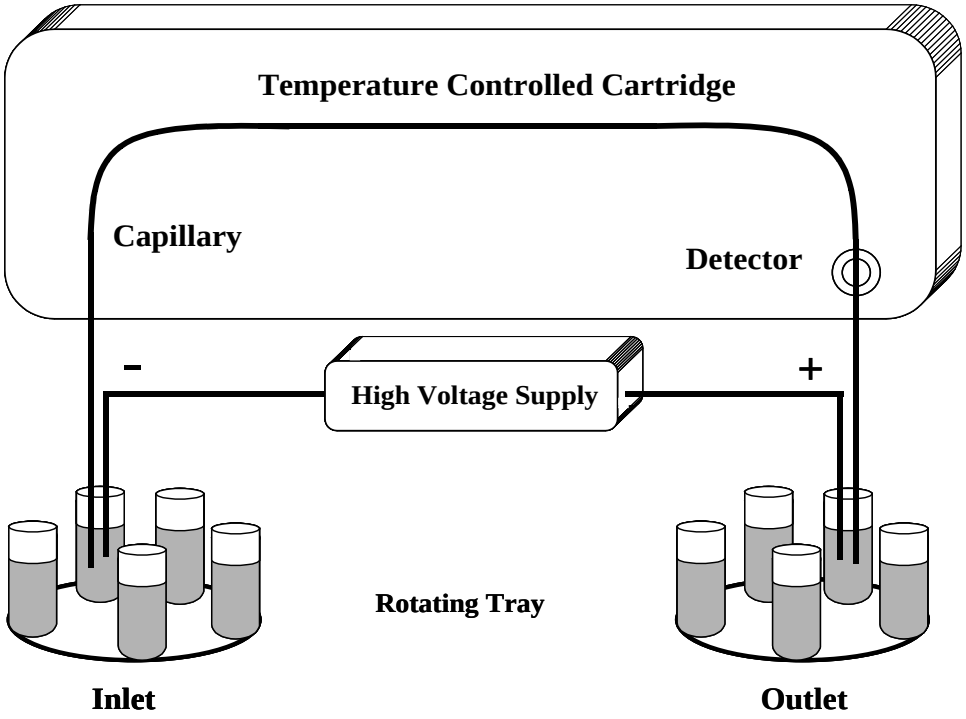


Figure 2.1 Capillary electrophoresis apparatus

In order to make interpretation easier, the output trace is inverted, so that troughs in detector output appear as peaks - this is purely a visual change and does not alter the data in any way. Detector output is plotted against time by the machine software, leading to traces showing peaks corresponding to each anion passing the detector. Peak area (in arbitary units) is proportional to anion concentration, a relationship demonstrated over several orders of magnitude later in this chapter for each of the important anions in tubular fluid and urine. Between samples the capillary is flushed either with the run buffer or with other solutions.

2.3.1 Capillary electrophoresis system

A Beckman P/ACE 5500 capillary electrophoresis system with a 254 nm filter and a photomultiplier tube UV detector, controlled by a Beckman P/ACE station version 1.1 (Beckman Coulter UK Ltd, High Wycombe, Bucks, UK), was used in all the CE experiments described in this thesis. This station and its software were also used for the analysis. The analysis capillary was fused silica: 50 μm internal diameter, 375 μm

external diameter, 37 cm total length [30 cm to detector] (Composite Metal Services Ltd, Hallow, Worcs, UK). The instrument parameters are shown in Table 2.1 and the run conditions in Table 2.2. Prior to each batch of measurements, the capillary was flushed with run buffer at 20 p.s.i. for 10 minutes, followed by a series of five blank samples. This procedure was found to be sufficient to produce a stable baseline, which was confirmed at the beginning of each day's work. Large volume samples, such as urines and calibration samples, were loaded into the machine in 0.5ml plastic vials. Tubular fluid samples were loaded in microvials, smaller vials which were conical in shape and held up to 50µl.

Separation parameters	
Voltage	8 kV
Temperature	25 ^{o}C
Detection parameters	
Wavelength	254 nm
Attenuated range	0.200 AU
Data collection rate	10 Hz
Rise time	1 sec
Signal type	Indirect

Table 2.1 Instrument parameters for capillary electrophoresis

Time point (min)	Function	Duration	Applied potential (kV)	Pressure (p.s.i)	Inlet vial	Outlet vial
Pre	Rinse	1min	0	20	Buffer	Waste
Pre	Inject	5 sec	0	0.5	Sample	Waste
0	Separate	4 min	8	0	Buffer	Buffer

Table 2.2 Capillary electrophoresis run conditions

2.4 Results and Discussion

2.4.1 Use of capillary electrophoresis to measure anions

The predominant anions in tubular fluid and urine are chloride, bicarbonate and phosphate. My initial work therefore concentrated on analysis and quantification of these anions, with a view to focusing on phosphate in the subsequent investigation.

As an example of the results produced by the capillary electrophoresis technique described above, an electropherogram demonstrating separation and detection of the anions chloride, phosphate and bicarbonate (and also nitrate and citrate) under the conditions described is shown in Figure 2.2. The trace shows a combined standard made up from commercially available solutions of these anions.

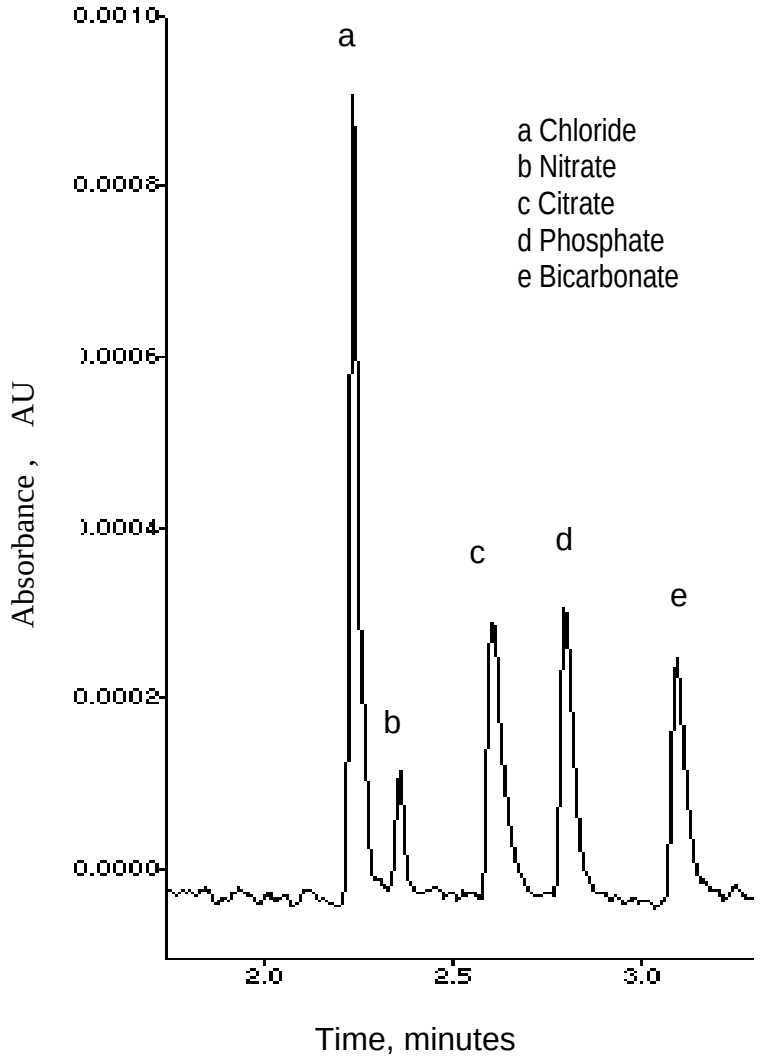


Figure 2.2 Separation of anions by capillary electrophoresis

A standard solution, containing chloride 50 mmol/L, nitrate 6 mmol/L, citrate 10 mmol/L, phosphate 11mmol/L and bicarbonate 15 mmol/L, was used.

Standard curves for phosphate and chloride covering the expected range in urine are shown in Figure 2.3 and Figure 2.4. For each of these anions the correlation coefficient (r) was 0.999. Figure 2.5 shows a standard curve for bicarbonate; the correlation coefficient was 0.997. These results demonstrate that the relationship between analyte concentration and peak area is linear through a wide range of concentrations for all three anions.

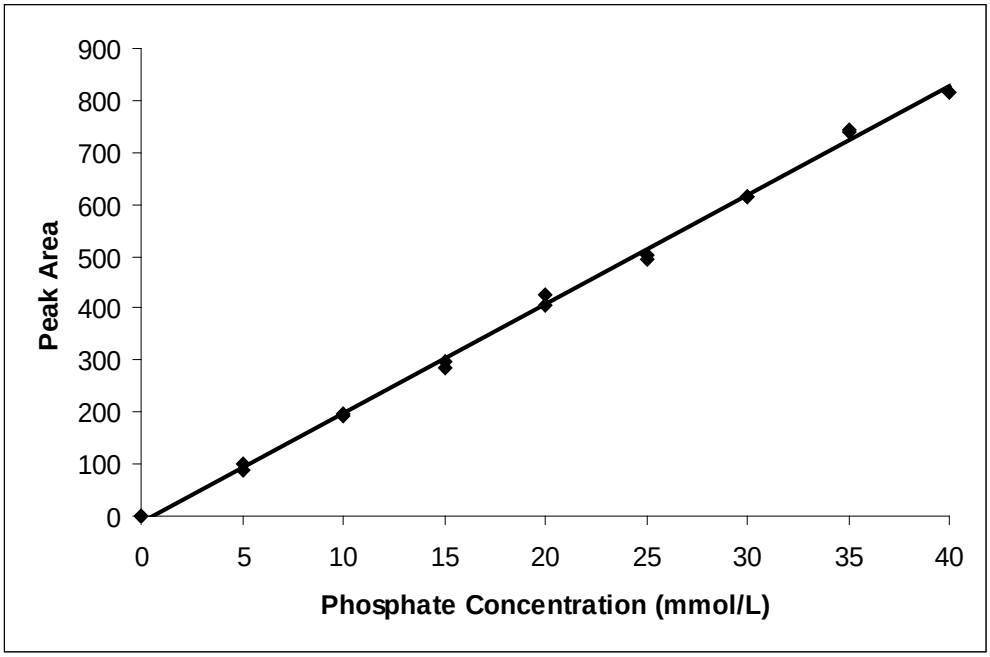


Figure 2.3 Calibration curve for phosphate

The solid line represents the best-fit line determined by linear regression. y=20.91x -10.08; r=0.999

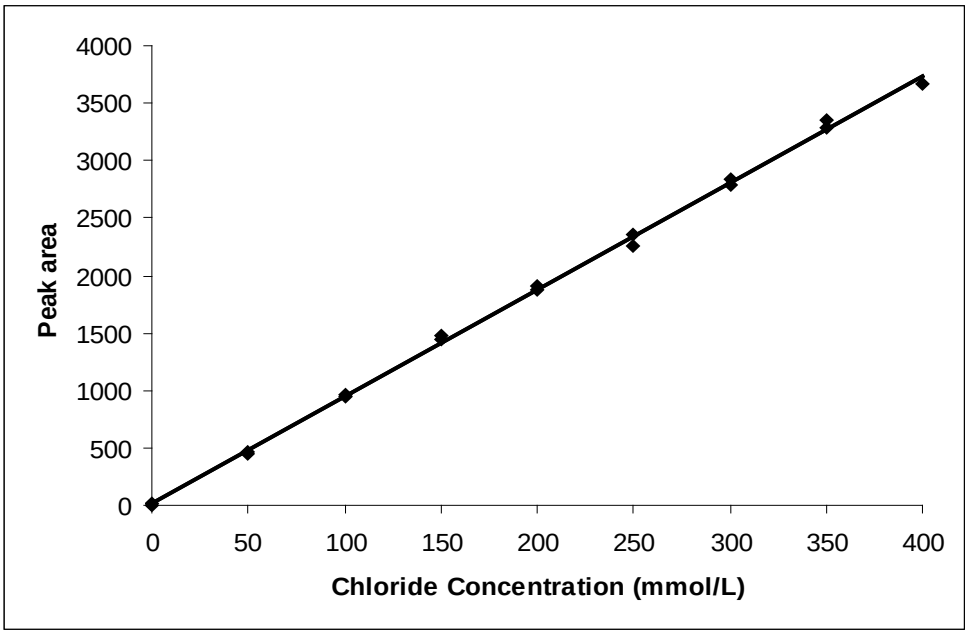


Figure 2.4 Calibration curve for chloride

y=9.30x+20.15; r=0.999

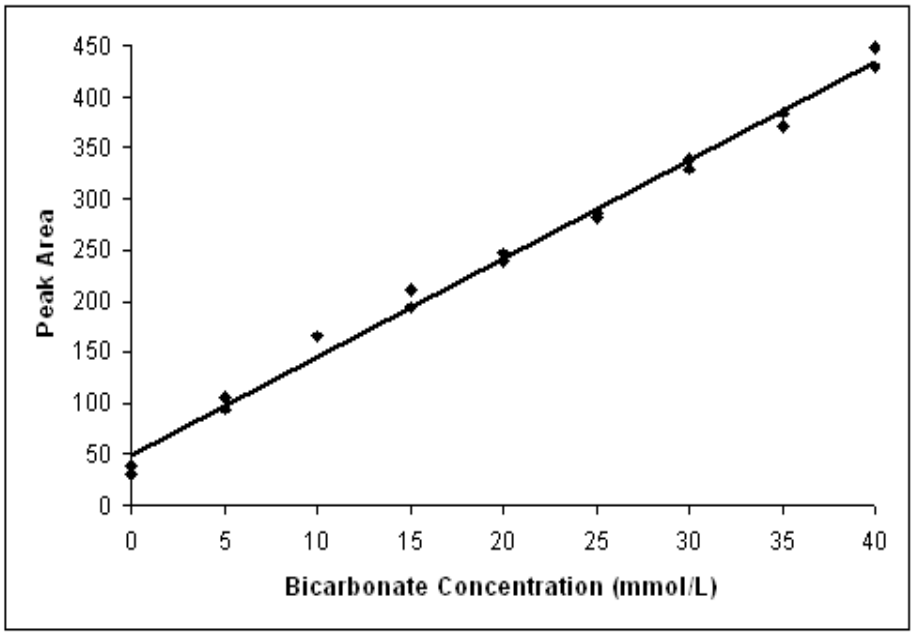


Figure 2.5 Calibration curve for bicarbonate

y=9.63x+48.6; r=0.997

The detection limit was calculated using the standard technique of determining the mean baseline noise plus three standard deviations of the baseline noise in a sample of de-ionized water. On this basis the detection limits in the original samples were 0.3 mmol/L for chloride, 0.3 mmol/L for phosphate and 1.8 mmol/L for bicarbonate, when samples were diluted by a factor of 200. These detection limits compare favourably with those of the NANOFLO system (see section 2.4.3.3.1 below). The actual volume of diluted sample loaded onto the column is only 8.0 nL (Beckman CE Expert Software); therefore, the amount of anion present in the capillary at the detection limit is 12 fmol for chloride, 12 fmol for phosphate and 72 fmol for bicarbonate. The use of the amount of substance in the analysed sample to define the detection limit is very widespread (for example, Zhelyaskov et al in their NANOFLO paper (Zhelyaskov et al, 2000) and Stocking et al in their capillary electrophoresis work (Stocking et al, 1999)). It can be rather misleading in the case of CE, because the system as a whole is unable to analyse amounts as small as this implies, as most of the sample remains in the vial. However, this value does represent a fundamental property of the detection system. In my view, the detection limits of micromethods are best expressed as concentrations in volumes, so as to eliminate ambiguity. For example, in this CE system when optimised for phosphate detection, we can comfortably detect 0.2mmol/L phosphate in a 30nL sample. Further work on reducing the vial size might allow a considerable improvement in practical detection limits, by reducing the proportion of the sample left behind in the sample vial.

The repeatability (intra-assay variation) of the method was determined by repeat analysis (n=6) of a commercially available quality control (chloride 76.6 mmol/L, phosphate 5.1 mmol/L and bicarbonate 3.4 mmol/L). The standard deviation in concentration was determined as 1.3 mmol/L for chloride, 0.3 mmol/L for phosphate and 0.4 mmol/L for bicarbonate. The standard deviation in migration time was determined as 0.084 s for chloride, 0.33 s for phosphate and 0.078 s for bicarbonate, in a total run time of 4 minutes. This small variability in migration time for a given species between runs means that peaks can be unambiguously identified by their migration time.

The reproducibility (inter-assay variation) of the method was determined using certified quality control material. Four levels of control material were analyzed with each calibration; the results are summarized in Table 2.3.

Analyte	Quality control material	Target mean, +/- SD (*mmol/L*)	Measured mean (*mmol/L*)	Standard deviation *(mmol/L)*
Cl	Sigma Urine level 1	79 (75-83)	81	4.0
	Sigma Urine level 2	220 (209-231)	228	21.0
	Accutrol Serum Normal	100 (89-111)	105	4.7
	Accutrol Serum Abnormal	113 (101-125)	116	10.4
PO_4	Sigma Urine level 1	11 (8-14)	11.8	1.0
	Sigma Urine level 2	28 (21-35)	29.2	3.2
	Accutrol Serum Normal	1.2 (0.9-1.4)	1.1	0.7
	Accutrol Serum Abnormal	2.3 (2.0-2.6)	2.4	0.5
HCO_3	Accutrol Serum Normal	16 (10-22)	16.6	2.8
	Accutrol Serum Abnormal	26 (18-34)	29.6	4.1

Table 2.3 Reproducibility of capillary electrophoresis results

For the urine control material, n=14, and for the serum control material, n=11.

2.4.2 Measurement of anions in urine

A pair of overlaid traces showing analysis of actual urine samples is shown in Figure 2.6. These samples came from one animal during a clearance experiment, during which MEPE

was infused. As can be seen from these traces, the predominant anion found in urine samples is chloride, with variable amounts of phosphate (in this case very little before, and significantly more after, MEPE infusion, see chapter 3).

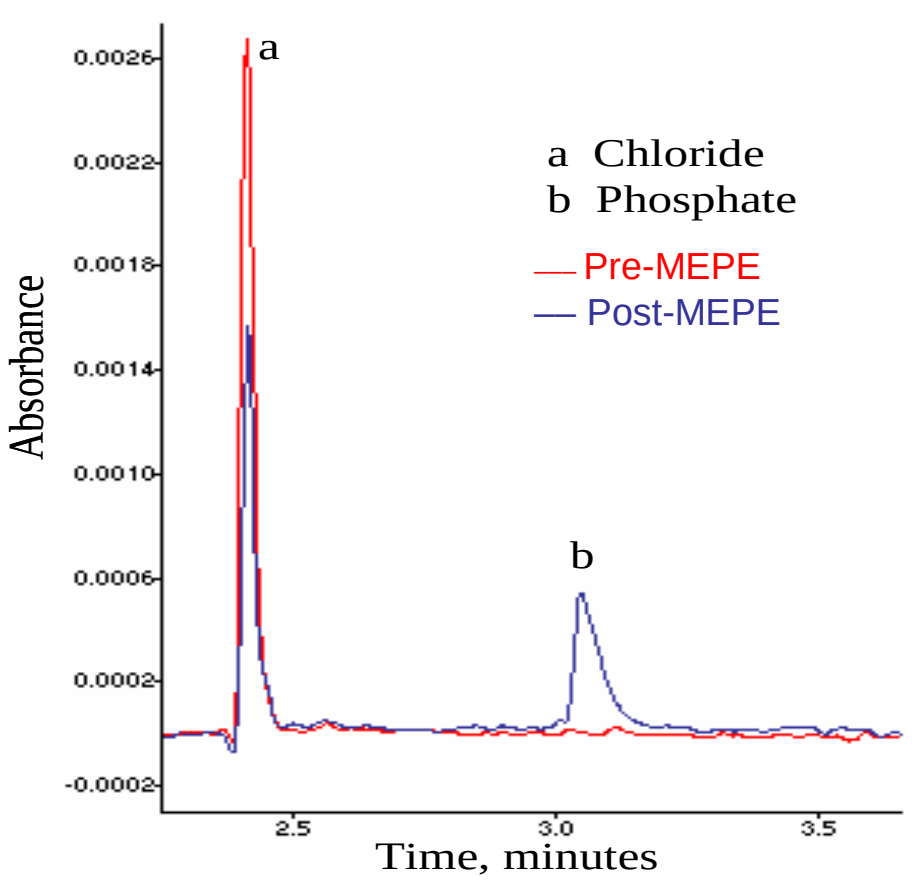


Figure 2.6 Capillary electrophoresis traces of rat urine

Two traces taken at different time points from the same animal are overlaid.

2.4.2.1 Unknown peaks

Various unknown peaks are seen in some of the urine samples, as well as the chloride and phosphate peaks identified above. These can be unambiguously identified only by addition of standard solutions of possible anions and demonstration of co-migration of the

resulting peaks. Despite performing this procedure with a range of plausible anions (including urate, oxalate, citrate, sulphate and sulphite), some peaks remain unidentified, although the presence of significant amounts of citrate and oxalate in rat urine was confirmed. Other workers also frequently find unidentified peaks (Wildman et al, 1991; Capasso et al, 2002).

2.4.2.2 Extension of the capillary electrophoresis technique to measurement of other anions

2.4.2.2.1 Iothalamate

Radiolabelled iothalamate has been widely used as a marker of GFR, as this substance is cleared from the plasma by glomerular filtration without significant reabsorption or secretion by the renal tubules (Levey et al, 1993). Direct comparison with DTPA and inulin clearances, two other standard techniques for determining GFR, showed virtually identical results in patients with renal disease (Perrone et al, 1990). Because of the advantages of avoiding radioactivity use, there has been interest in using non-radiolabelled iothalamate, measuring iothalamate in the urine by a range of techniques, e.g. high-performance liquid chromatography (HPLC) (Agarwal, 2003). Iothalamate determination by CE has also been used to determine GFR in humans (Wilson et al, 1997). These workers injected iothalamate subcutaneously and measured it in serum and urine by CE, reporting excellent correlation with an isotopic method. It is not clear whether the lack of subsequent reports on this technique represents indifference or widespread acceptance, but the former seems more likely, despite advantages including avoidance of radioactivity and possible cost reduction.

Iothalamate detection by capillary electrophoresis has also been used to determine single-nephron tubular fluid reabsorption in isolated rat tubular preparations, in which the iothalamate was added directly to the tubular fluid (Capasso et al, 2002).

I therefore performed experiments to determine whether this anion could be detected using our method, as an addition to the detection of other anions. Figure 2.7 shows an electropherogram of a chloride, bicarbonate and phosphate multi-anion standard with iothalamate added at 8 μmol/L (equivalent to 1.6 mmol/L before 200 fold dilution). The

iothalamate peak is quite distinct from all the other anions; there is no overlap or interference. The iothalamate peak is negative rather than positive in orientation because the other anions are detected indirectly (by displacement of chromium ions in the running buffer) whilst iothalamate is detected directly from its absorbance at 254nm. Figure 2.8 shows an overlay of peaks produced by a range of concentrations of iothalamate, and demonstrates that peak area is proportional to iothalamate concentration. These results indicate that the described technique can potentially be expanded to allow detection of iothalamate simultaneously with the other anions.

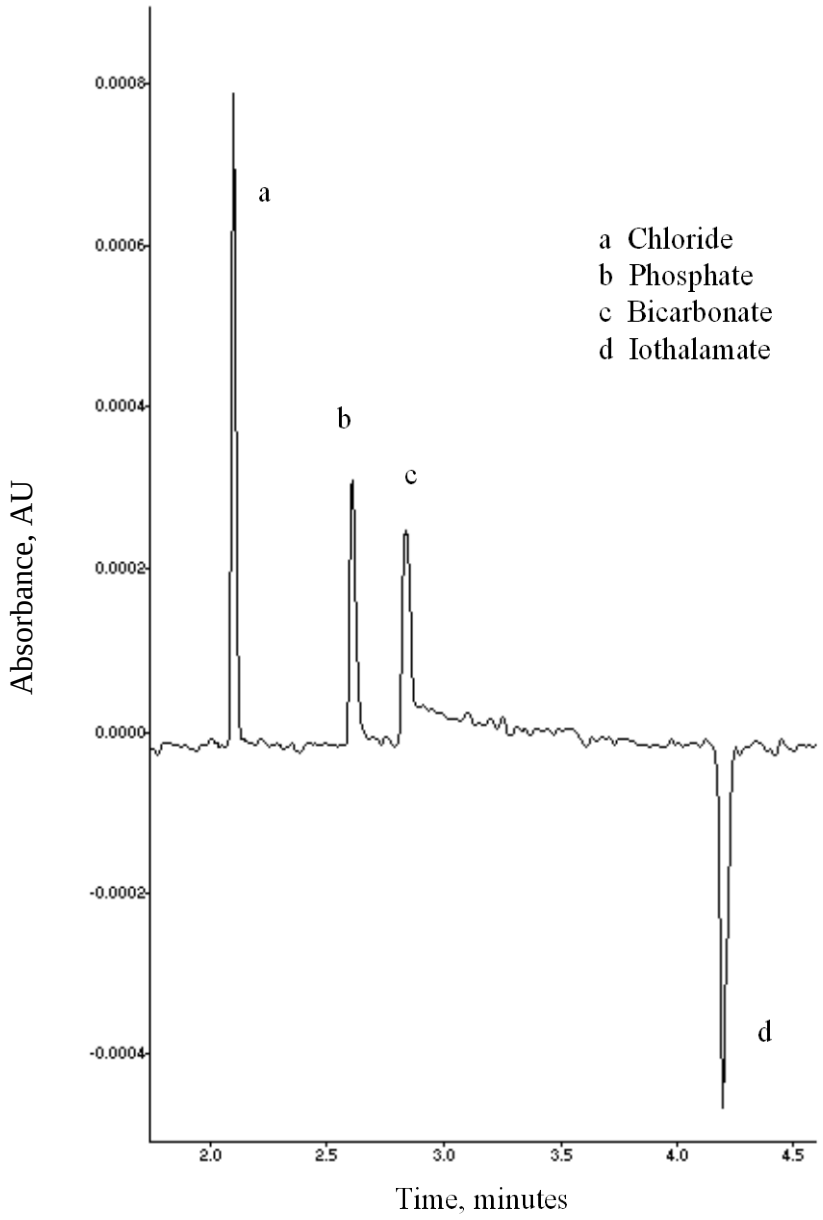


Figure 2.7 Electropherogram of a standard solution containing iothalamate, chloride, phosphate and bicarbonate.

The standard solution contained chloride 50 mmol/L, phosphate 11 mmol/L, bicarbonate 15 mmol/L and iothalamate 1.6 mmol/L.

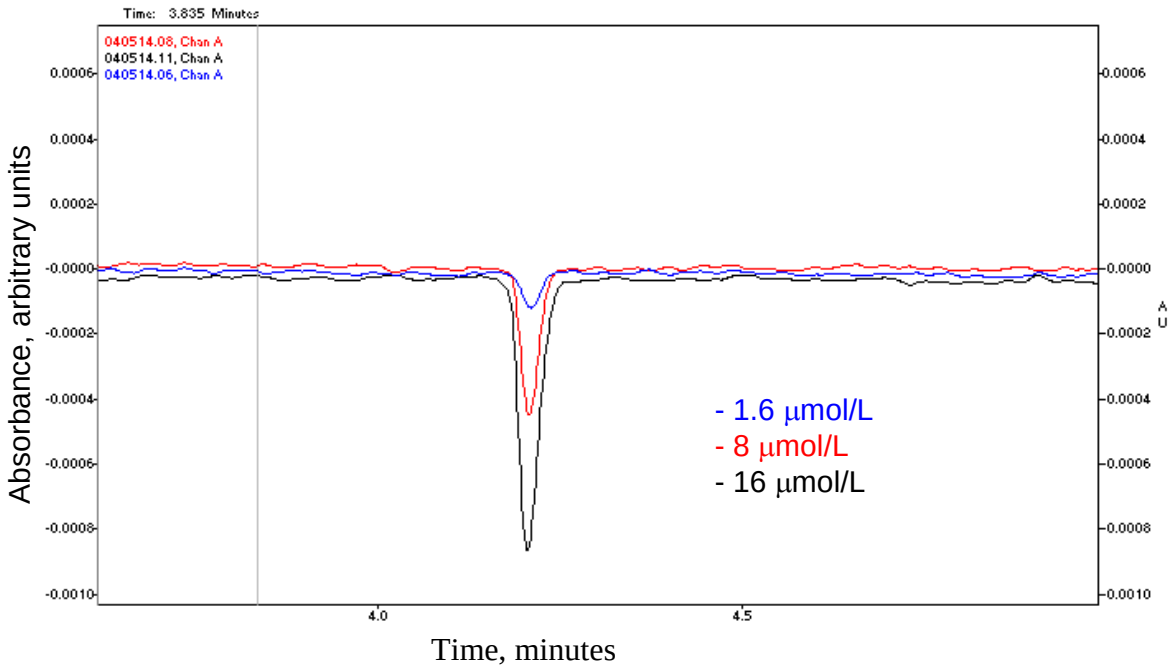


Figure 2.8 Overlay of iothalamate peaks at a range of concentrations

2.4.2.2.2 Citrate and oxalate

Citrate and oxalate are clinically important anions in urine, because they are significant in the development of kidney stones. Citrate acts to reduce the risk of calcium-containing stones, whilst excessive oxalate can lead to calcium oxalate stones. Capillary electrophoresis has been used to assay these anions in clinical urine samples as part of a stone screening process (Garcia et al, 2001). Direct detection was used by these workers as they were interested in organic acids only. I looked for citrate and oxalate not only in urine but also tubular fluid using indirect detection and found it was possible to demonstrate citrate in both, as shown below. Urinary oxalate was usually too low to be detected by this method, although the method will allow detection of high levels of oxalate. The identity of peaks was confirmed by spiking samples with known

concentrations of the anions in question; see Figure 2.9 for an illustration of the identification of a citrate peak in urine.

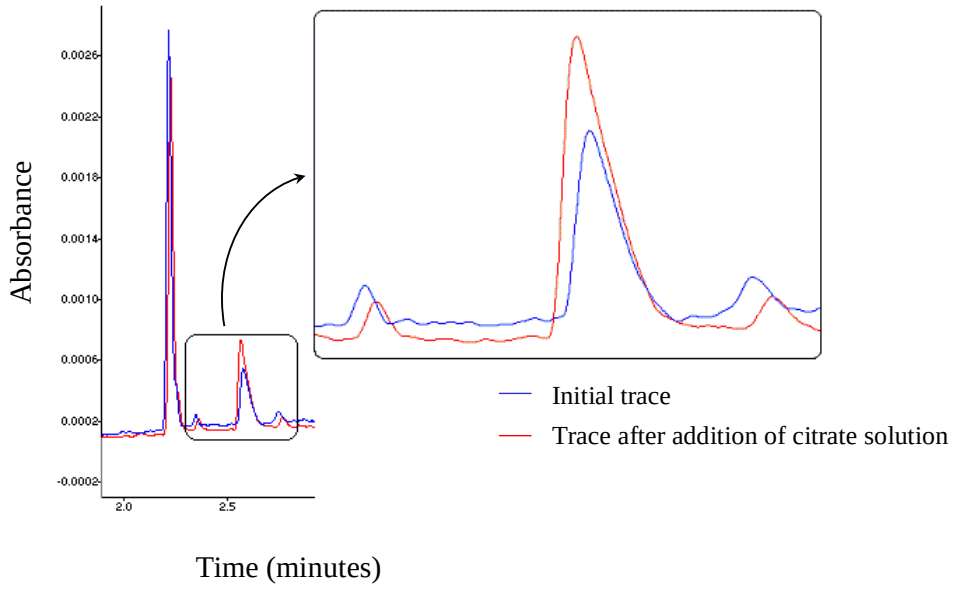


Figure 2.9 Illustration of identification of citrate peak in a urine sample

The peak is positively identified by spiking the sample with a citrate solution and repeating the analysis; the citrate peak is increased whilst other peaks are little changed.

2.4.2.2.3 Urate

Urate represents the final means of disposal of purines and is often present in significant concentrations in the urine, at or near its solubility limit. I tested for the presence of urate peaks by spiking samples with urate solutions. Because urate is not very soluble in aqueous solutions, I used a few drops of 10M sodium hydroxide to help get the urate into solution (final hydroxide ion concentration approximately 5mmol/L in the urate standard

solution); the initial traces showed a pair of peaks when I was expecting only one. Subsequent work demonstrated that these two peaks corresponded to hydroxide and urate, respectively. Examination of urine samples for urate confirmed that capillary electrophoresis was able to detect urate in urine. As my main interest was in phosphate measurement, I did not attempt to quantify urinary urate levels, but was able to confirm that the urate peak lay well away from the areas of interest for phosphate, chloride or bicarbonate.

2.4.3 Use of capillary electrophoresis to measure anions in tubular fluid

2.4.3.1 Adaptations to the technique

There are a number of important differences between tubular fluid and urine that have a bearing on analysis of tubular fluid. The most obvious and important is the question of sample volume. Urine samples in rats are usually available in volumes measured in millilitres (baseline urine output in a free-living rat is quoted as approximately 1ml/hr [e.g. in Shirley et al, 1989], although experimental urine output depends largely on the rate of intravenous fluid infusion during the procedure). In contrast, tubular fluid obtained by micropuncture is available in volumes measured in nanolitres, with end-proximal tubular flow rates of 10-20nl/min and distal tubular flow rates of around 5-10nl/min (Walter et al, 1988).This means that a range of techniques for working with and analyzing such small volumes is required. Capillary electrophoresis is suited to the analysis of such small volumes because it uses (indeed requires) diluted samples - for example, a 30nl sample becomes a more manageable 6μl after dilution to 200 times its original volume in water.

2.4.3.2 Microvials

When analyzing a sample, the vial containing the sample is placed in a rotating tray in the CE machine. The machine is able to use either standard 1ml vials, holding 200-500μl of diluted sample, or microvials, holding 5-50μl of diluted sample, which allow smaller

sample volumes to be analysed. These microvials have a different shape from normal vials, and one consequence is a considerable increase in surface-to-volume ratio (see Figure 2.10 for a diagrammatic representation of this). This has implications for analyses of ions that might be susceptible to interactions with the atmosphere - in particular bicarbonate - and might lead to concerns about evaporation.

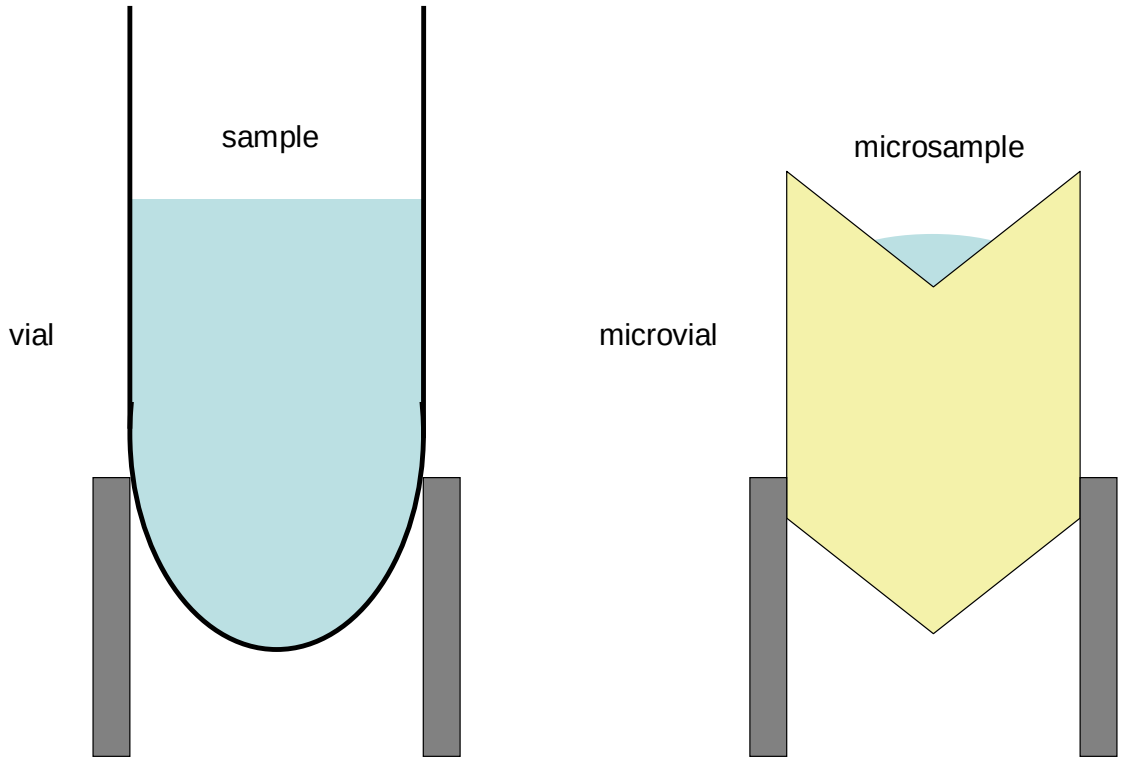


Figure 2.10 Conventional and microvials used for capillary electrophoresis

Figure 2.11 shows two standard curves for bicarbonate; one is with conventional vials (and has already been shown in Figure 2.5) whilst the other is with the microvials. It can be seen that there is a somewhat greater degree of scatter of the data when using microvials; with microvials the correlation coefficient was 0.981, and with standard vials

it was 0.997. In contrast, chloride and phosphate gave identical curves with the two types of vial; for a comparison between the results for phosphate with the two types of vial, see Figure 2.12. A further difference to note between the analysis of bicarbonate and of phosphate and chloride is that for the latter two ions the calibration curve passes through zero, while for bicarbonate it does not: when the blank (water) was tested a (small) peak was seen. These observations suggest that there is significant uptake of atmospheric carbon dioxide by the sample. It may be that this uptake of carbon dioxide from the atmosphere is more variable in microvials than in standard vials, leading to the less accurate determination of bicarbonate in microsamples.

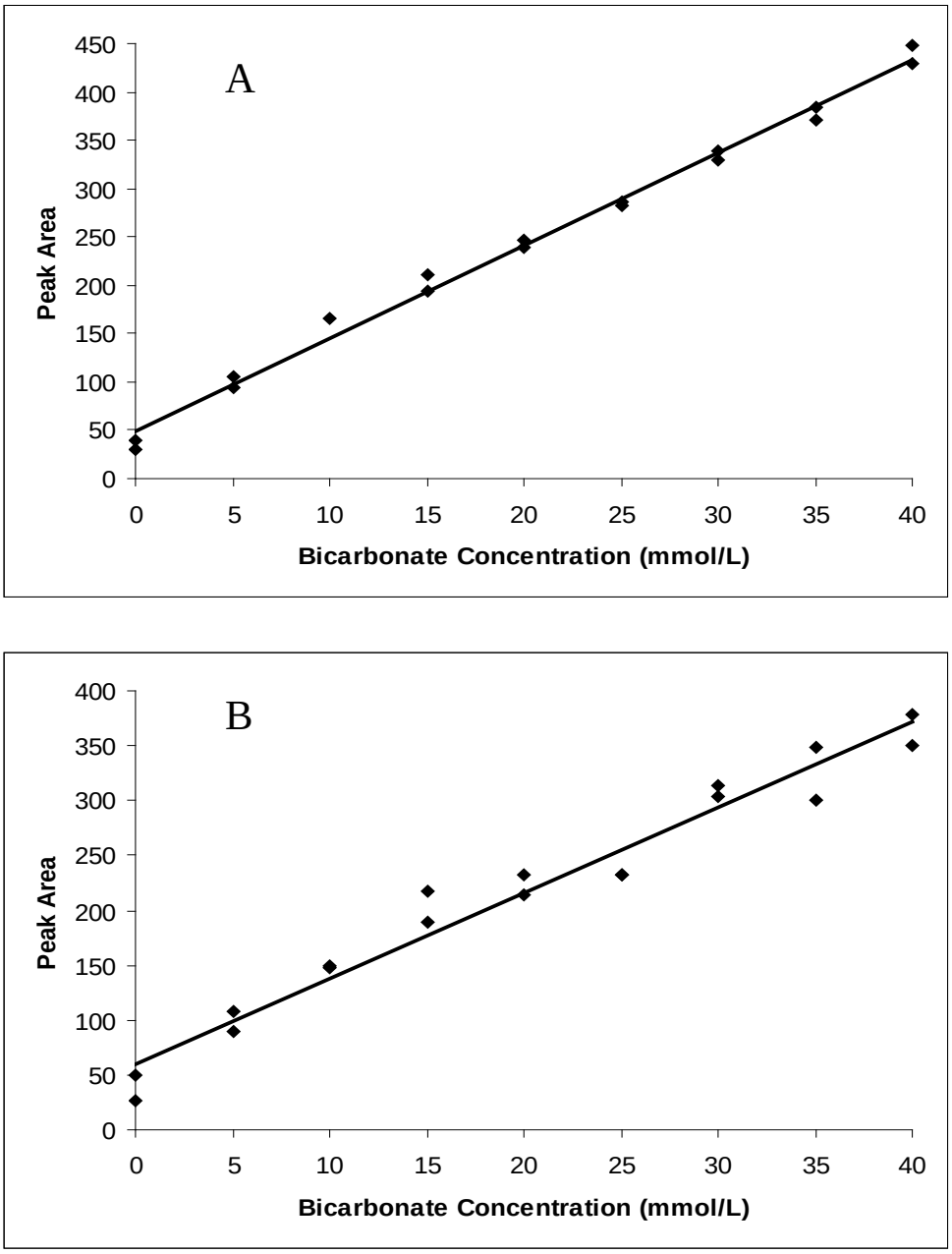
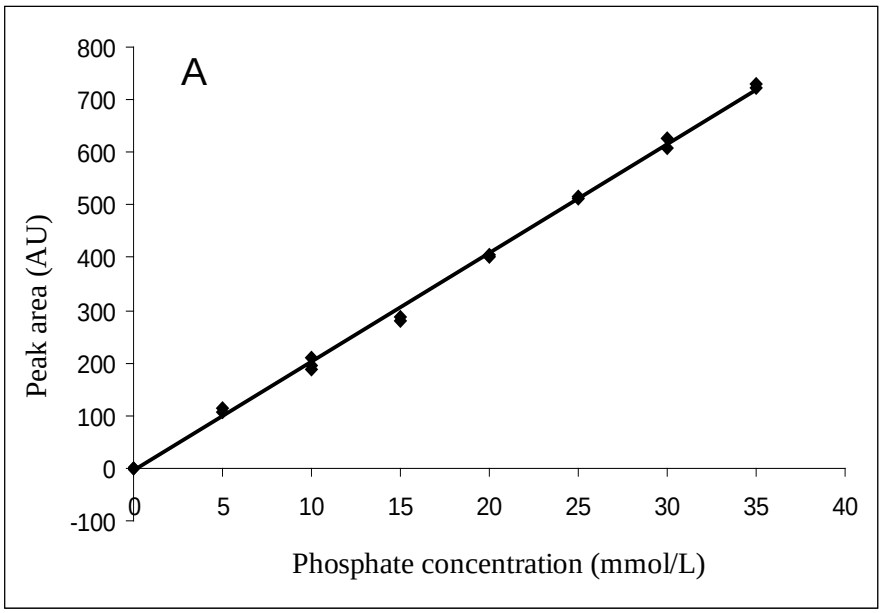


Figure 2.11 Calibration curves for bicarbonate in standard vials and in microvials

The solid lines represent the best-fit line determined by linear regression.

(A) standard vials ; y=9.63x+48.62; r= 0.997

(B) microvials ; y=7.78x+60.10; r=0.981



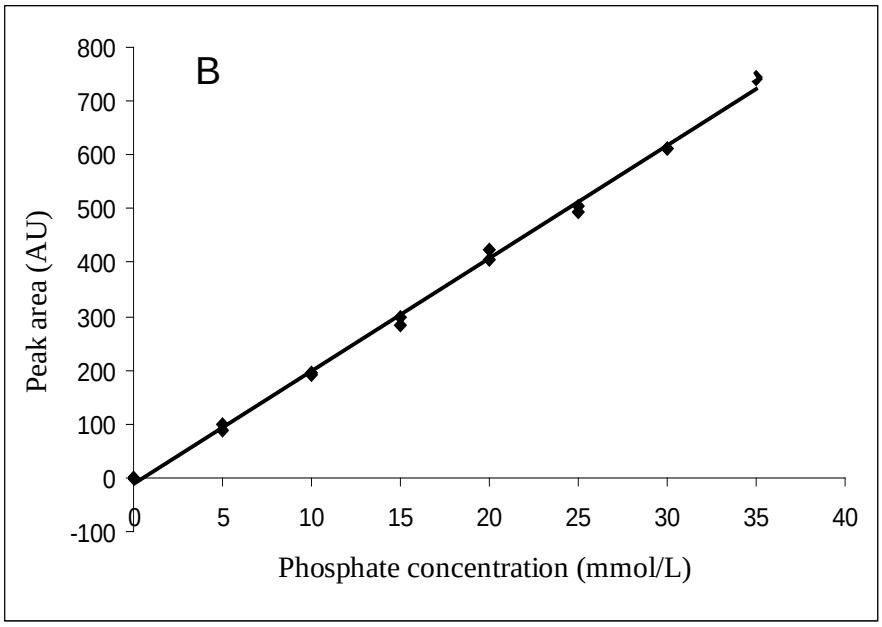


Figure 2.12 Calibration curve for phosphate in standard vials and in microvials

The solid lines represent the best-fit line determined by linear regression.

(A) standard vials; y=20.68x-5.65; r=0.999

(B) microvials; y=21.02x-11.4; r=0.999

Figure 2.13 shows the results from a series of tubular fluid samples collected by free-flow micropuncture from intact and thyroparathyroidectomised rats (for micropuncture methods, see chapter 4; the thyroparathyroidectomised rats were purchased from Harlan UK, Bicester, Oxfordshire, UK), indicating that the CE techniques described in this chapter are applicable to biological micro-samples and can demonstrate changes in phosphate and chloride concentrations under experimental conditions. Note that, in contrast to the findings in intact animals, phosphate was undetectable (as can be seen in Figure 2.13 C) in the distal tubular fluid of thyroparathyroidectomised animals. Note also that the results demonstrate that distal tubular fluid chloride concentrations are less than half those found in the proximal tubule, in keeping with current knowledge of tubular function.

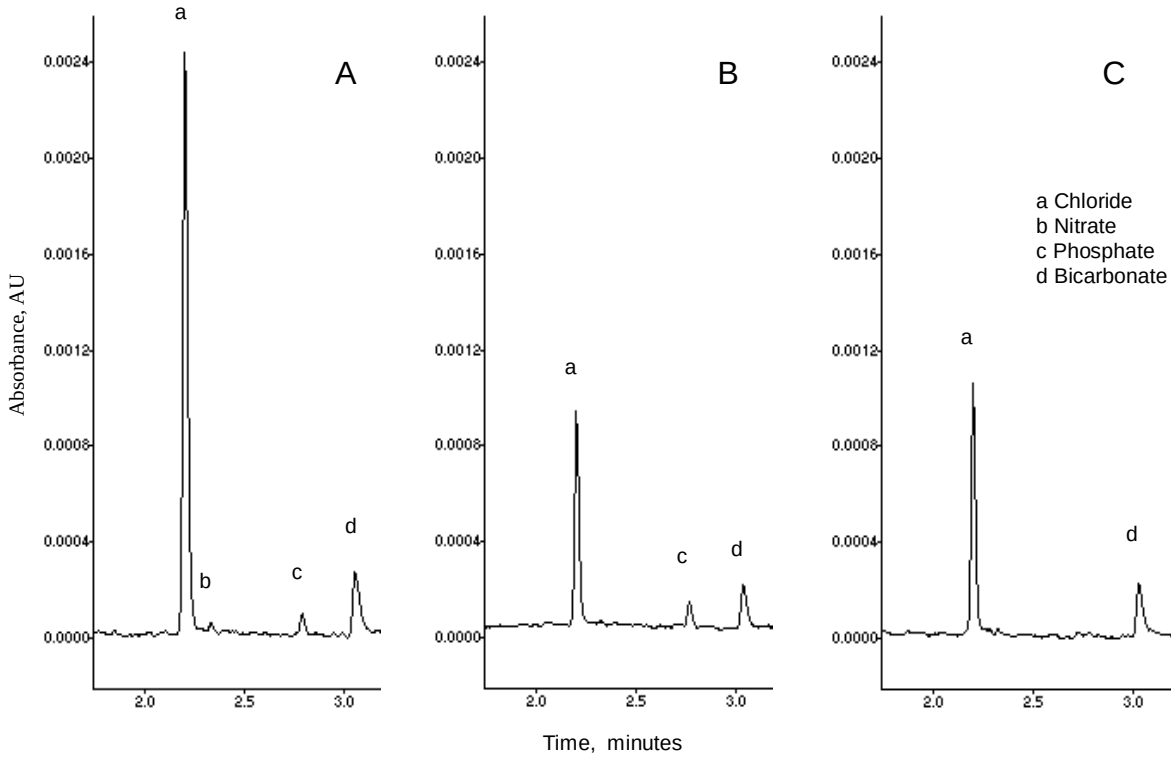


Figure 2.13 Tubular samples obtained during free-flow micropuncture

(A) Proximal tubular fluid from an intact animal. Chloride is 134 mmol/L and phosphate 1.7 mmol/L. (B) Distal tubular fluid from the same animal. Chloride is 47 mmol/L and phosphate 2.1 mmol/L. (C) Distal tubular fluid from a thyroparathyroidectomised animal. Chloride is 55 mmol/L and phosphate is below 0.3 mmol/L; this corresponded with undetectable urinary phosphate excretion.

Figure 2.14 shows a urine sample from an intact rat, collected at the same time as the tubular samples in Figure 2.13 A and B. Note that, in comparison with the tubular samples, urine has a much higher chloride concentration, no bicarbonate and detectable amounts of nitrate and citrate; the identity of these last two peaks was confirmed by 'spiking' the urine samples with standard solutions.

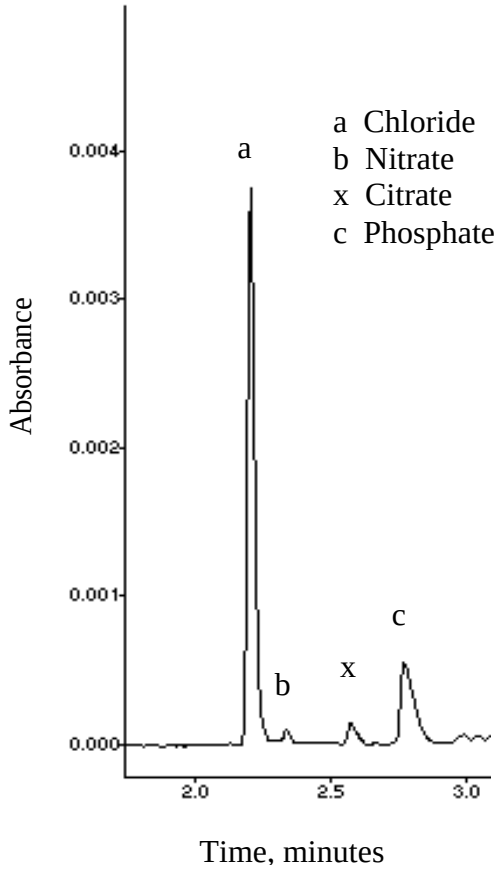


Figure 2.14 Urine sample from a normal rat, collected simultaneously with tubular fluid samples shown in Figure 2.13 A and B.

In this sample, chloride is 239 mmol/L and phosphate 29 mmol/L. Note that bicarbonate is undetectable in the urine, and that the urine contains detectable amounts of nitrate and citrate.

2.4.3.3 Direct comparison with the NANOFLO technique

Other than CE, no straightforward method of determining non-radioactive phosphate in biological microsamples using off-the-shelf equipment is available. I wanted to assess the CE technique for anion measurements by comparison with an established technique, and therefore compared CE results for chloride (rather than phosphate) with those obtained using an established alternative technique for the determination of chloride in tubular fluid samples - the NANOFLO, a commercially available system based on flow-through microfluorometry of directly injected samples (Zhelyaskov et al, 2000). The latter technique uses the fluorescent reaction of lucigenin with chloride ions to generate a signal. The fluorescent dye lucigenin reacts with halides to produce quenching of fluorescence, which is greatest around the 500nm wavelength (Wissing & Smith, 2000). The dye also responds to other halides, but as none of these is present in significant quantity in tubular fluid it is reasonable to assume that all the reaction is due to chloride ions. There is also some potential quenching with citrate, but again the concentration difference expected between chloride and citrate in tubular samples means that this is not likely to be a significant source of error.

Samples of known volume are injected into a capillary system, through which there is a continuous and constant flow of lucigenin in buffer. The sample is then carried past a detector, where the quenching reaction between chloride ions and lucigenin produces a change in detector output. The change is related to the amount of chloride in the injected sample, and chloride concentration can thus be calculated from a standard curve. The standard curve is generated by injection of solutions of known chloride concentration and plotting of trough height over background.

This method of detecting chloride in microsamples is used routinely in several micropuncture laboratories.

2.4.3.3.1 Technical details of the NANOFLO method

The NANOFLO instrument is commercially available, produced by World Precision Instruments Inc (Sarasota, Florida, USA). Results were acquired and analysed using the

DUO18 software, also produced by WPI. Solutions of saline and lucigenin were produced from analytical grade chemicals (Sigma-Aldrich, Poole, Dorset, UK) and reverse-osmosis purified water. An illustration of the machine is shown in Figure 2.15.

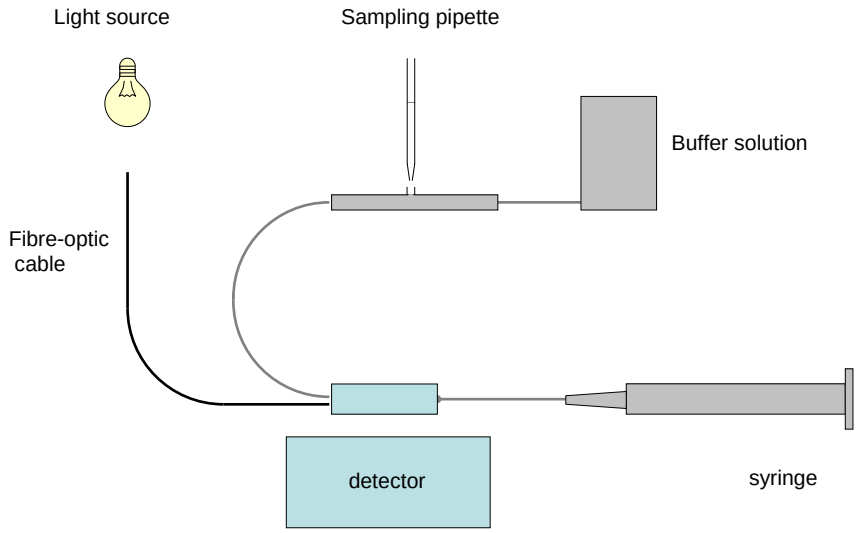


Figure 2.15 Schematic diagram of the NANOFLO instrument

Buffer solution (containing lucigenin) is continuously pulled through the apparatus by the syringe at a rate of 0.3ml/hr. Samples are injected, using a calibrated sampling pipette, through a small port in the top of the apparatus and then mix with the lucigenin, before being passed in front of the detector and illuminated with light at the desired wave-length.

I followed exactly the published NANOFLO method of Zhelyaskov et al (Zhelyaskov et al, 2000) but found that this gave very large biphasic peaks which overwhelmed the sensitivity of the detector. Subsequently, I tried using much lower concentrations of lucigenin, but then had difficulty with the detection limit. After personal correspondence with the author of the previous work, a wratten (neutral) filter was placed in the light path (Eastman Kodak, Rochester, New York, USA) and this produced results which closely duplicated the previously published work. Results with the NANOFLO showed greater variability than with CE and the machine is technically challenging to use. In addition, the detection limit is significantly higher than with CE, at about 10mmol/L chloride in a 10nl sample. One further point to make is that the original paper on the NANOFLO does not include any biological samples, but only pure standards made up in water. My experience

with the NANOFLO was that it was a great deal easier to get reproducible results with standards than with biological samples, perhaps because the standards were not stored under oil.

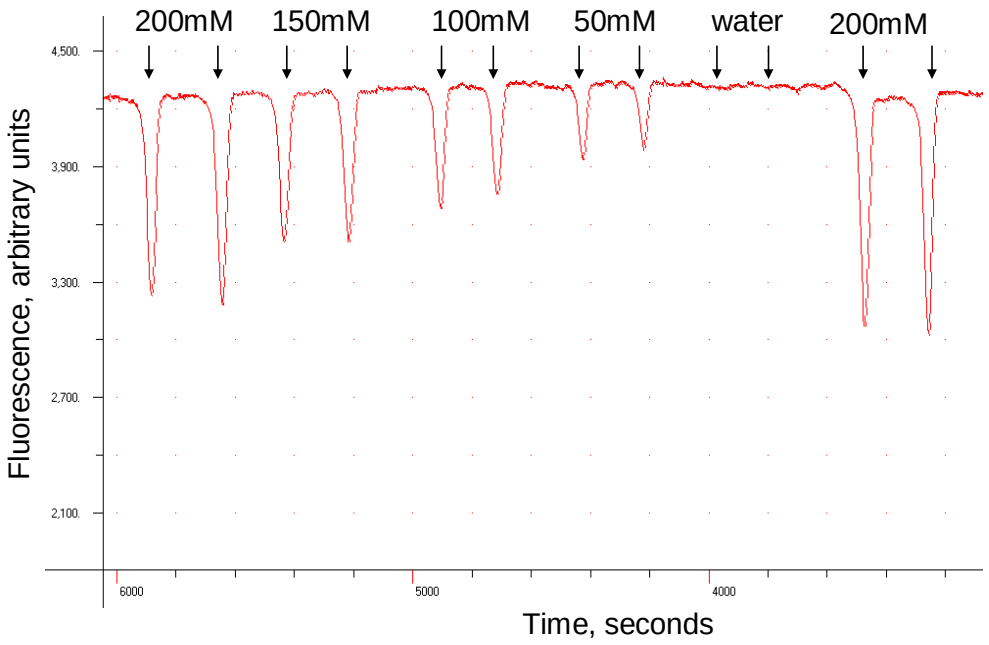


Figure 2.16 Illustrative trace showing results from the NANOFLO using saline standards

An illustrative trace showing a series of samples used to construct a calibration curve using the NANOFLO is shown in Figure 2.16. These samples were made from standard sodium chloride solutions made up in HEPES buffer. Representative traces from the machine in operation are shown in Figure 2.17. Note that chloride is detected as a negative peak, and that the depth of each trough compared to the preceding background signal is used to calculate the ratio F1/F0. Injection of water or buffer alone gave no trough. The calibration curves were generally satisfactory, with $r>0.99$, but there was a tendency for the baseline to fall with time as the fluorescence became bleached. Consequently, the method is limited in the number of samples per day that can be analysed. All samples were analysed in duplicate, as can be seen from the example traces shown.

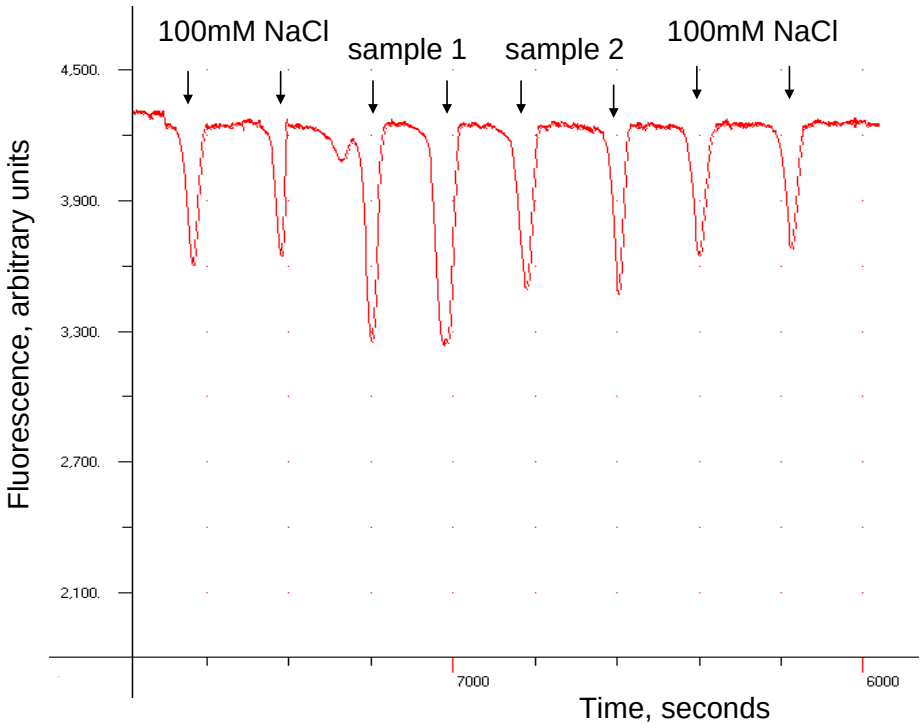


Figure 2.17 Illustrative NANOFLO trace with two samples and a standard

2.4.3.3.2 Comparison of results between the NANOFLO and capillary electrophoresis methods

Comparison of results between the two methods (with respect to chloride concentration) showed reasonable agreement. Results are presented directly against one another in Figure 2.18. Each point represents a biological sample (*not* a standard solution), either urine or tubular fluid, analyzed by both methods. The correlation coefficient was 0.980.

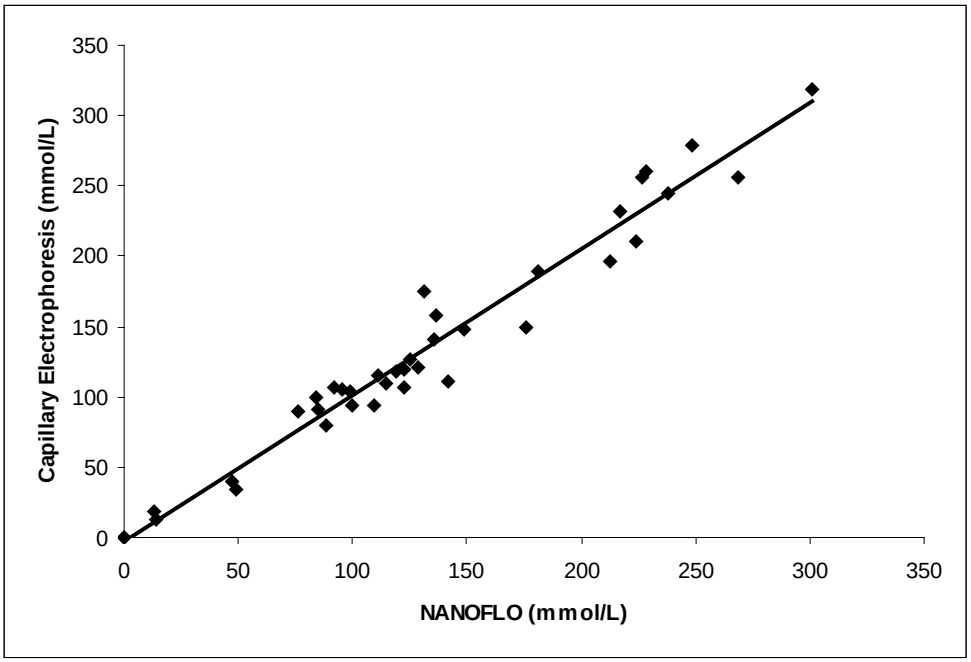


Figure 2.18 Comparison between values obtained for chloride concentration (in urine and tubular fluid) by capillary electrophoresis and NANOFLO

y=1.041x-2.856; r= 0.98.

Correlation coefficients alone say little about the level of agreement between two measurement methods. Given that the two methods are attempting to measure the same thing (in this case chloride ion concentration), it is almost inevitable that they will be correlated. For this reason, results were also compared by the use of a Bland-Altman plot (Bland & Altman, 1986) in Figure 2.19. This contains the same data as Figure 2.18, but for each data point the difference between the values found using the two methods (calculated by subtracting the value found by CE from the value found by NANOFLO) was plotted against the average value. This form of analysis allows both a better measure of the agreement between the two methods and a visual check of the scatter of the data. It also allows a check for any systematic bias in which scatter depends on the value being measured. As all but two points fell between the lines indicating two standard deviations, and as these two points are in the middle of the range of values, this demonstrates an absence of systematic bias between the two methods.

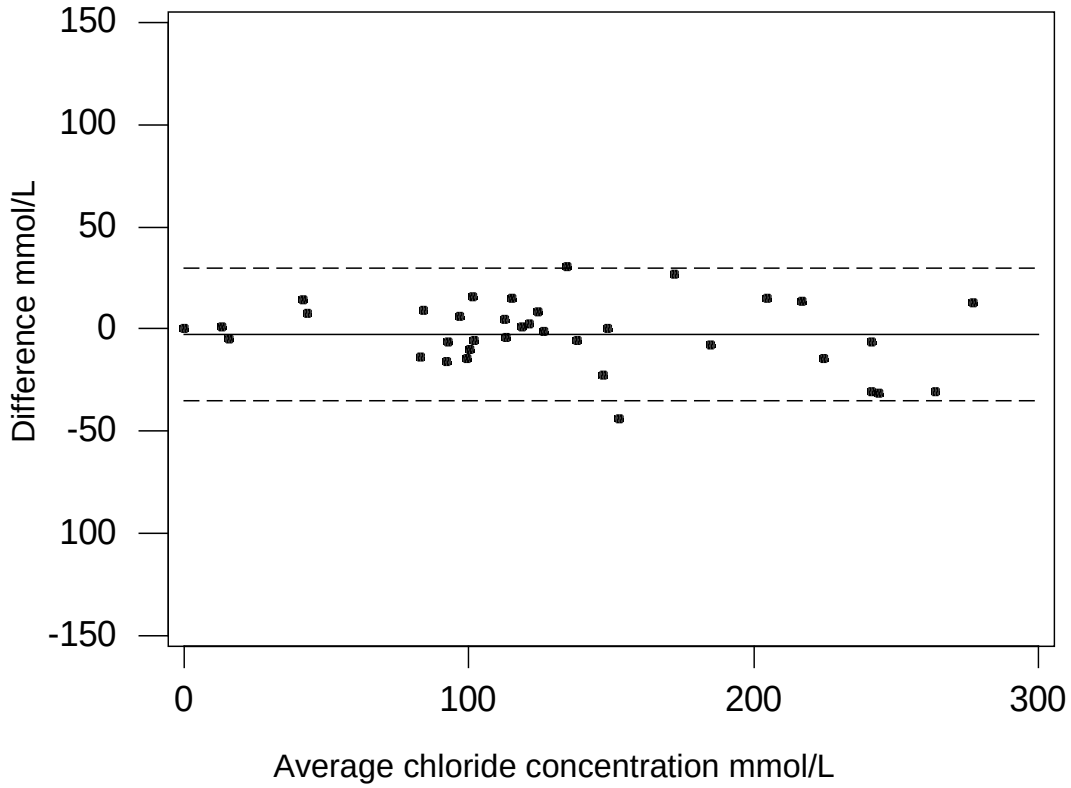


Figure 2.19 Bland-Altman plot showing difference against mean for chloride concentration determined by capillary electrophoresis and by NANOFLO

The solid line represents the mean difference (in this case, close to zero) while the dashed lines represent two standard deviations of the difference.

2.4.4 Use of capillary electrophoresis to measure anions in plasma

2.4.4.1 Adaptations to the technique

There is a limit to the volume which can be taken from a rat's circulation (typical blood volume 15-20ml) during an experiment without disturbing renal and systemic

haemodynamics. If serum phosphate is to be measured repeatedly during an experiment, it must be measured in very small volumes, as blood is also being taken hourly for GFR determination. Many workers get round this by measuring the serum chemistry only at the end of the experiment, when larger volumes of blood can be obtained by aortic puncture and there is no concern about affecting the GFR. However, this obviously cannot measure changes in phosphate during the experimental period. I therefore modified the capillary electrophoresis method to measure anions in small samples of plasma.

The major difficulty encountered in the analysis of anions in plasma samples by capillary electrophoresis is the presence of plasma proteins in the samples. These proteins coat the capillary, altering transit times and peak heights. I decided against trying to deproteinate the samples, as has been reported by others (Wilson et al, 1997), on the basis that repeated washings with sulphuric acid and sodium hydroxide would introduce too many potential contaminants. I decided instead to wash the capillary after each run. Repeated experimentation showed that highly reproducible results could be obtained by rinsing the capillary with 0.1 M sodium hydroxide between each sample and by alternating protein-containing samples (for example, plasma) with non-protein containing samples (for example, standards). Davydova and coworkers, using direct UV detection, measured plasma at a dilution of 1:10 (Davydova et al, 1999). Despite a deproteination step, they also found that plasma assays required extensive rinsing with sodium hydroxide and water, steps not required when assaying tubular fluid or urine.

2.4.4.2 Results for plasma

My principal interest was in plasma phosphate. Plasma samples were taken simultaneously with the urine (and tubular fluid) samples; plasma phosphate determinations allowed calculation of phosphate clearances. However, I also determined plasma chloride routinely as it provided a check on the dilution of the samples - dilution error being a potential source of misleading results. As plasma chloride in rats is around 105mmol/l (Cowley et al, 1997), samples in which chloride values differed substantially from this were rejected, and duplicate samples reanalysed.

A typical trace of normal rat plasma is shown in Figure 2.20. The main anions present are chloride, bicarbonate and phosphate, in that order.

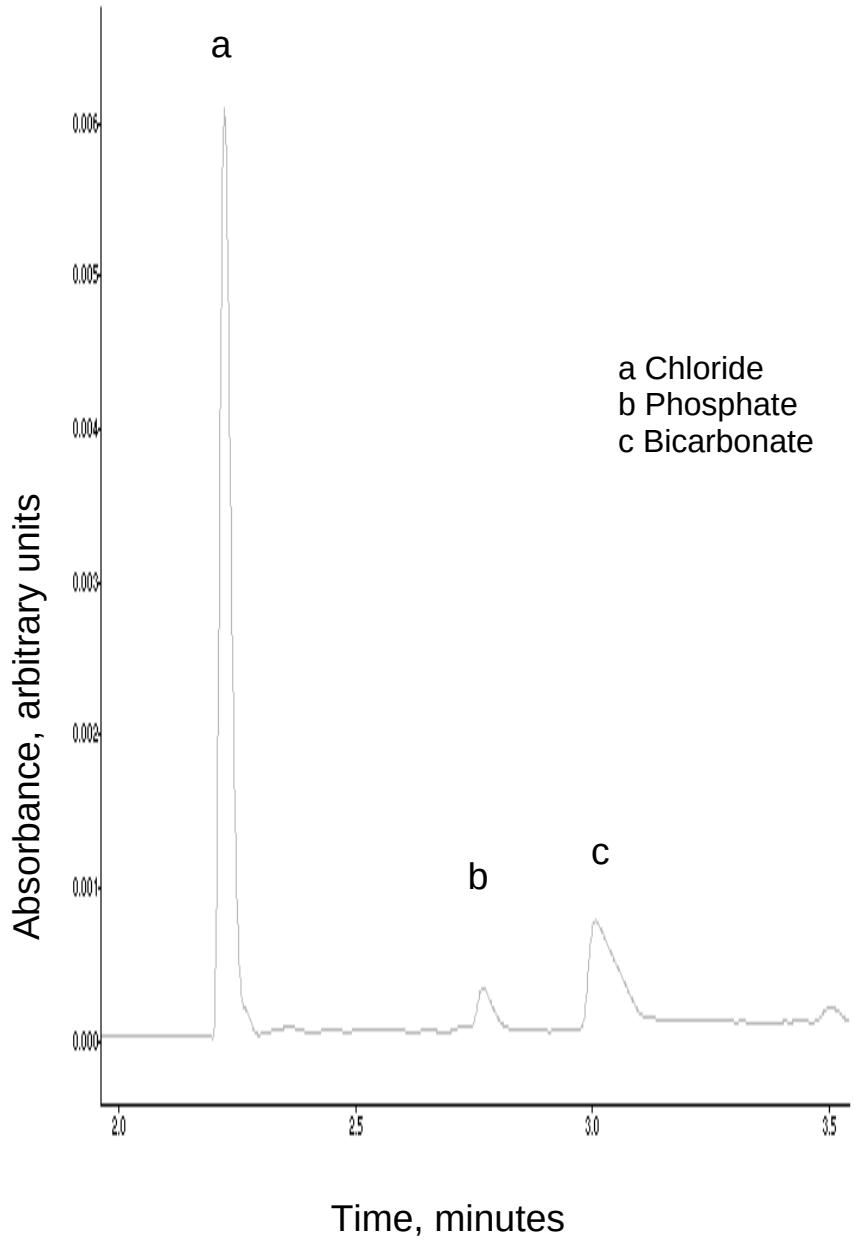


Figure 2.20 Illustrative capillary electrophoresis trace of normal rat plasma

Chloride concentration is 104 mmol/L, phosphate 2.1 mmol/L and bicarbonate 22 mmol/L

2.5 Conclusions

The capillary electrophoresis technique described has been developed to the point where it is an accurate and reproducible means of measuring numerous anions in small biological samples, including plasma, urine and tubular fluid. It has particular advantages for very small samples, allowing multiple assays on a single sample, although there are problems with the measurement of bicarbonate, possibly owing to carbon dioxide uptake by microsamples. The method is nevertheless extremely useful for the measurement of phosphate in tubular fluid samples, a requirement of a major section of this thesis.

Chapter 3

Investigation of the renal effects of MEPE: clearance studies

3.1 Introduction

Clearance experiments allow quantification and analysis of the effects of postulated regulators of renal function on the handling of substances by the nephron. The renal clearance of a marker substance that is freely filtered and neither secreted nor reabsorbed by the kidney (for example, inulin) is a measure of GFR. Comparison of this with the clearance of the substance of interest, for example phosphate, allows calculation of net reabsorption or secretion.

Clearance experiments have been used extensively to investigate renal phosphate reabsorption, going back to the original demonstration that the process could be saturated. Frick (Frick, 1968) infused increasing concentrations of phosphate into anaesthetised rats and demonstrated that, as plasma phosphate levels were increased, reabsorption of phosphate increased and eventually reached a maximum level. The inference drawn by this author (and subsequently shown to be correct, see Chapter 1) was that, rather than being reabsorbed by simple diffusion, phosphate transport must involve carrier proteins that can be saturated.

As discussed in Chapter 1, Rowe and colleagues have previously reported on the effects of MEPE on phosphate excretion in mice (Rowe et al, 2004). In this work they gave MEPE by repetitive intraperitoneal injection and measured urine and serum phosphate and creatinine. Their results suggested that MEPE increased fractional phosphate excretion. However, creatinine clearance is not a reliable marker of GFR in rodents, as creatinine has been shown to be both secreted and extensively reabsorbed (Namnum et al, 1983). Furthermore, because the urine samples in Rowe's study were of small volume, urine samples from all the animals were pooled for analysis. The experiment was performed twice and the results described as 'the same', but no statistical measure of variability was provided. Therefore, it is not possible from this previous work to be sure that tubular reabsorption of phosphate was altered.

The key issue to be investigated initially in the present study was whether MEPE is in fact a phosphaturic substance. Since PTH is known to be phosphaturic and its action has been extensively studied, I chose to use it as a positive control. The chief biological actions of PTH are due to its first 34 amino-acids (reviewed in Mannstadt et al, 1999), so most workers in this field use PTH (1-34); this compound was used in the present study.

3.1.1 A note on rat models in the study of human physiology

Some areas of phosphate physiology are relatively well studied in human populations - for example, the hereditary hypophosphataemias and phosphate balance in renal failure and dialysis patients - usually when abnormalities of phosphate balance lead to obvious disease states. Other areas of phosphate physiology will never be straightforward to study in humans, for example the nephron sites involved in phosphate reabsorption, or changes in phosphate transporter numbers in response to stimuli. In these areas, research is performed in animals - historically in rats, more recently (because of greater ease in genetic manipulation) also in mice. The rat (Rat Genome Consortium, Gibbs et al, 2004) and mouse (Mouse Genome Consortium, Waterston et al, 2002) genomes have been sequenced, as, of course, has the human genome. The similarities between genomes are more striking than the differences, and almost every significant disease-causing gene in humans has been found to have mouse and rat homologues. Care should however always be taken when extrapolating from rodents to humans - clearly there are *some* significant differences!

3.2 Aims of the present study

As discussed in Chapter 1, MEPE is a novel protein first isolated from patients with oncogenic osteomalacia, a syndrome in which there is a circulating phosphaturic substance. I aimed to discover whether MEPE would cause phosphaturia when infused into intact animals. To this end, I infused a range of doses of MEPE so as to construct a dose-response curve. In addition, I measured GFR and the filtered phosphate load, so as to allow calculation of the tubular reabsorption of phosphate.

3.3 Methods

3.3.1 Experimental protocol

Prior to the clearance surgery, rats were kept in the university's animal facility and fed a standard diet (Rat and Mouse No.1 maintenance, Special Diets Services, Witham, Essex UK). They were allowed unlimited access to food and water, and the food contained 0.5% total phosphorus and 600 IU/kg of Vitamin D_3.

Adult male Sprague-Dawley rats, of mean body weight 247g (range 225-300g), were anaesthetised with intraperitoneal sodium thiopentone (100mg/kg body weight; Link Pharmaceuticals, Horsham, Sussex). Animals were monitored throughout the experiments for any sign of responsiveness by assessing the reaction to pinching the feet, and further anaesthetic was given as required in the form of intravenous boluses of 5-10 mg/kg body weight of sodium thiopentone. Temperature was monitored continuously by means of a rectal thermometer; animals were placed on a thermostatically controlled warming table which maintained their temperature at 37^0C. At the end of the experiments, animals were killed by overdose of sodium thiopentone without recovery of consciousness.

The right jugular vein was cannulated with two cannulae made from polyethylene tubing (Portex Plastics, Hythe, Kent), which were used for infusions of saline, tritiated inulin, MEPE or PTH, as required. NaCl 0.9% solution was infused throughout the experiment at 4ml/hr. The skin was divided in the anterior neck and a tracheotomy was performed using cautery; the trachea was supported for the remainder of the experiment by means of a polyethylene tube placed under it. A lower abdominal incision was used to expose the bladder, which was then catheterised using shaped polyethylene tubing. This bladder catheter was sutured into place, with the sutures round the bladder ensuring a minimal residual volume. The left femoral artery was cannulated using polyethylene tubing and the arterial catheter was primed with saline containing heparin. Arterial blood pressure was monitored continuously throughout the experiment using a blood pressure transducer

(model 1050.1, UFI, Morro Bay, CA, USA) and MacLab recording system (AD Instruments, Chalgrove, Oxon, UK) attached to the arterial catheter.

Thirty minutes after surgery was completed, animals were given a bolus of tritiated inulin (2 μCi, Amersham Biosciences, Little Chalfont, Bucks, UK) followed by a continuous infusion of tritiated inulin at 2 μCi/ hour. Urine output was determined hourly by weighing the urine produced (and assuming a specific gravity of 1.0).

After a further hour of equilibration, excretion rates and clearances were determined for one hour (control period). Blood samples were collected hourly from the arterial catheter. Each sample was approximately 100μl and was collected by unclamping the catheter and allowing blood to flow slowly under arterial pressure into a sample collection vial. The arterial catheter was then re-primed with a small volume of heparinised saline. Blood samples were spun at 1500g for five minutes and the plasma samples thus generated were split for tritium and phosphate analysis. Urine samples obtained from the bladder catheter were mixed briefly, and then aliquots were taken for tritium and phosphate analysis.

After the one-hour control period, the infusion was changed to include PTH (20 μg/h; PTH 1-34, Sigma Ltd, Watford, Herts UK) or MEPE (at doses of 30 μg/h [MEPE 1], 100 μg/h [MEPE 2] or 300 μg/h [MEPE 3]; recombinant full-length human MEPE, raised in insect *Spodoptera Frugiperda* cells, a gift from Acologix, Hayward, CA, USA), or continued as saline alone (vehicle controls), for the following two hours (n=6 rats per group). The second of these two hours was designated the experimental period, during which time excretion rates and clearances were determined again. The experimental procedure is summarised in Figure 3.1.

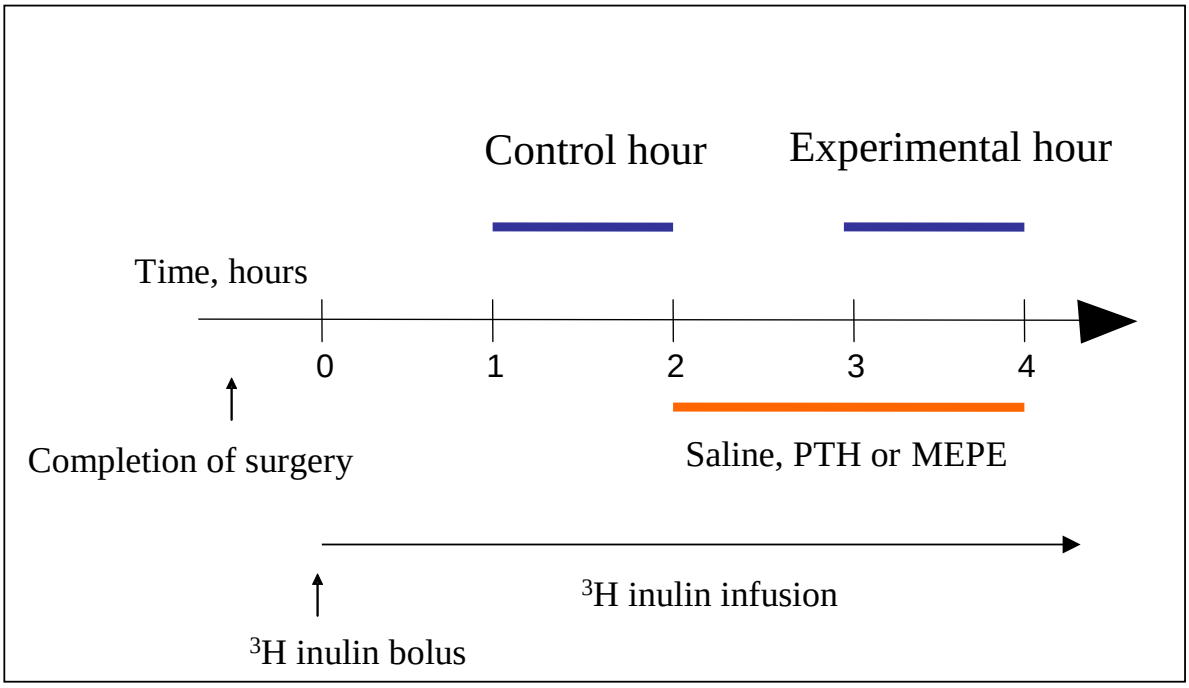


Figure 3.1 Clearance protocol

3.3.2 Analyses

The activities of tritiated inulin in plasma and urine were determined by scintillation counting of 10 µl aliquots of spun plasma and urine (Packard Tricarb, Model 2900TR; Pangbourne, Berks, UK) after dispersal in Ultima Gold scintillation fluid (PerkinElmer, Beaconsfield, Bucks, UK). Three aliquots were taken from each sample; values obtained from the three were averaged unless one was clearly much different from the other two, in which case the two agreeing values were averaged. Plasma and urine phosphate levels were measured by capillary electrophoresis (CE) as described in Chapter 2. Urine samples were diluted 1:200 in ultrapure water, then frozen for later CE analysis. Plasma samples were diluted 1:100 in ultrapure water, then frozen for later CE analysis.

Sodium concentrations in urine were measured by flame photometry (model 543, Instrumentation Laboratory, Warrington UK), following appropriate dilution if required.

3.3.3 Calculations

Glomerular filtration rate was calculated as inulin clearance:

$$GFR = C_{in} = [U_{in}] \times V_U / [P_{in}], \text{ where}$$

C_{in} = inulin clearance

$[U_{in}]$ = urinary inulin count

V_U = urine flow rate

$[P_{in}]$ = plasma inulin count.

Phosphate clearance (C_{Pi}) was calculated as:

$$C_{Pi} = [U_{Pi}] \times V_U / [P_{Pi}], \text{ where}$$

$[U_{Pi}]$ = urinary phosphate concentration

$[P_{Pi}]$ = plasma phosphate concentration.

For clearance values during the control period, the plasma phosphate concentration in the first blood sample was used; for clearance values during the experimental period, the plasma phosphate concentration in the final blood sample was used.

Fractional excretion of phosphate (FE_{Pi}) was calculated as:

$$FE_{Pi} = C_{Pi}/GFR.$$

Filtered load of phosphate at the glomerulus was calculated, on the assumption that phosphate is freely filtered, as:

$$\text{Filtered load} = GFR \times [P_{Pi}].$$

3.3.4 Statistics

Results are presented as mean values ± standard error of the mean (SEM). Comparisons between the results during the control and experimental periods in each group of animals were made using Student's paired t-test. One-way ANOVA was used to compare the five groups during the control period and during the experimental period. Two-way ANOVA was used when groups in both control and experimental periods were compared simultaneously, followed, where appropriate, by Bonferroni's test for multiple comparisons. Comparison of the change in FE_{Pi} in each MEPE-treated group with that in the vehicle group was made by one-way ANOVA followed by Dunnett's multiple comparisons test. In all cases, a p- value of less than 0.05 was considered statistically significant.

3.4 Results

3.4.1 Blood pressure

Figure 3.2 shows the mean arterial pressure in each group of animals. There was no significant difference between any of the groups during either the control period or the experimental period.

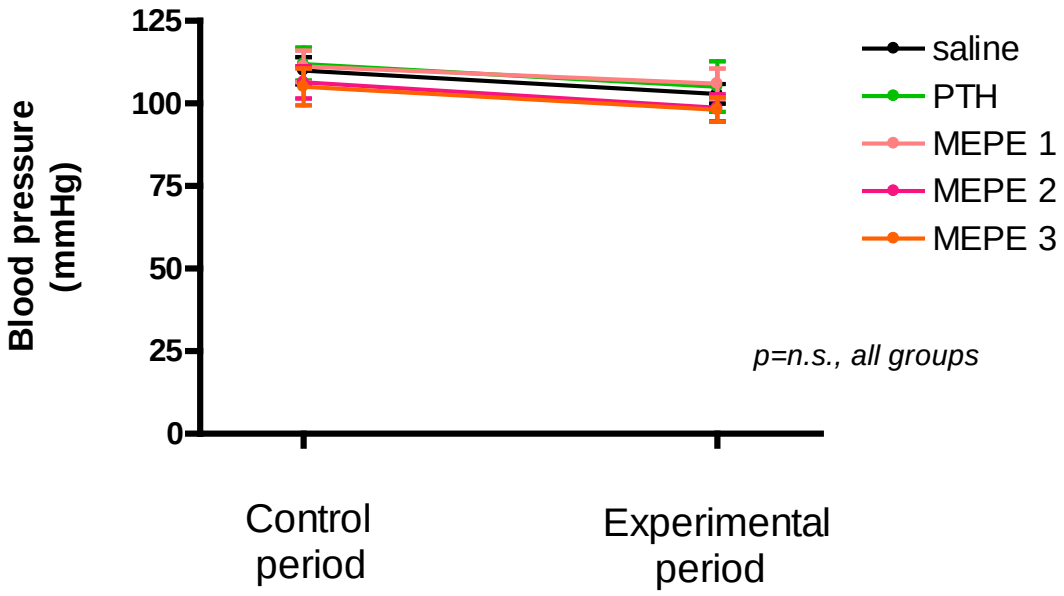


Figure 3.2 Mean arterial pressure

Results are presented as mean values ± SEM. In each group, n=6 rats.

3.4.2 Glomerular filtration rate (GFR)

GFR was measured during the control and experimental period in each animal. Figure 3.3 shows the mean (± SEM) values for GFR for each of the five groups of animals.

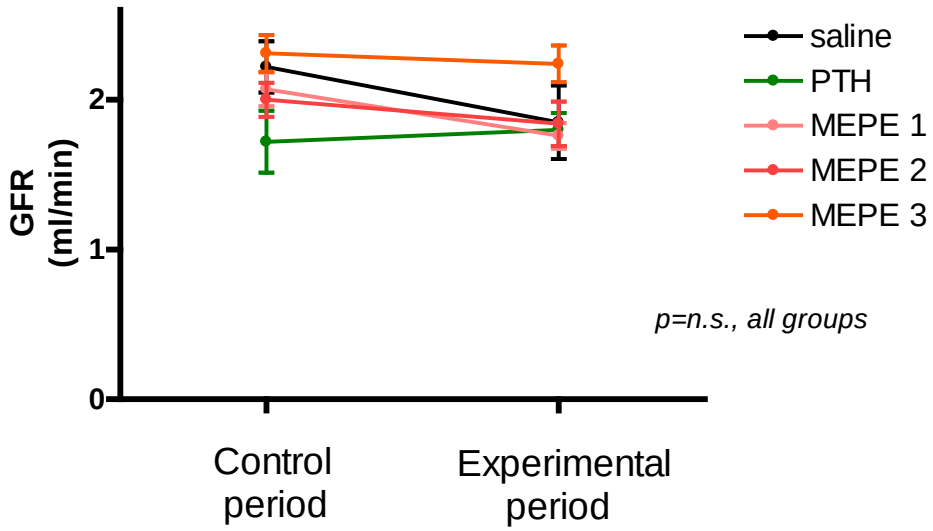


Figure 3.3 Glomerular filtration rate

Results are presented as mean values ± SEM. In each group, n=6 rats

There was no significant difference between groups either before or after treatment. Although there was a slight tendency for GFR to fall over the period of the experiment in some animals, this did not reach significance in any of the groups.

3.4.3 Sodium excretion

These data are shown in Table 3.1.

	Control period	*P*	Experimental period
Saline	219 ± 46	0.74	240 ± 40
PTH	193 ± 22	0.63	241± 88
MEPE 1	189 ± 56	0.37	257 ± 26
MEPE 2	175 ± 44	0.05	213 ± 40
MEPE 3	222 ± 46	0.34	237 ± 40

Table 3.1 Sodium excretion

Results (μmol/h) are given as mean values ± SEM; n=6 rats in each group.

P values refer to differences between the control and experimental periods within each group, compared using Student's paired t-test.

There was no significant difference in urinary sodium excretion between groups before or after treatment. Mean sodium excretion rose in every group during the course of the experiment, but the variance was large and the only statistically significant increase in any group was in that receiving the intermediate dose of MEPE.

3.4.4 Absolute phosphate excretion

Baseline (control period) urinary phosphate excretion was highly variable between animals, but the mean values in each group were similar; see Figure 3.4.

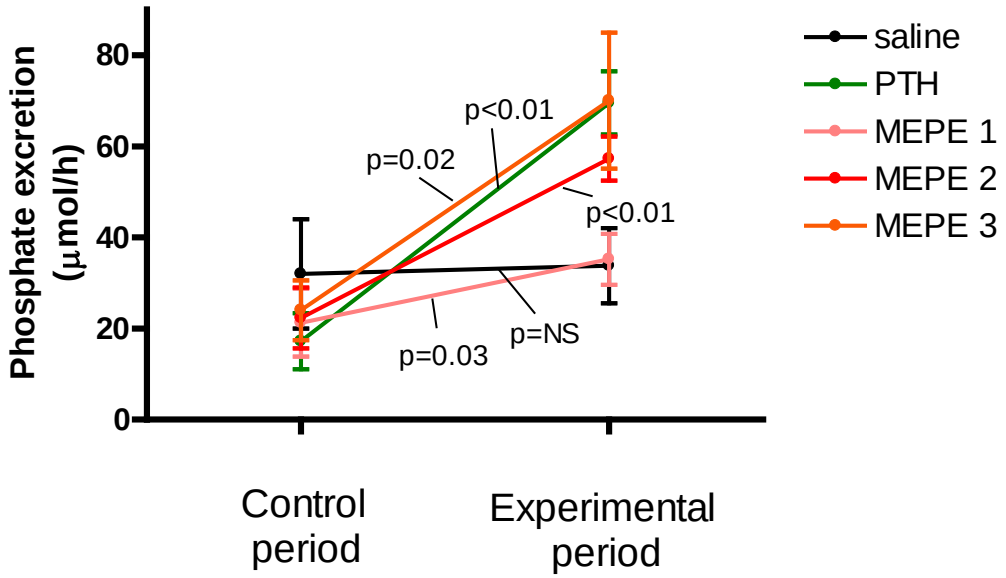


Figure 3.4 Absolute phosphate excretion

Results are presented as mean values ± SEM. In each group, n=6 rats.

Absolute phosphate excretion did not change between control and experimental periods in control (saline-infused) animals. In animals treated with PTH, there was a marked increase in phosphate excretion, as expected. In animals infused with the lowest dose of MEPE, there was a small (but statistically significant) rise in phosphate excretion, and in those treated with the two higher doses there was a large increase in phosphate excretion, which was statistically significant in both cases.

3.4.5 Plasma phosphate

Plasma phosphate did not differ between groups during the control period or during the experimental period. Although there was a trend towards a fall in plasma phosphate between control and experimental periods in all groups, this did not reach statistical significance (see Table 3.2). The largest fall was in the PTH-treated rats, but even here it just failed to reach significance.

	Control period	*P*	Experimental period
Saline	2.18 ± 0.09	0.37	2.00 ± 0.07
PTH	2.15 ± 0.12	0.09	1.92 ± 0.15
MEPE 1	2.14 ± 0.09	0.63	2.05 ± 0.06
MEPE 2	2.12 ± 0.12	0.84	2.02 ± 0.11
MEPE 3	2.10 ± 0.03	0.55	2.02 ± 0.12

Table 3.2 Plasma phosphate concentrations

Results are in mmol/L, expressed as mean values ± SEM. In each group, n = 6 rats.

3.4.6 Filtered load of phosphate

The measurements of GFR and plasma phosphate concentration indicated that the PTH- and MEPE-induced increases in phosphate excretion could not be attributed to the filtered load of phosphate. However, in order to confirm that changes in urine phosphate excretion were due to a change in renal tubular phosphate handling, the filtered load of phosphate was calculated for each animal. These results are shown in Figure 3.5. The filtered load fell slightly between control and experimental periods in every group of animals, but only in the MEPE 1 group did this fall reach statistical significance.

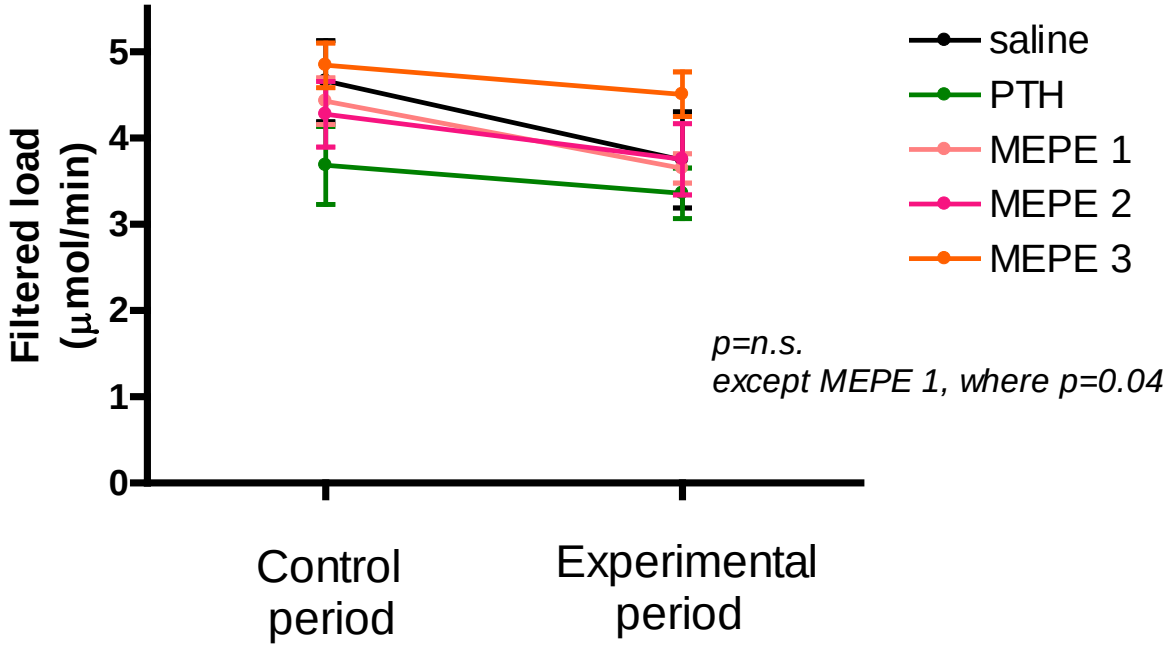


Figure 3.5 Filtered load of phosphate

Results are presented as mean values ± SEM. In each group, n= 6 rats.

3.4.7 Fractional phosphate excretion

Fractional phosphate excretion (FE_{Pi}; Figure 3.6) echoed the absolute excretion rates. Thus, FE_{Pi} remained unchanged in the control animals; there was a marked increase in FE_{Pi} in animals given PTH; and there was a dose-dependent increase in FE_{Pi} in animals infused with MEPE.

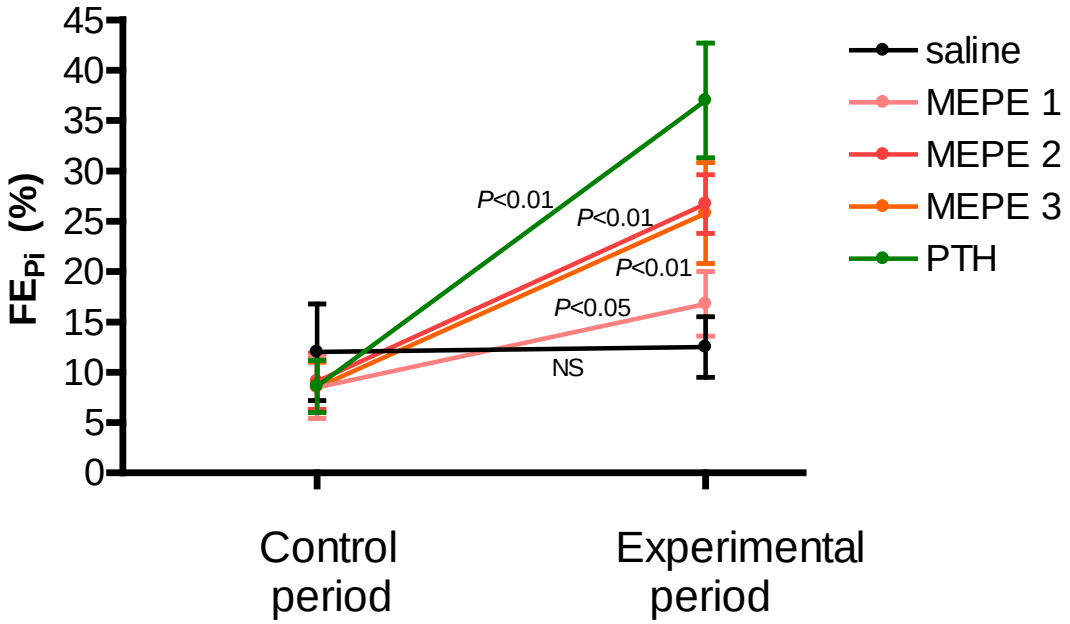


Figure 3.6 Fractional phosphate excretion

Results are presented as mean values ± SEM. In each group, n=6.

In order to determine the dose-response curve for MEPE, the results shown in Figure 3.6 were recalculated, taking the change in FE_{Pi} between the control and experimental periods for each rat (ΔFE_{Pi}). These data are shown in Figure 3.7.

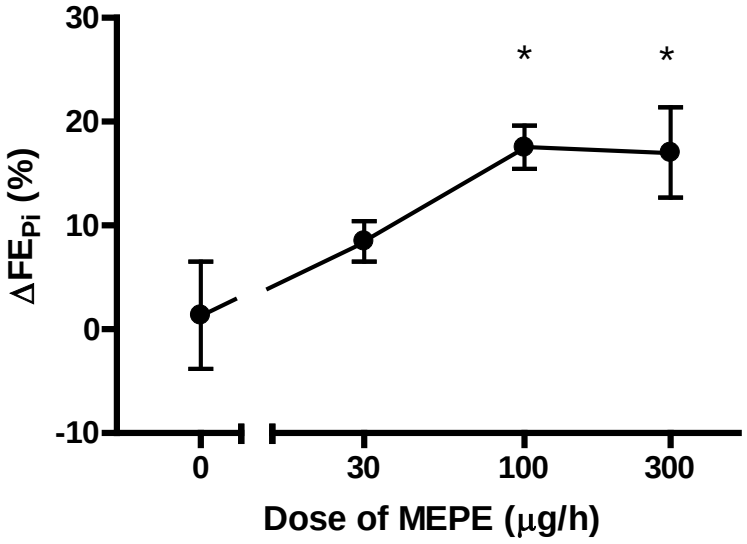


Figure 3.7 Dose-response curve for the effect of MEPE on FE_{Pi}

Results are expressed as mean values ± SEM. In each group, n=6.

Note that the x-axis of this graph is a log scale.

** p<0.01 compared with vehicle- infused rats.*

The change in fractional phosphate excretion was significantly larger than that seen in the control animals ($p<0.01$) for both of the higher doses of MEPE, but not for the low dose.

Statistical note

In addition to using paired and unpaired t tests, all the data in this chapter were analysed by two-way ANOVA, examining the interaction between time and treatment. For blood pressure, GFR, sodium excretion, filtered phosphate load and serum phosphate, there were no significant changes for either time or treatment effects. However, significant changes were seen for absolute and fractional phosphate excretion. Post-hoc testing of these data

using Bonferroni's multiple comparisons test showed significant treatment effects for PTH and both of the larger doses of MEPE, but not (for fractional phosphate excretion) for the low dose of MEPE (see Table 3.3) .

Experimental group	p-value for Bonferroni post-hoc testing of absolute phosphate excretion	p-value for Bonferroni post-hoc testing of FE_{Pi}
Vehicle	ns	ns
PTH	<0.01	<0.01
MEPE 1	<0.05	ns
MEPE 2	<0.01	<0.01
MEPE 3	<0.01	<0.01

Table 3.3 Post-hoc testing of results significant by 2-way ANOVA

3.5 Discussion

Typical GFR values for clearance experiments using adult Sprague-Dawley rats are in the range 1.5-3 ml/min (Webster & Haramati, 1985). Many authors used slightly larger animals than I did (for example Burnatowska-Hledin et al, 1985: average weight 300g, GFR 2.3ml/min; Carney and Thompson, 1982: average weight 330g, GFR 2.6ml/min). For animals of 250g, as used in the experiments described in this chapter, a mean GFR of around 2ml/min is well within the range of values quoted by others.

Fractional excretion of phosphate (FE_{Pi}) at the start of an experiment depends in part on the animals' diet, and might vary between workers depending on this; mean values in control animals (male Sprague-Dawley rats) used in the clearance experiments of Berndt et al (Berndt et al, 2003) were 10%, with some increase over time in saline-infused animals. Other workers have found values for FE_{Pi} in control animals ranging from 10 - 15% (Burnatowska-Hledin et al, 1985; Rybczynska et al, 2006) to almost 30% (Pflueger et al, 1998). The values I found (around 10%) are therefore at the lower end of these previously reported observations.

The dose of PTH used in the present experiments is similar to that used by Murer and colleagues (Kempson et al, 1995), and in their hands it also caused a highly significant change in urinary phosphate excretion, with fractional excretion of phosphate roughly doubled by PTH infusion. Some workers (e.g. Zhang et al, 1999) have reported that PTH infusion into rats causes a significant diuresis and natriuresis. In addition, clearance experiments using PTH infusions have shown significant effects on GFR in humans (Jespersen et al, 1997). However, these reports are offset by other clearance studies (Harris et al, 1979; Gloor et al, 1979) in which no significant effect of PTH on either GFR or fractional sodium excretion were observed. I found no significant effect of PTH infusion on either GFR or urine sodium excretion. Although there was a tendency for urine sodium excretion to increase in all groups of animals over the course of the experiment, this increase was only statistically significant in one of the MEPE-infused groups.

In previous work on mice, Rowe and his group used MEPE intraperitoneally (Rowe et al, 2004). They used a protocol of repeated injections at two doses, totalling 40μg/kg and

400μg/kg. At the lower of these two doses they reported no significant effect of MEPE on 'fractional' phosphate excretion (assessed by use of a single, pooled urine sample). At the higher dose, significant effects were seen. After correspondence with this group (P. Rowe, personal communication), I performed pilot experiments using an intravenous dose of MEPE (30 μg/hr over two hours), which is less (per kg body weight) than the higher of Rowe's two doses. After obtaining modest increases in fractional phosphate excretion using this dose, I went on to construct a dose-response curve using this dose and successive multiples of it.

The clearance results presented in this chapter demonstrate that MEPE infusions cause a dose-dependent phosphaturia and a dose-dependent increase in fractional phosphate excretion, without any significant change in GFR, serum phosphate concentration or filtered load of phosphate. The increase in phosphate excretion must therefore be due to a change in tubular phosphate handling. It is notable that the phosphaturia following the highest dose of MEPE approached that achieved by PTH.

MEPE was originally isolated from patients with tumour-induced hypophosphataemia. The results presented here demonstrate that this protein might at least partly account for the renal phosphate wasting seen in these patients, and MEPE can therefore be put forward as a putative phosphatonin under these circumstances. This is not to ignore the fact that other substances have also been identified as putative phosphatonins in these patients (see Chapter 1); it may be that different patients have different proteins circulating or that numerous proteins circulate in some or all of these patients. It is more difficult to comment on a possible physiological role of MEPE (if any) as there is little information about the plasma concentration of MEPE in healthy humans or in healthy animals. Preliminary work has indicated that significant levels of MEPE do circulate in humans and suggests wide variations in plasma MEPE concentration between individuals (Jain et al, 2004).

3.6 Conclusions

MEPE has been shown in this chapter to be phosphaturic in anaesthetised rats. A dose-response curve has been obtained, and comparisons made with the phosphaturic effect of PTH, a substance known to be of physiological importance in the regulation of serum phosphate. The MEPE-induced phosphaturia was due to a reduction in fractional phosphate reabsorption rather than to changes in the filtered load of phosphate.

The site of action of MEPE along the nephron could not be ascertained from the experiments described in this chapter, but, given the magnitude of the response and the fact that the bulk of phosphate reabsorption occurs in the (early) proximal tubule, this nephron segment must be the most likely candidate. In support of this, *in vitro* studies by others have shown that phosphate uptake in cultured renal proximal tubule cells is reduced during incubation with MEPE (Rowe et al, 2004), while a recent study has reported MEPE immunoreactivity and mRNA in the proximal tubule (Ogbureke & Fisher, 2005). However, in order to assess this issue directly, examination of segmental phosphate transport using micropuncture experiments is required.

Chapter 4

Localisation of the site of action of MEPE

4.1 Introduction

4.1.1 Clearance methods for assessment of segmental reabsorption

As indicated in Chapter 3, measurement of GFR can tell us how much of a substance is filtered at the glomerulus, and comparison with the amount excreted in the urine allows calculation of the overall whole-kidney and whole-nephron handling of filtered substances. Localisation of the nephron segment(s) responsible for the reabsorption or secretion of a substance requires the use of further techniques. A number of indirect methods, based on renal clearance measurements, have been suggested for assessment of proximal tubular function without recourse to invasive techniques.

4.1.1.1 Urate clearance

Urate is freely filtered at the glomerulus and is reabsorbed only in the proximal tubule. However, it is an unsatisfactory marker of proximal tubular function because urate clearance measures the balance of reabsorption and secretion in the renal tubule, and urate secretion in the proximal tubule is highly variable (see Shirley & Walter, 1993).

4.1.1.2 Lithium clearance

Renal lithium clearance has been widely used as an indirect way of assessing segmental tubular function, as it has been shown that lithium clearance acts as a reasonable marker of sodium and water delivery from the proximal tubule (reviewed in Thomsen & Shirley, 1997). This is based on the assumptions that lithium is reabsorbed in the proximal tubule in the same proportion as sodium and water, and is neither secreted nor reabsorbed in the remainder of the nephron. These assumptions have been extensively tested and are not absolutely valid (about 10% more lithium than water is in fact delivered to the end of the proximal tubule, while there is also a small amount of lithium uptake in the loop of Henle), but the errors are modest and in opposite directions, tending to cancel one another out; thus lithium clearance can provide reliable qualitative information (Shirley & Walter, 1993). Use of lithium clearance has the advantages of allowing experiments on conscious

animals and in humans, and the technique has been widely used to study renal sodium and water handling.

In contrast to sodium, phosphate makes up only a small proportion of total ions and osmolality in tubular fluid (less than 5% even under extreme conditions), and therefore even large changes in proximal phosphate handling will not alter end-proximal water (and lithium) delivery substantially; for this reason lithium clearance is unlikely to allow detection of specific changes in renal phosphate handling. However, if there are large changes in proximal solute and water handling in general, phosphate is likely to be affected.

4.1.1.3 Phosphate clearance

At one time it was suggested that phosphate itself could be used as a marker of end-proximal fluid delivery. However, although most phosphate reabsorption undoubtedly does occur in the proximal tubule (see below), it is now clear that the amount of phosphate absorbed in the proximal tubule is not related to sodium and water reabsorption in any fixed way, but depends on a number of regulatory factors (see Chapter 1). Therefore, the ratio of tubular fluid-to-plasma phosphate (TF/P_{Pi}) varies enormously, depending on the circulating concentration of PTH (see Figure 4.1) and other phosphaturic factors. In addition, there is evidence for at least some phosphate reabsorption beyond the late proximal tubule.

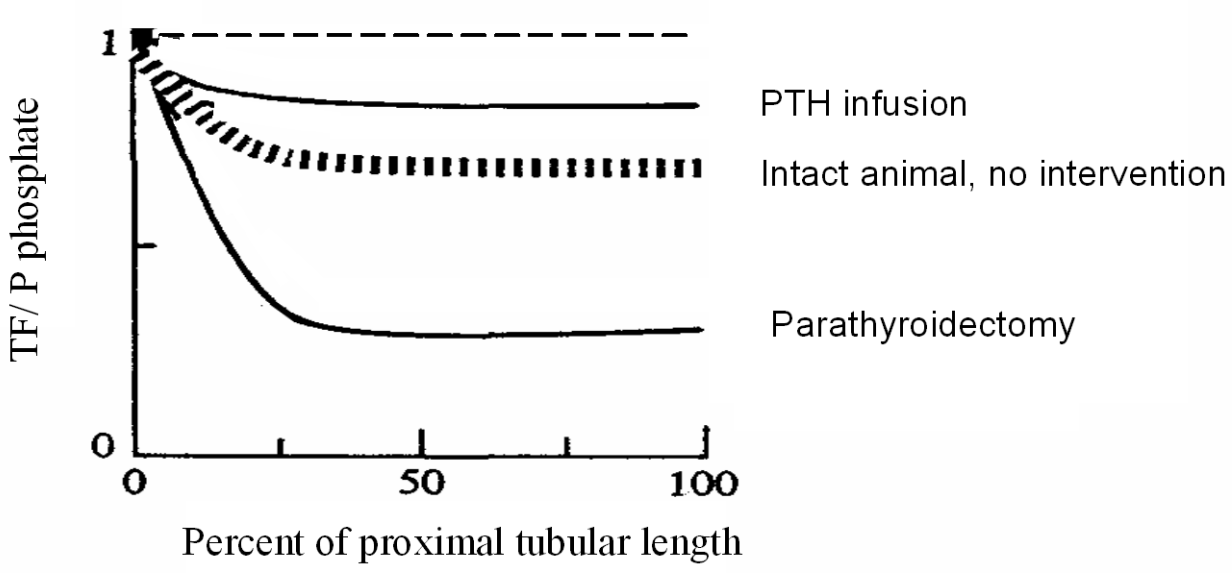


Figure 4.1 Approximate ratio of proximal tubular fluid-to-plasma phosphate (TF/P_{Pi}) concentrations under various conditions

The figure is adapted from Knox et al, 1973

It is clear, therefore, that indirect methods as outlined above are not likely to be of help in assessing the sites(s) of altered phosphate transport. Even the most reliable clearance technique (lithium clearance) can only point to changes in proximal tubular reabsorption of water (and sodium); a selective change in phosphate reabsorption will not be detectable.

4.1.2 Micropuncture

Renal tubular micropuncture allows direct access to renal tubular fluid, and thus provides direct information about the functions of discrete sections of the nephron. The techniques of renal micropuncture were developed in the 1920s and 1930s by the pioneering figures of Wearn and Richards (Wearn & Richards, 1924, reviewed in Gottshalk, 2000). These techniques are time-consuming and technically difficult (Velazquez & Wright, 1992) and there is also a requirement for accurate determination of very small volumes and analytes within these small volumes. A further problem with the technique is that it is invasive and can only be performed on anaesthetised animals. Despite these difficulties, micropuncture techniques have the overwhelming advantage of allowing direct *in vivo* demonstration of

changes in tubular fluid composition in response to stimuli in intact animals, information which cannot be obtained in any other way.

Micropuncture has identified the site of reabsorption and secretion of all physiologically important ions and molecules along the nephron, and has told us most of what we know about how the kidney actually works, from elucidating the counter-current mechanism for generating concentrated urine (Gottschalk & Mylle, 1959) to direct evidence for tubulo-glomerular feedback (Thurau & Schnermann, 1965). Micropuncture has also provided direct demonstration of the filtering role of the glomerular membrane, by allowing comparison of the filtered fluid in Bowman's space with plasma.

It must be emphasised that only certain parts of the nephron - those on the surface of the kidney - are generally accessible by means of micropuncture techniques. Further, it is usually only possible to access those nephrons which lie at the surface, which may not be typical of all nephrons. Therefore, only the first two-thirds of the proximal tubule of superficial nephrons (the proximal convoluted tubule; pars convoluta) is accessible; the final third (the pars recta) is not. Moreover, in most animals the glomeruli are not visible on the renal surface and so, other than in certain strains of animals in which a small fraction of glomeruli are found on the surface, the initial segment of the proximal convoluted tubule (the S1 segment) is not accessible. See Figure 4.2 for a diagrammatic representation of a typical superficial and deep nephron, illustrating these points.

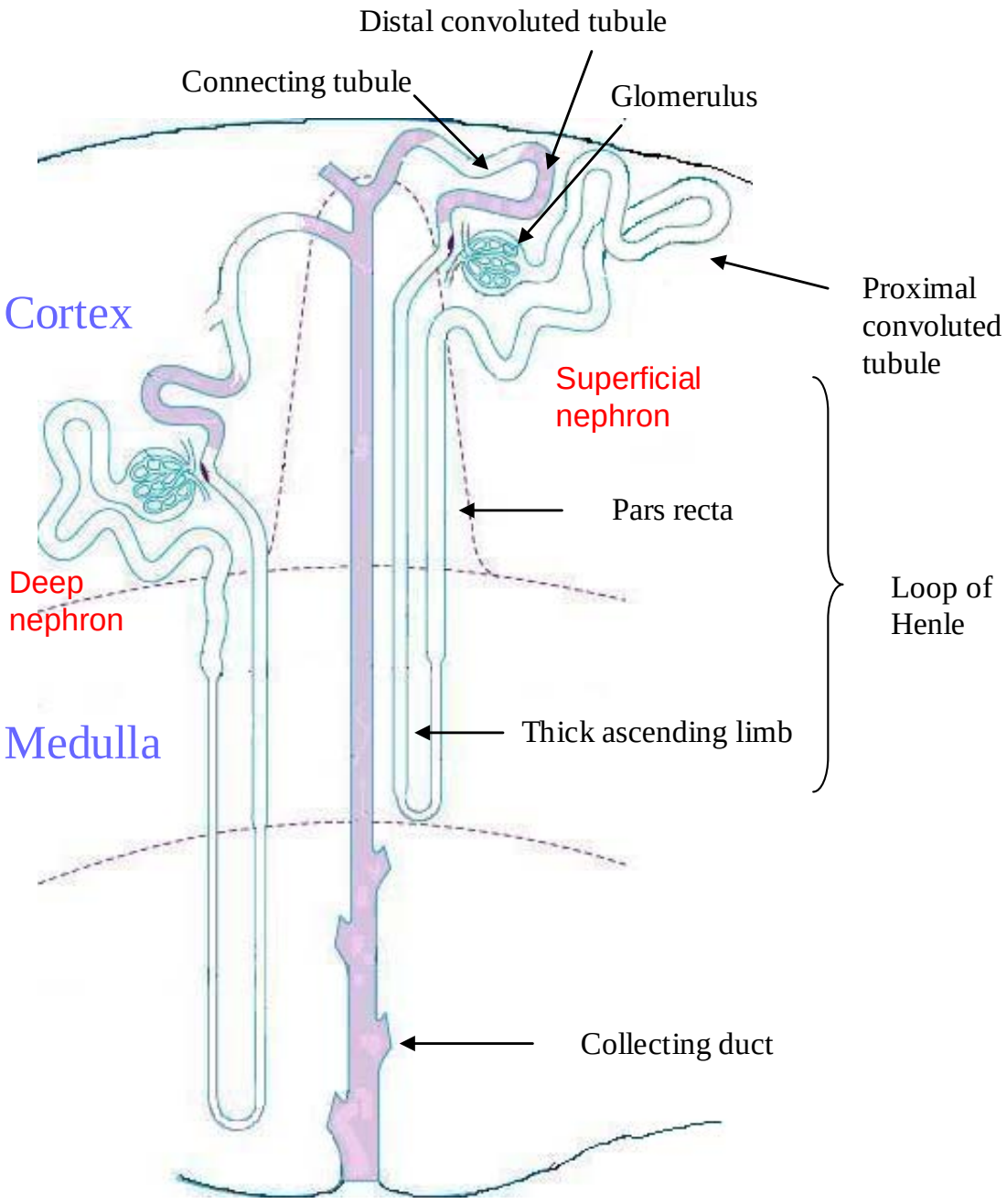


Figure 4.2 Diagram of structure of a superficial and a deep nephron

(Adapted from Kriz & Bankir, 1988)

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4.1.2.1 Micropuncture assessment of renal phosphate handling

Glomerular filtration

If the glomerular membranes provided no barrier to phosphate (that is, if phosphate were freely filterable), the concentration of phosphate in Bowman's space would be slightly higher than in plasma, for two reasons. Firstly, plasma contains (non-filterable) proteins and is only about 94% water. Secondly, the Gibbs-Donnan effect means that filterable anions will be present on the tubular side of the membrane in higher concentration than on the plasma side, because at plasma pH non-filterable proteins are negatively charged and so their overall negative charge is retained in the plasma, balanced by retention of a slight excess of cations. Set against these two effects is the fact that about 10% of plasma phosphate is protein-bound and not filterable (Suki & Rouse, 1996). In the event, data obtained from Munich-Wistar rats (in which some of the glomeruli are located superficially and so can be directly accessed by micropuncture) indicate that the phosphate concentration in glomerular ultrafiltrate is on average slightly lower than that in plasma water (Harris et al, 1974). Surprisingly, these authors found that the glomerular fluid/plasma water phosphate concentration ratio was different in male and female rats, averaging 0.96 in female rats and only 0.79 in male rats, whereas no significant differences between males and females in the filterability of sodium, calcium, inulin or chloride were demonstrated. In contrast, another group (also working on Munich-Wistar rats) found that the glomerular fluid/plasma water phosphate concentration ratio was almost exactly unity in male rats and around 0.95 in female rats (Grimellec et al, 1975). In view of these conflicting micropuncture findings, I have elected to follow most others in this field and equated filtered phosphate concentration with that of plasma.

Tubular handling

Strickler and colleagues (Strickler et al, 1964) reported in the 1960s that proximal tubular fluid /ultrafiltrate phosphate ratios were 0.73 in the rat, indicating that phosphate was normally reabsorbed in excess of water in the proximal tubule. They reported that 24% of filtered phosphate was delivered to the distal tubule and 22.5% was present in the final urine, suggesting little distal reabsorption or secretion of phosphate, at least under the conditions of this experiment.

Free-flow micropuncture experiments in the 1970s (Boundry et al, 1975) confirmed that most phosphate reabsorption takes place in the proximal tubule, particularly the early proximal tubule, with a steady decline in the fraction of phosphate remaining further along the proximal tubule. These authors did find, however, that the fractional excretion of phosphate in the urine was lower than the fractional delivery to the late part of the distal tubule, suggesting that there might be some net reabsorption of phosphate along the terminal nephron. Indeed, free-flow micropuncture experiments in dogs (Knox & Lechene, 1975) suggested that part of the phosphaturic effect of PTH in these animals was due to actions in the distal nephron. An alternative (or additional) explanation is that inaccessible deep nephrons might reabsorb proportionately more phosphate than the accessible superficial ones. These deeper nephrons feed into the same collecting ducts as the superficial nephrons (see Figure 4.2), potentially explaining why there appears to be less phosphate in the urine than is delivered to the superficial distal tubules.

Microinjection studies using ^{32}P (Brunette et al, 1973) agreed that the majority of phosphate reabsorption was in the proximal convoluted tubule, while a significant proportion was reabsorbed in the inaccessible part of the nephron between late proximal convoluted tubule and early distal tubule (that is, in the anatomical loop of Henle); no phosphate reabsorption occurred distal to injections into the early distal tubule. A technically very demanding stationary microperfusion study in rats also provided evidence for phosphate reabsorption in the proximal convoluted tubule and additionally in the adjacent descending part of the proximal tubule (pars recta). Again, no evidence was found for phosphate reabsorption in the distal tubule; nor was any reabsorbed in the ascending limb of the loop of Henle (Lang et al, 1977).

Rouffignac's group has addressed the issue of phosphate reabsorption in the distal nephron in more detail by means of injection of ^{32}P into early distal tubules of various strains of rats and analysis of urinary recovery (Poujeol et al, 1977). In the Munich-Wistar strain there was no phosphate reabsorption beyond the distal tubule, whilst for two other strains (Sprague-Dawley and Long-Evans) significant reabsorption was seen. In a subsequent series of experiments in the Munich-Wistar strain (Poujeol et al, 1980), microinjection experiments were performed in juxtamedullary (deep) nephrons by

exposing the papilla through a small hole in the ureter. It was shown that these nephrons reabsorbed a considerable amount of phosphate between the tip of the loop of Henle and the urine, whereas in superficial nephrons there was no net phosphate reabsorption when radiolabelled phosphate was injected into the distal tubule. These experiments suggest that in the Munich-Wistar strain of rats, tubular phosphate handling differs between superficial and deep nephrons.

Immunohistochemistry also reveals some differences between superficial and deeper nephrons: Segawa et al (2002) showed that staining for the NaPT2c transporter is much greater at the proximal tubule brush border of juxtamedullary nephrons than in superficial or midcortical nephrons, whereas NaPT2a is found more widely and uniformly.

In summary, although there is some equivocal evidence for a small amount of phosphate reabsorption in the distal nephron, the consensus is that the vast majority of renal phosphate reabsorption normally occurs in the proximal tubule. Immunolocalisation data and results from knock-out animals suggest that most of this regulated renal phosphate reabsorption is due to the activity of the NaPT2a co-transporter (see Chapter 1).

4.1.3 Location of the action of MEPE

As discussed in Chapter 1 and above, most currently recognised regulated phosphate reabsorption takes place in the proximal convoluted tubule, particularly the S1 segment. PTH acts by altering the number of NaPT2a co-transporters in the proximal tubule brush-border membrane (see Chapter 1). Most other factors (for example, thyroxine and growth hormone) known to affect renal phosphate reabsorption are also believed to act on the proximal tubule, but little direct evidence for this has been produced. No data concerning the location of the site of activity of MEPE are currently available. A preliminary report by Bresler and colleagues (Bresler et al, 2004) found that antibodies to ASARM peptides (the ASARM motif is a putative active part of the MEPE molecule, see Chapter 1) bind to the proximal tubules, and this suggests that there could be an interaction between MEPE and the proximal tubule. In addition, immunohistochemisty of renal sections showed strong staining for MEPE in the proximal tubule, and particularly around the area of the

apical surface of the proximal tubule, which was suggested as showing possible binding of MEPE to an as yet uncharacterised receptor in this area (Obureke & Fisher, 2005). Nevertheless, no study has assessed whether MEPE can act at this nephron site.

4.2. Aim of the present experiments

The present experiments were designed to investigate the mechanism of the phosphaturia induced by MEPE, using micropuncture to assess directly proximal tubular function. For consistency, and so as to demonstrate the maximal change in tubular fluid phosphate handling, the most distal accessible part of the proximal tubule was used for sampling in every experiment. Experiments were performed using saline infusion as a time control and PTH infusion as a positive control. The dose of MEPE was based on the dose-response curve constructed in the clearance experiments (Chapter 3). Each experiment included an initial baseline control period, that is, a period before infusion of MEPE or PTH, to take into account the considerable between-animal variation in baseline phosphate handling.

4.3 Methods

4.3.1 Experimental protocol

Adult male Sprague-Dawley rats, of mean body weight 249g (range 240-260g), were anaesthetised with intraperitoneal sodium thiopentone (100mg/kg body weight; Link Pharmaceuticals, Horsham, Sussex) and prepared surgically for micropuncture studies. The surgery was as described in Chapter 3 for the clearance experiments, but with the addition of a flank incision to expose the left kidney. Perirenal fat was removed and the kidney was placed in a specially designed Perspex dish, with a gap for entry and exit of the renal artery and vein and the ureter; the dish was clamped to the operating table in order to minimise movement of the exposed kidney during respiration. The kidney was bathed continuously in mineral oil heated to 37^{o}C. The left ureter was catheterised with polyethylene tubing (Portex Plastics, Hythe, Kent). Animals were infused with 0.9% NaCl solution at 4ml per hour throughout. Experiments were begun after a two-hour

equilibration period; after the first hour of the equilibration period a bolus of tritiated inulin (60 μCi) was given, followed by an infusion of tritiated inulin at a rate of 60 μCi/hour. During an initial one-hour control period, urine was collected from the ureter and 3-4 micropuncture samples were collected from late proximal convoluted tubules (see Figure 4.3). Samples were obtained using micropipettes, tip internal diameter 9-10μm, inserted into candidate proximal tubular loops. Microdroplets of Sudan black-stained oil were then injected into the tubules; no reappearance of the droplet or only one reappearance was taken to indicate that the puncture site was appropriate, i.e., in the late proximal convoluted tubule. Multiple reappearances of the droplet indicated placement too proximally, in which case the pipette was withdrawn and a new nephron was selected for a further attempt. Once a suitable nephron segment had been entered, a column of oil 4-5 tubular diameters in length was injected and allowed to flow just distal to the puncture site. Gentle aspiration was then applied to the pipette so that tubular fluid arriving at the collection site was drawn into the micropipette. By maintaining the position of the oil column, it was ensured that the tubular fluid was collected at the same rate as it arrived at the puncture site. Thus, complete collection of tubular fluid was confirmed. Tubular fluid was collected continuously for periods of six to twelve minutes; once each collection was completed the pipette was withdrawn and the tubular sample deposited under oil onto a watch-glass. After any puncture, the next puncture site was selected following a systematic pattern that ensured that no nephron was sampled more than once. At the end of each collection, Microfil (Flow-Tec, Carver, MA, USA) was injected into the nephron through another micropipette, filling the nephron lumen. This substance hardens into a cast of the nephron which can be examined under the microscope at the end of the experiment. Subsequent examination of these Microfil casts confirmed late proximal location of tubular fluid collection points. Immediately following the control period, the intravenous infusion was changed to MEPE in seven animals (100μg/hr), or changed to PTH in seven animals (20μg/hr) or continued as saline alone in another seven animals, for the next two hours (see figure 4.3). During the second of these two hours (the experimental period), urine and micropuncture collections were performed again, using identical methodology.

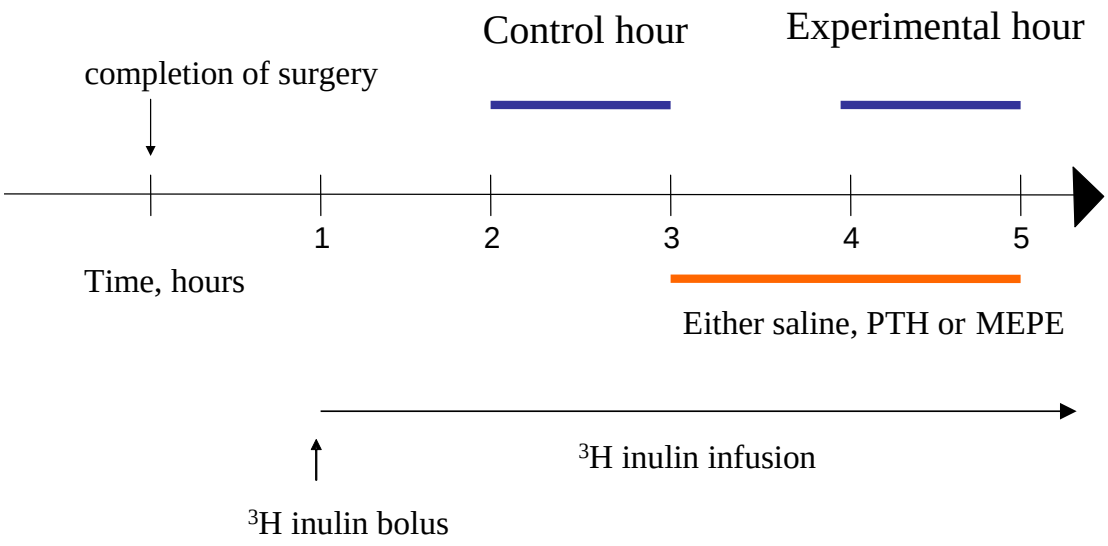

Figure 4.3 Protocol for micropuncture experiments

Blood samples were collected hourly from the arterial catheter. Each sample was approximately 100µl and was collected by unclamping the catheter and allowing blood to flow slowly under arterial pressure into a sample collection vial. Blood samples were spun at 1500g for five minutes and the plasma samples thus generated were then split for tritium and phosphate analysis.

4.3.2 Analyses

Urine, plasma and tubular fluid [^{3}H] inulin activities were determined using β–scintillation spectroscopy (Packard Tricarb, Model 2900TR; Pangbourne, Berks, UK) after dispersal in Ultima Gold scintillation fluid (PerkinElmer, Beaconsfield, Bucks, UK). Urine [^{3}H] inulin was determined by triplicate counts of 5µl samples after first diluting the urine 200-fold. Plasma [^{3}H] inulin was determined by triplicate counts of 5µl samples of

the plasma fraction of centrifuged blood samples (aliquoted using ‘Supracaps’, Scientific Laboratory Supplies, Nottingham, UK); plasma inulin at any given time (for comparison with corresponding tubular fluid samples) was calculated assuming that counts altered linearly between successive hourly determinations. The tubular fluid samples under oil were subdivided; using previously calibrated constriction pipettes, duplicate samples were deposited in ultrapure water in CE microvials for subsequent ion analysis (62 nl sample in 200 times dilution of ultrapure water) and duplicate samples (of 38nl) were deposited in scintillation vials for [^{3}H] inulin determination. Urine samples were diluted 1:200 in ultrapure water and then frozen for later CE analysis. Plasma samples were diluted 1:100 in ultrapure water and then frozen for later CE analysis.

Sodium concentrations in urine were measured by flame photometry (model 543, Instrumentation Laboratory, Warrington UK), following appropriate dilution if required.

4.3.3 Calculations

GFR was calculated as inulin clearance, where

$GFR = C_{in} = [U_{in}] \times V_U/[P_{in}]$, where

C_{in} = inulin clearance

$[U_{in}]$ = urinary inulin count

V_U = urine flow rate

$[P_{in}]$ = plasma inulin count.

Single-nephron GFR (SNGFR) was calculated correspondingly as single-nephron inulin clearance:

$SNGFR = [TF_{in}] \times V_{TF}/[P_{in}]$, where

$[TF_{in}]$ = tubular fluid inulin count

V_{TF} = tubular fluid flow rate.

Phosphate clearance was calculated as in Chapter 3.

The fractional excretion of phosphate (FE_{Pi}) was calculated as

$$FE_{Pi} = C_{Pi}/C_{in},$$ where C_{Pi} = phosphate clearance.

The fractional delivery of phosphate to the late proximal convoluted tubule (FD_{Pi}) was calculated as the tubular fluid: plasma concentration ratio for phosphate divided by that for inulin:

$$FD_{Pi} = TF/P_{Pi/in}.$$

4.3.4 Statistics

Results are presented as means ± SEM. Comparisons between the micropuncture and contralateral kidney in each group were made using Student's paired t-test. Comparisons between results obtained during the control period and the experimental period in each group were likewise compared using Student's paired t-test. One-way ANOVA was used to make comparisons between all three groups during the control period and during the experimental period. Two-way ANOVA was used when groups in both control and experimental periods were simultaneously compared, followed (if significance was indicated) by Bonferroni's test. In all cases, a *P* value of less than 0.05 was considered statistically significant.

4.4 Results

4.4.1 Whole-kidney GFR

GFR data were collected to allow calculation of the amount of phosphate filtered during each collection period. Values were obtained for both the micropuncture kidney and the contralateral kidney, in order to assess whether the extra micropuncture surgery altered the overall function of the exposed kidney. The results are shown in Figure 4.4 and Table 4.1.

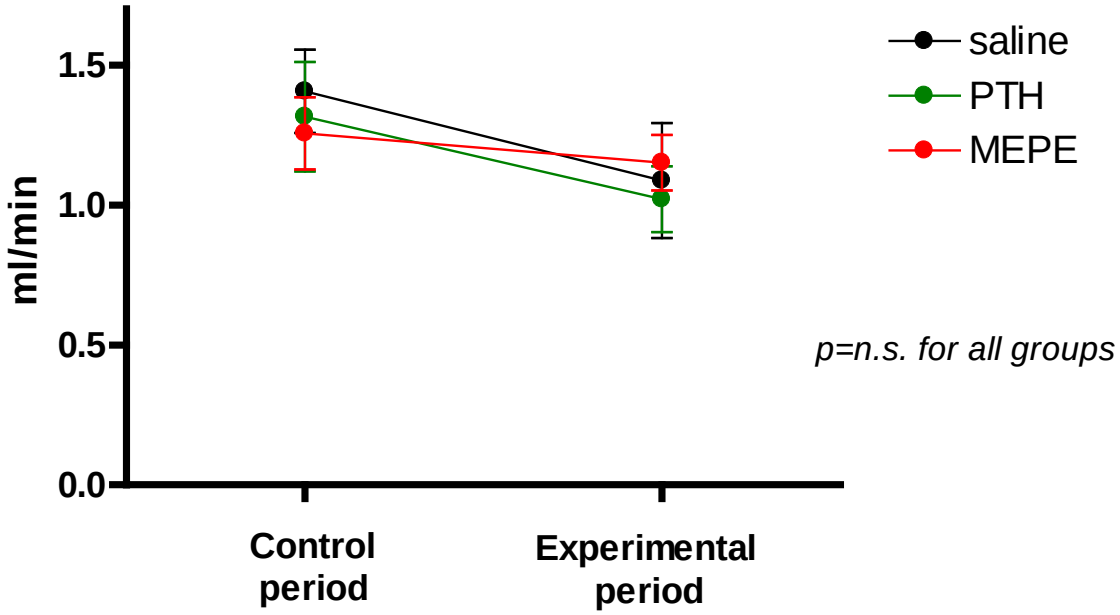

Figure 4.4 Single-kidney GFR

Values are presented as means ± SEM; there were 7 rats in each group.

Results are for the micropuncture kidney (only)

	Control period			Experimental period		
	Micropuncture kidney	*p*	Contralateral kidney	Micropuncture kidney	*p*	Contralateral kidney
Saline	1.40 ± 0.14	0.89	1.43 ± 0.18	1.09 ± 0.21	0.79	1.14 ± 0.26
PTH	1.32 ± 0.20	0.76	1.52 ± 0.28	1.02 ± 0.12	1.00	1.02 ± 0.16
MEPE	1.26 ± 0.13	0.33	1.16 ± 0.12	1.16 ± 0.10	0.99	1.15 ± 0.08

Table 4.1 Single-kidney GFR

Values (means ± SEM) are in ml/min; there were 7 animals in each group

The mean GFRs were not significantly different between the three groups, either during the control period or the experimental period. In all groups there was a modest fall between control and experimental periods, though this was not statistically significant. There was good agreement between the GFRs obtained from the micropuncture side and the contralateral side, with no significant differences when analysed using paired t-tests (Table 4.1), providing some reassurance that exposure of the micropuncture kidney and placing it in a dish did not significantly alter its overall function.

4.4.2 Single-nephron GFR

Single-nephron GFR for each group is shown in Figure 4.5. The value used for each animal in each period was an average of several (at least three) determinations.

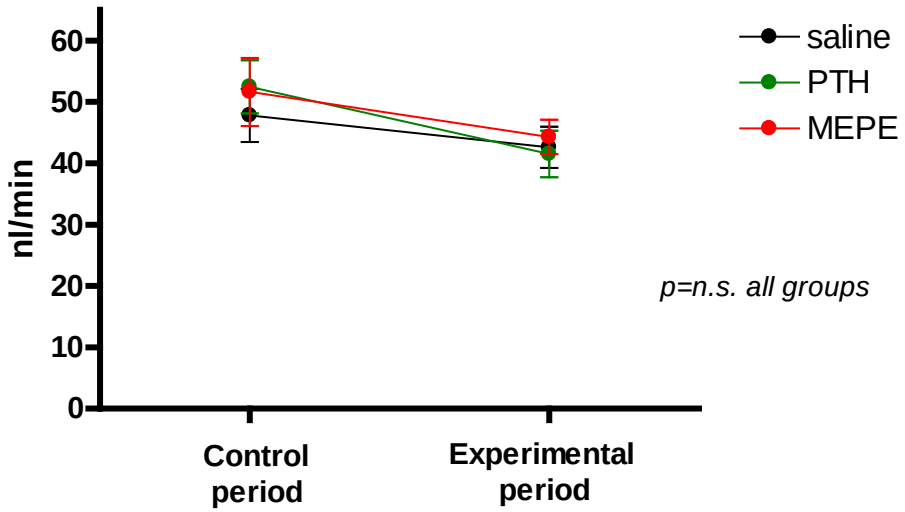

Figure 4.5 Single-nephron GFR (SNGFR)

Values are presented as means ± SEM; there were 7 animals in each group

Single-nephron GFRs were almost identical in the three groups during the control period. As with the whole-kidney GFR results presented above, SNGFRs were slightly (though

not significantly) lower during the experimental period, but there were no significant differences between groups.

4.4.3 Urinary sodium excretion

These results are shown in Table 4.2. Mean sodium excretion rose in every group during the course of the experiment (although the variance was large), but overall sodium excretion (left plus right kidneys) did not increase significantly in any of the groups. The only statistically significant increase was that in the contralateral kidney of MEPE-infused animals. There were no significant differences in sodium excretion between the three groups in either time period.

Finally, there were no significant differences in sodium excretion between micropuncture and contralateral kidneys, except in the saline group during the experimental period.

	Control period			Experimental period		
	Micropuncture kidney	*P*	Contralateral kidney	Micropuncture kidney	*P*	Contralateral kidney
Saline	94 ± 39	0.11	134 ± 59	110 ± 41	0.03	151 ± 46
MEPE	103 ± 29	0.69	95 ± 35	145 ± 47	0.77	138 ± 43*
PTH	84 ± 30	0.37	76 ± 27	90 ± 20	0.68	84 ± 25

Table 4.2 Single-kidney sodium excretion

Values are absolute sodium excretions in µmol/h, means ± SEM; n=7 rats in each group. P values refer to differences between urine collected from the micropuncture and contralateral kidneys, assessed by paired t-test.

** P=0.014 compared with the corresponding value during the control period; for other paired comparisons of control vs. experimental periods, P>0.05*

4.4.4 Plasma phosphate concentration

During the control period, plasma phosphate concentrations were 2.05 ± 0.08 mmol/l in the saline group, 2.03 ± 0.12 mmol/l in the PTH group and 2.15 ± 0.12 mmol/l in the MEPE group. In the experimental period the corresponding values were 1.93 ± 0.12, 1.87 ± 0.03 and 2.08 ± 0.06 mmol/l. There were no significant differences between the groups during either period, and no significant change in any group between control and experimental periods.

4.4.5 Urinary phosphate excretion

Results for absolute single-kidney phosphate excretion by the micropuncture kidney are shown in Figure 4.6. There was a wide variation in baseline urine phosphate excretion in individual rats, but closely comparable means in the three groups. When absolute phosphate excretion in the experimental period was compared with that in the control period in each group, there were significant increases in the PTH and MEPE groups, but no significant change in the saline group.

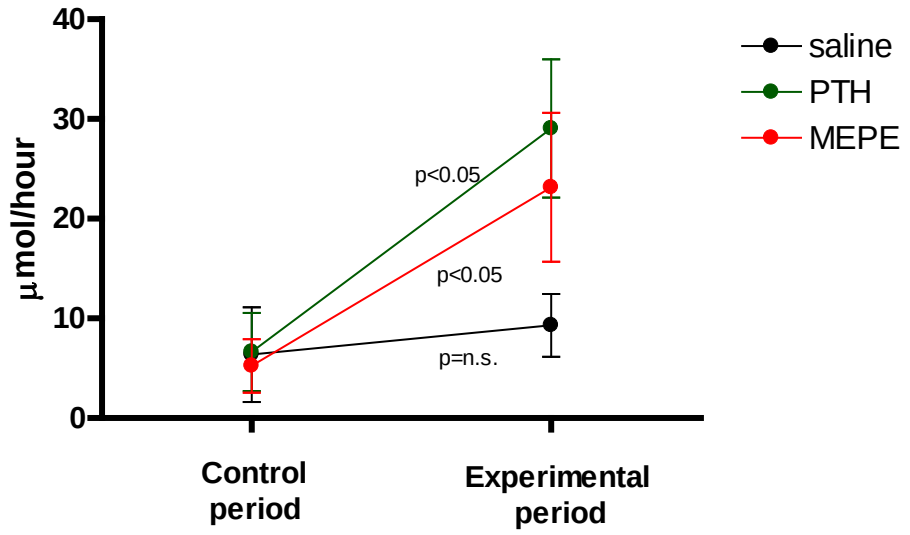

Figure 4.6 Absolute excretion of phosphate

Values are means ± SEM; n=7 rats in each group

When the results were re-analysed as fractional rather than absolute phosphate excretion, similar results were obtained (Figure 4.7). In this case, however, the change in excretion was significant in the saline-infused group as well as in the PTH and MEPE groups, although the size of the change was much smaller in the saline group.

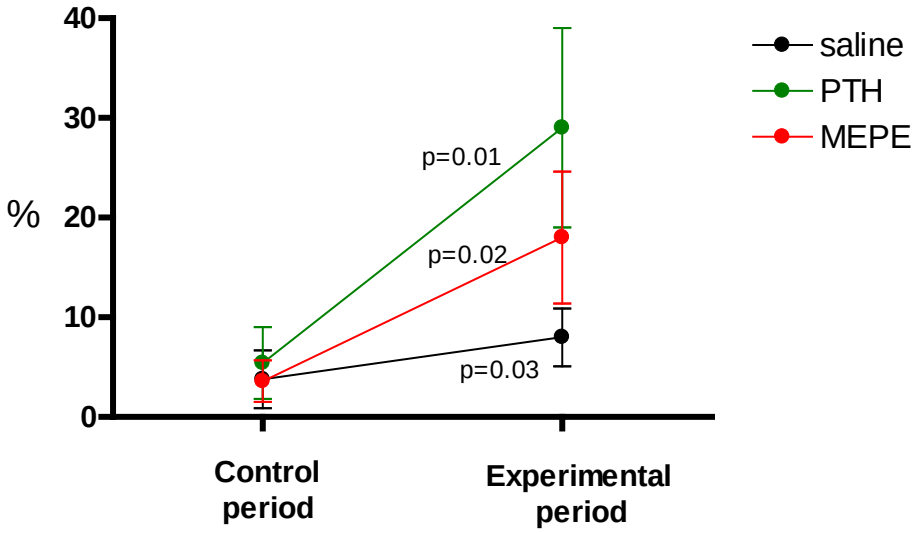

Figure 4.7 Fractional excretion of phosphate

Values are means ± SEM; n=7 rats in each group.

4.4.6 Tubular fluid phosphate

Tubular fluid/plasma phosphate concentration ratios are shown in Figure 4.8. Each data point is the mean (± SEM) calculated from seven animals, and the value in each animal is the average of at least three tubular fluid samples. Once again the raw data showed considerable variation between animals. Although there was a trend towards an increase in TF/P phosphate in the MEPE group, there was no statistically significant change in any of the three groups.

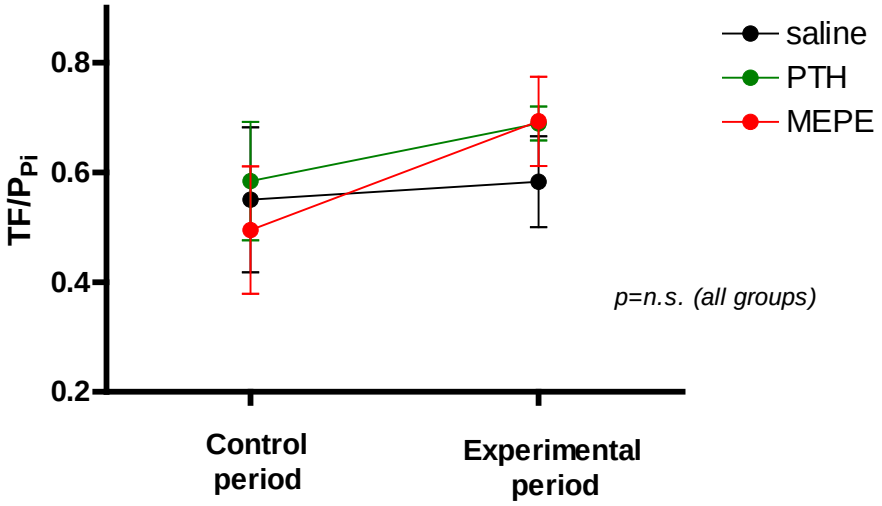

Figure 4.8 TF/P phosphate in the late proximal convoluted tubule
Values are means ± SEM; n=7 rats in each group.

In order to correct for fluid reabsorption along the tubule, the data are also presented as fractional phosphate delivery (FD_{Pi}), calculated as tubular fluid:plasma phosphate/inulin concentration ratios (as inulin is not reabsorbed by the tubules). In control animals infused with saline alone, there was no significant change in FD_{Pi} between the control and experimental periods. In contrast, there was a significant increase in FD_{Pi} in the animals infused with PTH and in those infused with MEPE. These data are presented both in the form of mean results for each individual animal (Figure 4.9), in which each line represents a different experiment and in the form of aggregated data with all the micropuncture results summed (Figure 4.10).

There was considerable variability between fractional phosphate deliveries in different nephrons of the same animal, which cannot be attributed only to time-dependent changes in phosphate transport. Even when tubular fluid collections were made within 15 min of each other there were sometimes large differences in tubular phosphate delivery, indicating that nephrons are markedly heterogeneous with regard to their phosphate handling.

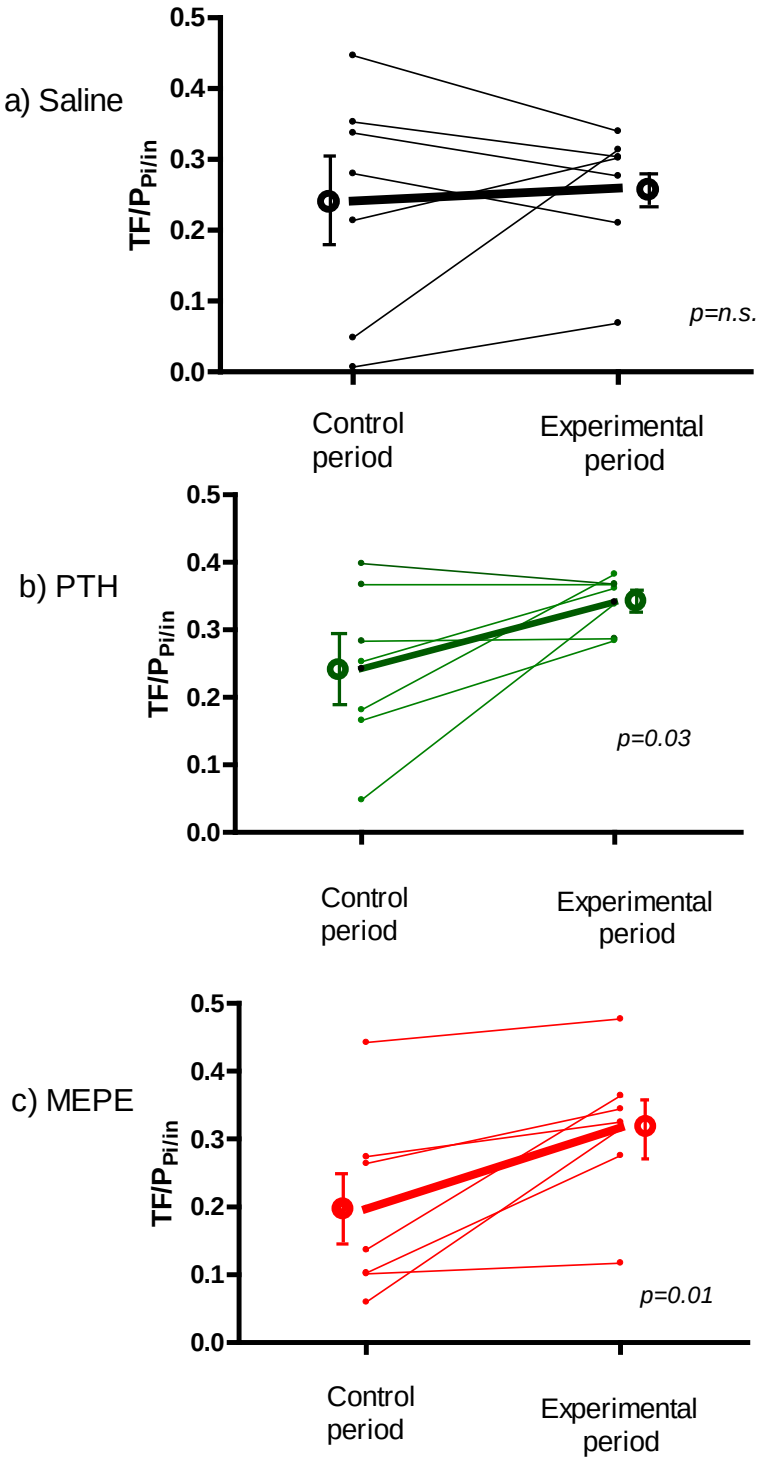

Figure 4.9 Fractional delivery of phosphate (calculated as TF/P $_{Pi/inulin}$) at the late proximal convoluted tubule

Rats infused with (a) saline alone, (b) PTH, or (c) MEPE. Each line connects the mean values in one animal; there were seven rats in each group. Mean (± SEM) data from each group of seven animals are also shown; the bold lines join up those means.

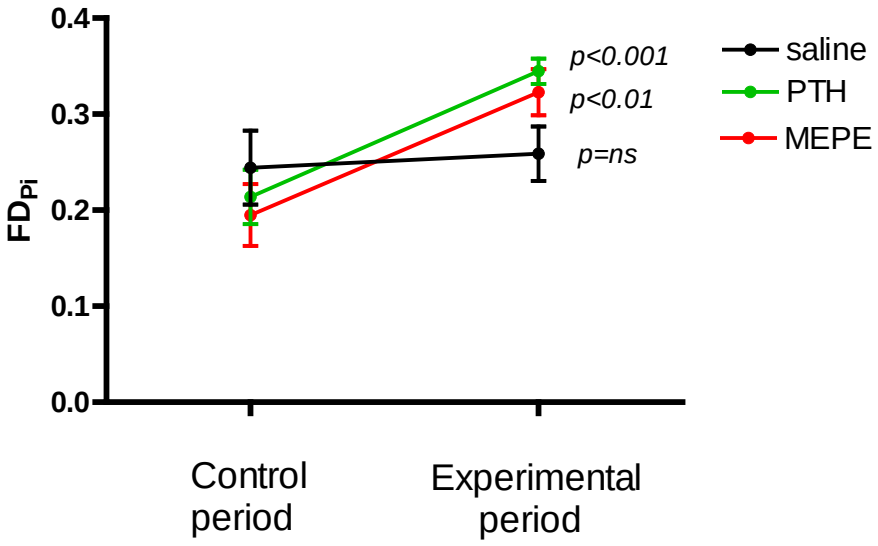

Figure 4.10 Fractional phosphate delivery, using all measured values individually

***In each group**, for each period, n = 21-24. Statistical comparisons between control and experimental periods were by means of Student's unpaired t test.*

Statistical note

In addition to using paired and unpaired *t* tests, all the data in this chapter were analysed by two-way ANOVA, examining the interaction between time and treatment. These analyses confirmed the significance of time but in no case did they show a statistically significant difference between any of the treatments for any of the variables measured.

4.6 Discussion

Most micropuncture workers have used Sprague-Dawley, Wistar or Long-Evans strains of rat for their experiments (Velazquez & Wright, 1992), in most cases (as in my work) using whichever strain is available in their local university colony. Important work has also been done on naturally occurring mutants of these strains, for example the Munich-Wistar rat with accessible surface glomeruli and the Brattleboro rat which is a Long-Evans rat with a hereditary lack of vasopressin. All research strains are highly inbred, in contrast to most human populations which are outbred, and even colonies of the same strain of rat in different centres will tend to diverge over time. Thus, results from different laboratories, even when ostensibly working on identical strains of animal, might differ significantly.

Values for TF/P_{in} in the late proximal convoluted tubule (PCT) were in the range 2-3. These values are similar to those reported by others in rats, and indicate that between 50% and 66% of filtered water is reabsorbed between the glomerulus and the collection site. Control values for TF/P_{Pi} at the late PCT were approximately 0.6, which again agrees with previously reported values (Asplin et al, 1996).

The values for SNGFR are also broadly in line with previously published data (Greger et al, 1977; Woda et al, 2001). SNGFRs in the present experiments were approximately 40-50nl/min. The rat kidney contains about 30,000 nephrons (Bonvalet, 1978; Larsson et al, 1980), so a rough calculation (ignoring differences between the filtration rates of superficial and deep nephrons) would equate an SNGFR of 40nl/min with a single kidney GFR of about 1.2ml/min. This is in fact approximately the GFR found in the present experiments. If anything, the SNGFR/GFR ratio is a little higher than would be expected on this basis, but this small discrepancy may be accounted for by the fact that proximal tubular collections disrupt the tubulo-glomerular feedback mechanism by stopping distal flow. This has the effect of slightly increasing the measured filtration rate in the sampled nephron, but does not alter whole-kidney values (Navar et al, 1974).

Saline was infused into the animals at 4ml/min throughout the experiment, equivalent to an infusion of 600μmol of sodium/hour. The urine sodium data demonstrated modestly

increasing urine sodium excretion during the course of the experiments and a wide range of baseline urine sodium excretions. However, since the average excretion rates were approximately 200μmol/hour, the animals were clearly in a state of overall sodium retention. Urinary sodium excretion has previously been shown to be reduced after surgery in a wide range of situations, in both humans (Paut & Lacroix, 2006) and experimental animals (Maddox et al, 1977; Shirley et al, 1990). Shirley et al (1990) showed, using lithium clearance as a marker of end-proximal fluid delivery, that the anaesthesia/surgery-induced antinatriuresis resulted from enhanced sodium reabsorption in the distal nephron.

I found large variations in the phosphate concentration of tubular fluid sampled from adjacent nephrons even when the sampling was only a few minutes apart. It is worth noting that immunostaining work by Murer's group (Levi et al, 1994) showed very considerable differences between even directly adjacent nephrons in the amount of NaPT2a present in the brush-border membrane. Moreover, data reported by Baum et al (Baum et al, 2005) from experiments on isolated proximal convoluted and straight tubules *in vitro* show phosphate transport varying by at least 50% between tubules. On the basis of these reports, the large variations in tubular phosphate found between spatially and temporally proximate collections are perhaps not surprising.

The phosphaturic effects of MEPE (and PTH) were demonstrated not to be due to increases in filtered phosphate load. Neither single-kidney GFR nor single-nephron GFR was significantly altered by MEPE in the dose used in these experiments, and there was no significant change in serum phosphate concentrations (which in fact went down slightly). Therefore, as previously observed in the clearance experiments, the observed increases in urinary phosphate excretion could only be due to altered tubular handling of phosphate.

During the control period in all groups, and during the experimental period in saline-infused animals, fractional delivery of phosphate to the end of the PCT was found to average 20-25%, whereas overall fractional excretion was only 4-8%. Although this difference between fractional delivery to the late PCT and fractional excretion in the urine seems considerable, in fact it suggests that nearly 80% of filtered phosphate is reabsorbed in the PCT and only a maximum of 16% in more distal nephron segments. It is likely that

this difference represents the contribution of the (inaccessible) pars recta to phosphate reabsorption, although an element of phosphate reabsorption in the distal nephron cannot be ruled out. An additional potential explanation is nephron heterogeneity, with the superficial nephrons accessible to sampling perhaps not being typical of all nephrons.

One obvious difference between the data presented in this chapter and those in the preceding one is that baseline fractional phosphate excretion in the animals undergoing micropuncture was somewhat lower. This effect was consistent in all three groups of animals and occurred despite normal GFRs and SNGFRs in the animals subjected to micropuncture. If anything, GFRs were higher in the micropuncture experiments than in the clearance experiments. Both MEPE and PTH were demonstrated to be phosphaturic under micropuncture conditions, as they were in the clearance experiments, but the final values for fractional phosphate excretion were lower than in clearance experiments using corresponding doses, probably reflecting the lower baseline values. These differences most likely reflect changes related to the differences in surgery, as the animals used in the two series of studies came from the same colony, were the same size (and age), were on the same diet and were given the same anaesthetic.

Initial statistical analyses were by *t* testing, with comparisons between each variable in the control and experimental period, which showed significant effects of MEPE and PTH not only on overall phosphate excretion but also on phosphate reabsorption in the PCT. Further analyses by two-way ANOVA confirmed that there were differences between the initial and experimental periods, but were unable to demonstrate significant differences between the three treatments given. This underlines the difficulty of obtaining statistically robust effects when using a technically demanding method that is subject to significant experimental error as well as reflecting natural variation between animals and tubules. (It is notable that most previous micropuncture workers have reported *t* testing only.)

The main conclusion from the experiments described in this chapter is that MEPE appears to cause a reduction in fractional proximal tubular phosphate reabsorption, which would explain the effects of MEPE on renal phosphate handling demonstrated in the preceding

chapter. These experiments have also confirmed that PTH also acts proximally in a broadly similar fashion.

4.7 Conclusions

The phosphaturic effect of MEPE results largely from inhibition of fractional phosphate reabsorption in the proximal tubule, as does the phosphaturic effect of PTH. It is most likely that this is due to internalisation of the NaPT2a protein from the proximal tubular brush-border membrane.

Chapter 5

Concluding chapter

Concluding chapter

It has long been clear that the actions of PTH and vitamin D alone do not explain either normal phosphate balance or the abnormalities seen in the hereditary hypophosphataemias and oncogenic osteomalacia. It was consequently hypothesised that a single mysterious substance - 'phosphatonin'- existed which could explain the otherwise inexplicable data. In recent years a number of candidate phosphatonins have been described; it is becoming clearer that several of these substances act on the kidneys and/or bones to regulate serum phosphate. It is therefore more accurate to talk of 'phosphatonins' in the plural.

The most widely studied phosphatonin is FGF-23. As indicated in Chapter 1, it has been demonstrated that in some patients with oncogenic osteomalacia FGF-23 levels are greatly elevated, and that the syndrome of autosomal dominant hypophosphataemia is due to mutations in the gene coding for this protein. FGF-23 levels are also elevated in some patients with other phosphate-losing conditions, including fibrous dysplasia of bone and post-transplant hypophosphataemia. However, it is clear that FGF-23 does not alone provide an explanation for all the clinical data. It has been unequivocally shown that it is possible to have phosphaturia in tumour-induced osteomalacia without a raised FGF-23 concentration (Jonsson et al, 2003). It is also possible to have phosphaturia and hypophosphataemia in fibrous dysplasia of bone, and after a renal transplant, without raised FGF-23 (Riminucci et al, 2003; Bhan et al, 2006). Moreover, it is possible to have very high FGF-23 levels without significant hypophosphataemia, at least after a renal transplant (Bhan et al, 2006). Other putative phosphatonins may therefore be playing a role in these situations, for example FRP-4, FGF-7 and MEPE - the subject of this present investigation.

Much previous work on renal phosphate handling has depended on the use of the radioactive isotope ^{32}P; this allows determination of small amounts of phosphate but is potentially dangerous to use and is associated with difficulties in safe acquisition and disposal as well as requiring extensive staff training. Previously published

micromeasurement techniques for non-radioactive phosphate have required specialist hand-made equipment. In the present investigation, a novel method of measuring phosphate concentrations in biological samples was developed, allowing the determination of phosphate along with other anions in samples as small as 20 nanolitres. This method, based on capillary electrophoresis and using only components available off the shelf, was shown to be reliable and reproducible. The capillary electrophoresis method was compared with an alternative method, the NANOFLO, which requires specialist custom-made equipment and can measure only certain ions (and these only one at a time); results using NANOFLO and the new method were comparable. The capillary electrophoresis method was adapted, and used, to measure phosphate in urine, tubular fluid and plasma. Preliminary experiments were performed to show that the method also detected citrate and oxalate, and could be used to determine the marker substance iothalamate; capillary electrophoresis could therefore have wider application in the study of tubular function in the future.

In the present investigation, renal clearance experiments were performed to determine whether MEPE is phosphaturic. It was demonstrated that MEPE causes dose-dependent phosphaturia when infused into rats. This phosphaturia is not caused by alterations in the filtered load of phosphate (as it was demonstrated that neither GFR nor plasma phosphate was significantly altered by MEPE infusion at the doses used) and must therefore be due to alterations in renal tubular phosphate reabsorption. The phosphaturic response to a range of MEPE doses was investigated, with evidence of a dose-response relationship and of a maximal effect; this maximal effect was similar to, but perhaps slightly less than, that seen with the well-studied phosphaturic hormone PTH.

The tubular effects of MEPE were studied more closely by means of micropuncture experiments in rats. It was confirmed that PTH reduced fractional proximal tubular phosphate reabsorption and it was found that MEPE, infused at a dose previously demonstrated to cause phosphaturia in clearance experiments, also reduced fractional proximal tubular phosphate reabsorption. Although both of these results were significant when assessed using t-testing, neither reached statistical significance when assessed by two-way ANOVA. Considerable variability in tubular phosphate reabsorption was found even in closely adjacent nephrons, as well as between individual animals, while even with

PTH (known previously, and demonstrated in Chapter 3 of this thesis, to cause a doubling or more in urinary phosphate when infused intravenously), changes in proximal tubular fluid phosphate were modest; in my view this is why I failed to demonstrate statistically significant changes by two-way ANOVA.

Following the completion of the present studies, immunoblotting of renal tissue obtained after infusion of MEPE into rats (performed by Dr Joanne Marks and reproduced here with her permission) has demonstrated a reduction in NaPT2a numbers at the proximal tubular brush-border membrane (see Figure 5.1); this finding is what would be expected, given that NaPT2a is generally considered the main regulated phosphate transporter in the proximal tubule and a key player in regulation of serum phosphate levels; and it parallels the previous demonstration by others that internalisation of NaPT2a is the mode of action of PTH in renal clearance experiments in animals. Nevertheless, it is reassuring to find that the MEPE-induced reduction in proximal tubular reabsorption described in this thesis is associated with apparent internalisation of apical NaPT2a.

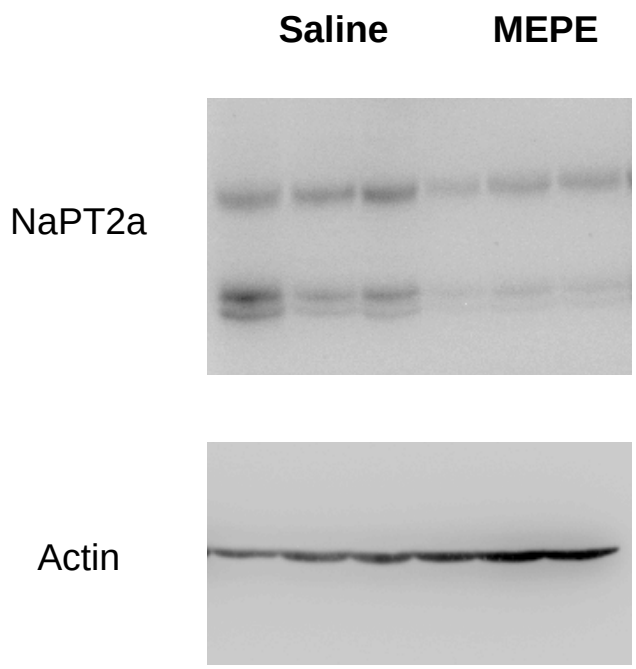

Figure 5.1 Western blot of renal brush-border membrane tissue obtained after MEPE infusion, or after infusion of saline alone, showing significant reduction in NaPT2a in the brush-border membrane after MEPE infusion *(data from Dr J Marks).*

Much work has been performed measuring phosphatonin levels in situations where they are likely to be in excess - such as in oncogenic osteomalacia. Results have often been variable or ambiguous. It would be interesting to measure levels of putative phosphatonins in situations in which they should be suppressed - for example, if MEPE and FGF-23 are involved in the regulation of serum phosphate, the plasma levels of both should be very low in patients with malnutrition or phosphate depletion. I do not think these measurements have yet been made, although it has been reported that in two patients with very low serum phosphate levels due to HHRH (caused by a known mutation in the NaPT2c gene) FGF-23 levels were normal, not suppressed (Yamamoto et al, 2005).

It is clear that there is a great deal of redundancy in the human phosphate control mechanisms. Thus, patients with inactivating mutations of the main regulated intestinal phosphate transporter (NaPT2b) have been reported as having normal serum phosphate concentrations (Corut et al, 2007), while a patient reported as having a dominant negative mutation of the main renal phosphate transporter (NaPT2a) *did* have a low serum phosphate and kidney stones but had normal growth and no other reported health problems (Prie et al, 2002). Patients with mutations in the PHEX gene, which causes XLH, often (but by no means invariably) have raised FGF-23 levels, but these have been demonstrated to move towards normal over time; this suggests that there are counter-regulatory mechanisms which can alter the postulated effects of PHEX on FGF-23 and perhaps on other phosphatonins.

It is interesting to note that two independent groups (Shimada et al, 2001; De Beur et al, 2002), using different methodologies, both identified a number of genes which might cause phosphaturia in tumour-induced osteomalacia, and that several of these candidate phosphatonins were identified by both groups. These techniques are based on looking for genes transcribed more frequently in TIO tumours than in normal bone. What is not clear is whether these genes directly regulate each other - in other words, whether one of them is abnormal as a primary abnormality and the others as a response to this. The search strategy used by Rowe et al (2000) was based on looking at circulating factors and found only two proteins, one of which was MEPE. This strategy might allow identification of

actual circulating proteins (‘phosphatonins’) as opposed to all proteins upregulated in cancer cells producing TIO, which could include up- or down-stream factors.

In conclusion, MEPE is a protein found in bone and over-expressed in at least some tumours causing phosphaturia. It has been found in the circulation of healthy humans, and in excess in some people with phosphaturia. I have demonstrated that it increases fractional phosphate excretion when infused intravenously. It does this mainly, perhaps entirely, by acting on the proximal tubule. Immunofluorescence experiments suggest that, like PTH, it inhibits proximal tubular phosphate reabsorption by triggering the internalisation of NaPT2a. It is conceivable, therefore, that MEPE might be a link between bone metabolism and renal phosphate handling, signalling changes in bone turnover which require alterations to the body’s phosphate balance.

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Abbreviations

ADHR	Autosomal dominant hypophosphataemic rickets (also known as autosomal dominant hypophosphataemia)
ANOVA	Analysis of variance
ARHR	Autosomal recessive hypophosphataemic rickets
ASARM motif	Acidic serine aspartate rich motif
BBM	Brush-border membrane (of the proximal renal tubule or the intestine)
cAMP	Cyclic AMP
CE	Capillary Electrophoresis
CRF	Chronic renal failure
CKD	Chronic kidney disease
DMP-1	Dentin Matrix Protein 1
DMP-2	Dentin Matrix Protein 2
ELISA	Enzyme-linked immunosorbant assay

ESRD	End-stage renal disease (that is, dependent on dialysis)
FD	Fibrous dysplasia (of bone)
FE_{Pi}	Fractional excretion of phosphate
FGF-23	Fibroblast Growth Factor 23
FRP-4	Frizzled-related Protein 4 (sometimes called sFRP-4)
GFR	Glomerular filtration rate
HHRH	Hereditary hypophosphataemic rickets with hypercalciuria
HPLC	High-performance liquid chromatography
HPT	Hyperparathyroidism
HSP-90	Heat Shock Protein 90
MEPE	Matrix extracellular phosphoglycoprotein
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid

n.s.	Not statistically significant (indicating a probability of >0.05 according to the relevant statistical test)
NHERF-1	Sodium/hydrogen exchanger regulatory factor 1
OF45	Osteoblast/osteocyte factor 45 (synonymous with MEPE)
OK cells	Opossum kidney cells
OMIM	Online Mendelian Inheritance in Man (online database of inherited diseases, hosted by the (US) National Centre for Biotechnology Information and freely available at http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim)
PCT	Proximal convoluted tubule
PEX	see PHEX
PHEX	Phosphate-regulating gene with Homology to Endopeptidases on the X chromosome (in some early work this gene is referred to as PEX)
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone related protein
SIBLING	Small Integrin Binding Ligand N-linked Glycoprotein

SAGE	Serial Analysis of Gene Expression
SEM	Standard error of the mean
SNGFR	Single-nephron glomerular filtration rate
STC1	Mammalian homologue of fish stanniocalcin 1
STC2	Mammalian homologue of fish stanniocalcin 2
TIO	Tumour-induced osteomalacia (synonymous with OHO)
XLH	X-linked hypophosphatemia, also known as X-linked hypophosphatemic rickets

Gene and protein nomenclature

As genes and their associated proteins are discovered by a range of workers, often near-simultaneously, each of whom names the results according to their own ideas and interests, there is much confusion and ambiguity in gene and protein naming.

It is generally agreed that genes and their proteins should take their names from the human homologue, if one exists.

In accordance with the recommendations of the Rat Gene Symbol Tracker (see ‘RatMap’, www.ratmap.org), I have used (or at least tried to use) consistent symbols for human and animal genes. Human genes are in italics and capitals. Animal (rat and mouse) genes are in italics with the first letter capitalized. Proteins are capitalized if human, and in lower case with the first letter capitalized if animal. Arabic numerals are favoured over Roman ones to avoid ambiguity (so most workers now use NPT2a rather than NPTIIa). Mixed capital and lower-case letters in human protein designations are discouraged but not always avoided - thus some workers use NPT rather than NaPT.

It is worth noting that these conventions are not universally followed, particularly in older work.

There is an additional problem when new genes or proteins are discovered which are closely related to a previously named gene. Thus NaPT2 becomes NaPT2a once a second NaPT2 type protein is described.