Parathyroid hormone (1-84): Assay development and application to the physiology and pathophysiology of calcium homeostasis

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SUMMARY

1. The initial aim of the thesis was the production of monoclonal antibodies for use in the development of an assay for PTH (1-84). The response to immunisation, using chemically synthesised PTH peptides as immunogen, was species and strain specific with DA rats consistently responding with high titre antibodies to PTH (1-34). Five monoclonal antibodies were produced to PTH (1-34), three of which (3B3, 4G3 and 6E3) were considered to be of potential use in assay systems.

2. A two-site immunometric assay specific for PTH (1-84) has been developed using 3B3 and a C-terminal monoclonal antibody - ESQ1. The assay development studies revealed 3B3 to be sensitive to the oxidation state of PTH. Hydrogen peroxide is therefore included in the assay to convert endogenous PTH (1-84) to the oxidised form prior to measurement. The validation studies confirmed the assay to be unaffected by PTH fragments. The assay has a minimum detection limit of 0.5 pmol/l, sufficient to measure PTH (1-84) in all normal subjects, and a range of 1.5-250 pmol/l with an intra-assay CV of less than 10% (2.8-250 pmol/l less than 5% CV). Studies on clinical samples indicate good discrimination between normal subjects (mean 2.1; range 1.0-5.0 pmol/l) and patients with primary hyperparathyroidism (mean 21.0; range 5.8-100 pmol/l) who in turn are well separated from patients with hypercalcaemia of malignancy (14/18 <0.5 pmol/l).

3. The measurement of PTH(1-84) over a 24 h period has shown the existence of circadian rhythm, characterised by an early evening rise and a broad peak through the night, in normal subjects which is absent in patients with primary hyperparathyroidism. The physiological significance of this observation is reflected in the presence of parallel changes in nephrogenous cyclic adenosine monophosphate in the normal subjects which are also absent in the

hyperparathyroid patients. Although transient falls in calcium were observed these studies the changes in PTH (1-84) over the 24 h period could not be fully accounted for by changes in serum adjusted calcium concentrations. An important observation from these studies was that discrimination between normal subjects and patients with primary hyperparathyroidism is time dependent. Thus for optimal discrimination between normal subjects and patients with primary hyperparathyroidism PTH (1-84) samples should be taken, and reference ranges established, between 1000 and 1600 h. The most significant finding of the circadian studies was that PTH (1-84) concentrations in normal subjects over a 24 h period show a stronger temporal correlation with other circadian hormones, notably prolactin, than with adjusted calcium concentrations. This observation suggests that factors other than plasma ionised calcium are involved in the tonic control of PTH (1-84) secretion.

4. PTH (1-84) concentrations in the circulation vary in a manner consistent with pulsatile secretion. The estimates of pulsatility obtained in normal subjects, pulses every 10-15 minutes, are in agreement with the frequency of pulsatility previously reported for bioactive PTH. This supports the concept of PTH (1-84) being the major bioactive form of PTH in the circulation. The response to an acute lowering of serum calcium confirms that, in common with other hormones, modulations of the amplitude and frequency of pulses are an integral part of the control of PTH (1-84) secretion. However, the pattern of pulsatility observed, at the frequencies studied, appears to be characteristic of the individual and does not correlate with the presence of primary hyperparathyroidism.

5. The response to treatment, with the bisphosphonate Pamidronate, was compared in patients with either Paget's disease of bone or hypercalcaemia associated with malignancy. In the patients with Paget's disease therapy with Pamidronate produced a small but significant drop in serum adjusted calcium

which was sustained throughout the study. This was accompanied by a significant rise in PTH (1-84). The concentration of PTH (1-84) continued to rise progressively despite no further fall in serum calcium. In patients with hypercalcaemia of malignancy Pamidronate produced the expected and prolonged decrease in serum adjusted calcium such that at the end of the study the mean calcium in these patients was within the reference interval. The PTH (1-84) concentrations rose progressively throughout the study to a mean concentration above the reference interval. More interestingly the initial increase occurred when the mean adjusted calcium was above the reference interval with no patient hypocalcaemic. The results suggest a resetting of the 'trigger' point for PTH (1-84) secretion in patients with hypercalcaemia of malignancy. An important conclusion from this study is that in the assessment of PTH (1-84) concentrations in the hypercalcaemic patient samples should be obtained prior to treatment being initiated.

6. A study of the treatment of patients with secondary hyperparathyroidism undergoing haemodialysis demonstrated the ability of the assay to measure PTH (1-84) specifically in the presence of large amounts of C-terminal PTH fragments. The study also showed that in patients with moderately severe secondary hyperparathyroidism, even after many years of renal replacement therapy, medical treatment can achieve marked parathyroid suppression. A combination of high oral doses of Calcitriol and low calcium dialysate was well tolerated and achieved higher concentrations of 1,25 dihydroxyvitamin D without causing hypercalcaemia than oral Calcitriol therapy alone. There was evidence of the direct effect of 1,25 dihydroxyvitamin D on the parathyroid gland since PTH (1-84) concentrations fell significantly during the first month of active treatment despite static serum calcium concentrations. An additive suppressive effect of PTH (1-84) secretion was observed when the serum calcium increased with higher doses of Calcitriol. Thrice weekly Calcitriol

was shown to be as effective as daily therapy which may allow simplification of therapy regimes.

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DECLARATION:

The work of this thesis was carried out solely by the author, except where stated.

TO:

Mum and Dad
(and the man on the galloping horse)

CHAPTER 1

INTRODUCTION

CHAPTER 1 : INTRODUCTION

1 BACKGROUND TO THE PROJECT

The Endocrinology Section at Glasgow Royal Infirmary has had a strong interest in the measurement of parathyroid hormone (PTH) for many years. The measurment of PTH has been central to the routine laboratory assessment of patients with disorders of calcium metabolism and to studies on the physiology and pathophysiology calcium homeostasis. The present project was conceived at the time when the PTH immunoassay method was based on a unique polyclonal antiserum raised in a guinea pig - AS 211/32. While the assay had been a significant improvement on previous methods it was cumbersome by today's standards (7 day incubation), had limitations in its ability to measure PTH (sensitivity and specificity), but more importantly the antiserum was in increasingly restricted supply. The time was appropriate, therefore, for a project to design and develop an assay for PTH.

2 INTRODUCTION TO PTH

2.1 Biosynthesis and structure

PTH in man and in other species studied is comprised of 84 amino acids in a single peptide chain (Potts et al 1982). PTH is secreted by the chief cells of the parathyroid glands and within the parathyroid cell the biosynthetic pathway is known to conform to that of other secretory peptides .

The biosynthesis of PTH is shown diagramatically in Figure 1.1. PreproPTH (115 amino acids) is the primary translation product of the PTH mRNA. The preproPTH is synthesised on the ribosome then transported across the membrane of the endoplasmic reticulum with concomitant cleavage of the pre or signal sequence to yield the proPTH form (90 amino acids). The cleavage is so rapid that very little intact preproPTH is found in parathyroid cells. The relatively more stable proPTH is

Biosynthesis of PTH (1-84)

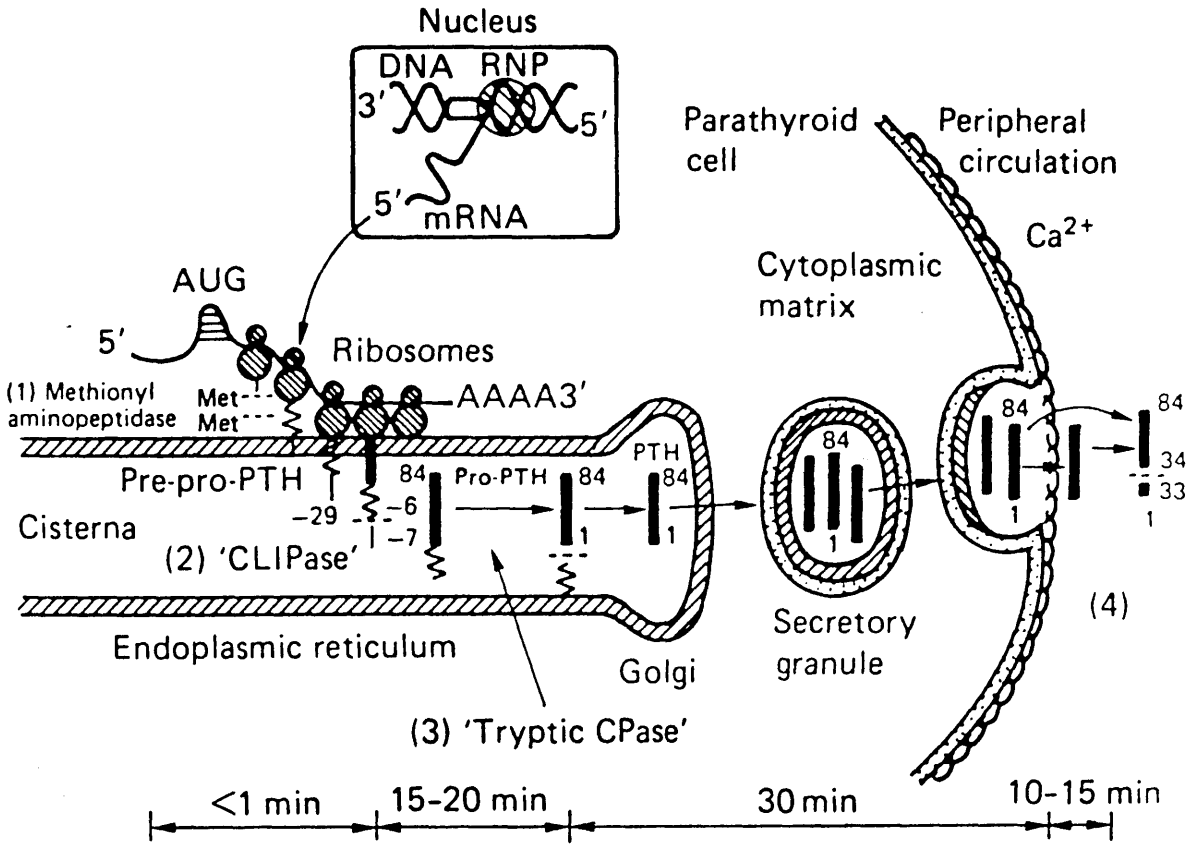

Figure 1.1

Scheme depicting the biosynthesis of PTH (1-84) (from Habener et al, 1977).

the major precursor protein. After a lag of several minutes, the proPTH moves to the Golgi apparatus where the prohormone is converted to PTH (1-84). The mature hormone PTH (1-84) is then packaged in secretory granules and leaves the cell by exocytic fusion of the granule with the plasma membrane (Habener et al 1977, Cohn and Elting 1983).

The PTH gene is represented only once in the haploid genomes of humans, rats and cows. The human PTH gene is located on the short arm of chromosome 11 and is closely linked to the b-globin gene. The PTH gene contains two introns. The first intron (3400 bp) comes 5 base pairs before the sequence encoding the preproPTH sequence. The second intron (103 bp) interrupts the codon encoding the fourth amino acid of the 'pro' sequence. The PTH gene introns therefore roughly separate discrete functional domains, the first separates the 5' noncoding region from the rest of the gene and the second separating the precursor specific 'prepro' region from that of mature PTH (1-84). A similar pattern of intervening sequence has been found in a number of genes encoding hormones such as gastrin and insulin (Kronenberg et al 1986).

The base sequences for the entire coding regions of the gene for human, bovine and rat PTH have been determined utilizing complementary deoxyribonucleic acid (cDNA) sequences to mRNA isolated from parathyroid cells (Vasicek et al, 1983, Heinrich et al 1984, Weaver et al, 1984). These analyses have confirmed the sequences for PTH obtained by amino acid sequencing techniques (Keutmann et al, 1978, Potts et al, 1982, Rosenblatt 1982). The primary structure of human preproparathyroid hormone is shown in Figure 1.2. The mature PTH (1-84) hormone contains no carbohydrates but there is evidence that up to 20% of hormone extracted from parathyroid glands is phosphorylated on the serine residues at positions three and seventeen (Goltzman et al, 1986).

The Primary Structure of Preproparathyroid Hormone

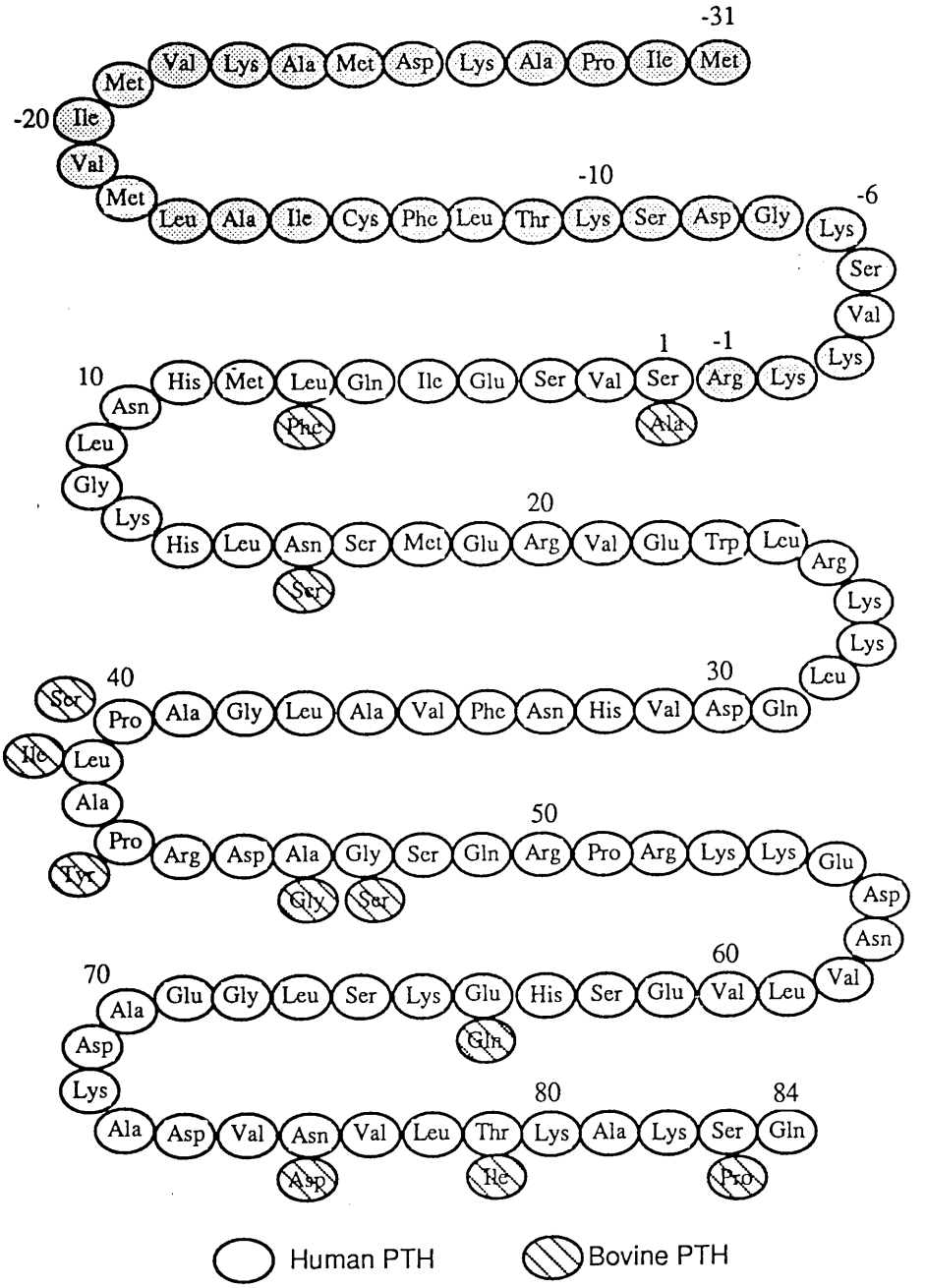

Figure 1.2

Primary structure of human preproparathyroid hormone. Also shown are the differences in primary structure between human and bovine PTH (from Potts et al, 1982).

2.2 Structure-Function Relationships

The biological activity of PTH (1-84) is associated with the N terminal third of the molecule. The sequence 1-27 is the minimum required for detectable biological activity (Cohn and Elting 1983). Deletion of residues 1 and 2 destroys the biological activity but not receptor binding such that the modified peptides 3-34 and 7-34 are competitive inhibitors of PTH action *in vitro* (Potts et al 1982, Horiuchi et al 1983). The interaction of PTH (1-34) with its receptor is also altered by oxidation of the methionine residues at positions 8 and 18 (Tashijian et al, 1964). Studies on the effects of oxidation on the biological activity of PTH peptides in animals have indicated either total inactivation (Galceran et al, 1984) or an alteration in the spectrum of biological activity (Pang et al, 1983). More recent work has indicated that oxidation alters the secondary structure of PTH (Zull et al, 1990).

The investigation of the secondary structure of PTH revealed two domains of secondary structure, one corresponding to the N-terminal region and the other to the C-terminal region, connected by a 'stalk' sequence in the region of residues 30-40 (Brewer et al 1975, Fiskin et al 1977). Using circular dichroism Zull et al (1990) have shown most of the secondary structure in PTH (1-84) to be associated with the N- terminal region. Within PTH (1-34) they showed that the secondary structure to be primarily generated by the first 18 residues. Further it was shown that oxidation of the methionine residues, particularly at position 8, was accompanied by a change in secondary structure and a reduction in affinity for PTH receptors. Deletion of residues from 1 through to 6 did not affect the secondary structure. Thus it was concluded that secondary structure is important for receptor binding and that oxidation alters the biological activity of PTH (1-84) by modifying the secondary structure. As deletion of residues 1 and 2 did not alter the secondary structure or receptor binding, but results in a loss of activity, it was concluded that these residues are important in the activation of the PTH receptor.

2.3 Mechanism of Action

PTH interacts with specific receptors on distinct cell types in target tissues. Classically the PTH-receptor complexes have been shown to stimulate cell function through coupling to adenylate cylase to produce the second messenger, cAMP, and subsequent phosphorylation of regulatory proteins by protein kinases (Chase and Aurbach 1967). The activation of adenylate cyclase is mediated through the guanine nucleotide coupling proteins - the G proteins. Two G proteins are involved in the regulation of adenylate cyclase, one stimulatory, Gs, and the other inhibitory, Gi (Hurley et al, 1984).

Recently PTH has been shown also to activate phospholipase C and act via the second messengers, inositol triphosphate (IP-3) and diacylglycerol (DAG) (Hruska et al, 1987). Both type 1, cAMP mediated, and type 11, IP-3 and DAG mediated, receptors have been described in various cells (Hesch et al, 1986, Hruska et al 1986, Hruska et al, 1987). The mechanism by which PTH and its receptor couple to both adenylate cyclase and phospholipase C may involve preferential activation of the Gi protein (Hruska 1987).The biological role for the interaction of these signals and remains to be established.

2.4 Control of secretion from the parathyroid glands

There is general agreement that the plasma concentration of calcium is the dominant regulator of PTH secretion. PTH secretion is increased in response to low calcium and decreased in the face of high calcium. There are at least two major mechanisms that control the secretion of PTH, one involving intracellular calcium, the other intracellular accumulation of cAMP.

The precise mechanism whereby calcium exerts its effects on PTH secretion is not fully established. It appears to be mediated in part through a decrease in the cAMP content of the cell (Brown 1982). Calcium does not acutely alter the rate of synthesis

of proPTH or its conversion to PTH but affects hormone production through enhancing intracellular degradation of PTH within the parathyroid cell. The major products of intraglandular degradation of PTH (1-84) are the C-terminal peptides PTH (34-84) and PTH (37-84). These C-terminal fragments are secreted with intact PTH (1-84) in a molar ratio of 2:1, indicating that the bulk of newly synthesised PTH (1-84) is degraded (Cohn and Elting 1983). The inabilty to find substantial amounts of N-terminal fragments in glandular secretion suggests that these fragments are further degraded within the gland probably by a mixture of enzymes including cathepsin B (MacGregor et al, 1979).

The availability of cDNA probes for preproPTH mRNA has made possible the study of the control of PTH synthesis together with its secretion. Acute experiments (4-7 h) on the effects of high extracellular calcium on bovine parathyroid cells showed changes in PTH secretion with no detectable change in mRNA concentrations (Heinrich et al 1983, Brookman et al 1986), whilst longer incubation periods (16-24 h) resulted in a decrease in mRNA in addition to a fall in secretion (Russel et al, 1983, Brookman 1986). Studies on human parathyroid adenomata in culture also showed suppression of mRNA in response to high calcium although this was not accompanied by a fall in PTH secretion (Farrow et al, 1988). A two-stage control of PTH synthesis and secretion has been proposed on the basis of these studies. Long term regulation would involve changes in mRNA concentrations within the cell while acute control would involve alterations in post-transcriptional events (Farrow et al, 1988). This model is consistent with the concept, discussed above, of acute changes in calcium controlling PTH secretion by altering intraglandular degradation of PTH (Cohn and Elting 1983).

Parathyroid glands are known to contain receptors for 1,25 dihydroxyvitamin D_3 (Mason et al 1980). Studies *in vivo* have indicated that that administration of 1,25 dihydroxyvitamin D_3 decreases parathyroid activity (Oldham et al, 1979). It was not clear, however, whether the parathyroid gland was a target tissue for 1,25

dihydroxyvitamin D_3 or whether suppression was as a result of raised circulating concentrations of calcium produced by the administration of 1,25 dihydroxyvitamin D_3. Recent studies, using a cDNA probe, have indicated that 1,25 dihydroxyvitamin D_3 regulates parathyroid gland activity by suppressing preproPTH mRNA concentrations and PTH secretion (Karmali et al, 1989). The receptor for 1,25 dihydroxyvitamin D_3 has now been shown to bind directly to the 5'-flanking region of the bovine parathyroid hormone gene (Farrow et al, 1990).

Magnesium inhibits PTH in a similar way to calcium although the affinity of magnesium for the inhibitory site is less than is the case for calcium. In addition profound hypomagnesaemia interferes with PTH secretion (Brown 1982). The monovalent ions lithium and potassium stimulate secretion (Brown et al, 1982). Sodium-potassium ATPase may also be involved in controlling secretion in that ouabain, an inhibitor of the ATPase, inhibits secretion (Brown et al, 1983).

In addition adrenergic agents, dopamine, secretin, prostaglandins, and several classes of drugs are also capable of influencing PTH secretion (Heath 1980, Brown 1982). Parathyroid cells contain the adenylate cyclase system and PTH secretion is intimately related to the cAMP content of of parathyroid tissue (Abe and Sherwood 1972, Dufresne and Gitelman 1972). Evidence from *in vitro* studies indicate that agents capable of altering the cAMP content of the parathyroid cell will alter the rate of PTH secretion. There is a linear relationship between measured total cAMP and the rate of PTH release regardless of which stimulator or inhibitor is applied (Brown et al, 1978).

Although the intracellular cAMP concentration is clearly an important modulator of PTH secretion calcium appears to be the more significant physiological regulator of secretion. Calcium is capable of completely suppressing PTH secretion regardless of the cAMP concentration within the cell. However, higher concentrations of calcium are required for half maximal inhibition as the cell content of cAMP rises

(Brown 1982).

2.5 Peripheral metabolism

Intact PTH (1-84) is rapidly cleared from the circulation (half life of less than 4 minutes) mainly by liver and kidney (Oldham et al, 1978, Martin et al, 1979, Bringhurst et al, 1988). In the liver, Kupffer cells metabolise PTH (1-84) by cleaving it at the region containing amino acids 33-41 (Bringhurst et al, 1982). In addition the kidney also clears PTH (1-84) from the circulation where both peritubular uptake and glomerular filtration are important. Inactive C-terminal fragments are then released into the circulation (Bringhurst et al, 1988). Liver and kidney cathepsin-like enzymes have been identified that are capable of cleaving PTH (1-84) at or near residues 33-44 and 36-57 (Bringhurst et al, 1982). Whether the N-terminal fragments are degraded, sequestered in tissue or secreted *in vivo* has not been completely resolved but most studies agree that if N-terminal fragments circulate then their concentration is much lower than that of PTH (1-84) (Goltzman 1986, Bringhurst et al, 1988).

Glomerular filtration is the main mechanism for removing inactive C-terminal fragments from the circulation. Impaired renal function prolongs the half-life of both intact PTH (1-84) and fragments. The concentration of the inactive C-terminal fragments is particularly affected as the half-life can be extended from less than an hour to days (Hruska et al, 1981).

In addition bone cells and skeletal tissue may metabolise PTH (1-84) and contribute to the heterogeneity of circulating PTH (Freitag et al, 1979).

2.6 Heterogeneity of Circulating PTH

As described in Sections 2.4 and 2.5 the metabolism of PTH (1-84) within the parathyoid gland and in liver, kidney and bone all contribute to the final heterogeneity of PTH in the circulation (see Figure 1.3). Generally less than 5% to 25% of total

Heterogeneity of circulating PTH

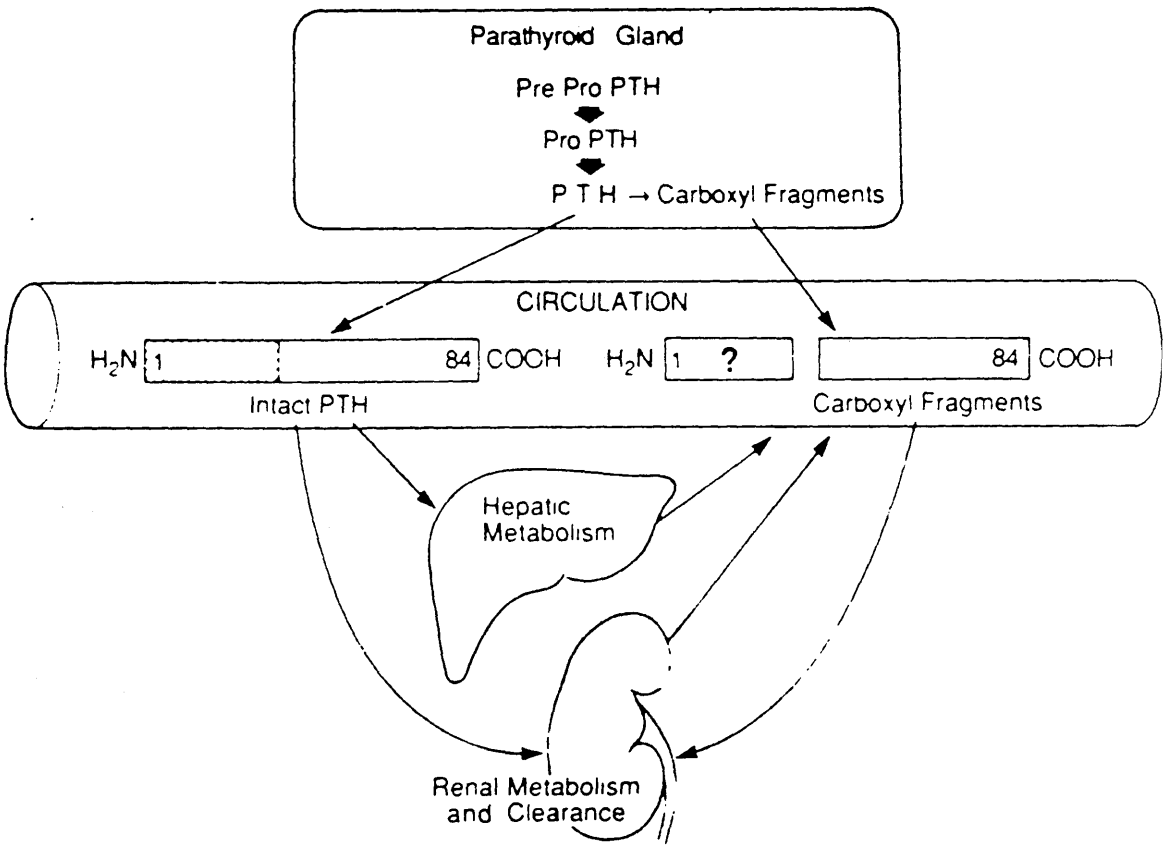

Figure 1.3

Scheme depicting the secretion, metabolism, clearance and circulating forms of PTH.

immunoreactive PTH is the major glandular form PTH (1-84) and the predominant circulating immunoreactive form consists of inactive C-terminal fragments (Bennet et al, 1981). In addition the presence of mid-molecule fragments in the circulation has been described (Roos et al, 1981, Marx et al, 1981). The available evidence suggests that, if present in the circulation, the concentration of N-terminal fragments is low relative to PTH (1-84) (Bringhurst 1988) although it may be increased in end stage renal failure (Goltzman 1986). These studies emphasise the dominant role of intact PTH (1-84) as the major biologically active form of the hormone *in vivo* .

3 PHYSIOLOGY OF PTH

3.1 Actions of PTH on the Kidney

Sites of action

The actions of PTH in target tissue are mediated by interaction with specific receptors on distinct cell types. The nephron represents a series of distinct cell types bearing receptors for several different hormones. PTH receptors are distributed in the cortical regions of proximal as well as distal tubules.In the proximal tubule sites are found in the cortical early convoluted as well as the 'bright' portions. In the distal tubule, PTH sensitive adenylate cyclase is found in the granular portion and the cortical ascending limb (Morel et al, 1982). The distribution found for PTH sensitive adenylate cyclase agrees with the physiological findings that PTH influences phosphate transport at proximal and distal tubular sites (Khlar and Peck 1980).

Calcium reabsorption

PTH directly affects reabsorption of calcium from the glomerular filtrate. Under the influence of PTH there is a decreased calcium clearance at any given calcium load, reduction in circulating PTH concentrations leads to an increased calcium clearance. The physiological mechanisms whereby calcium transport in the kidney is regulated by PTH have been delineated only partially. It is known that calcium and sodium

transport are coupled in the proximal tubule and that PTH can influence sodium transport in that segment of the nephron. However the major physiological effect of PTH on calcium reabsorption occurs beyond the proximal tubule in the thick ascending and granular portions of the distal tubule (Dennis and Brazy 1982). Direct effects of PTH on this segment have been identified *in vitro* and have been shown to be mediated through cAMP (Bordeau and Burg 1980).

Phosphaturic Effect

A rapid increase in the urinary excretion of phosphate was one of the first physiological effects of increased PTH secretion to be recognised. This action represents the effects of the hormone at two distinct loci within the nephron. Infusion of PTH reduces reabsorption of sodium and phosphate in the proximal tubule. Further, PTH reduces phosphate reabsorption in the distal tubule (Agus et al, 1981).

The effect of PTH on phosphate reabsorption in the proximal tubule may be secondary to its effect on sodium reabsorption. Dibutyryl cAMP has a similar influence on sodium and phosphate, and cAMP is known as a regulator of sodium transport in a number of tissues (Strewler et al, 1977). It has also been suggested that phosphaturia may be secondary to changes in intraluminal pH or proximal transport of bicarbonate. Increases in pH would change the ratio of HPO_4^{2-} to $H_2PO_4^-$ and consequently decrease the rate of phosphate reaborption as monvalently charged phosphate is more readily translocated across cell membranes than is divalently charged phosphate.

Other effects on the kidney

PTH causes a net inhibition of bicarbonate reabsorption in the proximal tubule. The mechanism is not clear. In conditions of excessive PTH secretion, such as marked hyperparathyroidism, proximal tubular acidosis can develop (Aurbach et al, 1985).

The effects of PTH on renal bicarbonate clearance may be one of the factors causing the phosphaturia discussed above.

Isotonic fluid reabsorption in the proximal tubule is also impeded by PTH. This may be secondary to decreased sodium transport in the proximal tubule. The sodium thus excluded from reabsorption in the proximal tubule gives rise to a net increase in sodium delivery to the distal tubule carrying with it an increase in associated water. The sodium is reabsorbed in the distal tubule leaving the water to be excreted. This is reflected an an increase in the free water clearance (Aurbach et al, 1985).

3.2 Actions of PTH on Bone

Early and late effects

The effect of PTH in mobilising calcium from bone occurs in two phases. The early phase represents mobilisation of calcium from areas of bone and the enhanced transfer of this calcium into the ECF; this effect does not require protein synthesis. The later phase is associated with an increase in in synthesis of bone enzymes, particularly lysosomal enzymes that promote bone resorption and influence bone remodelling. Bone remodelling, the resorption of older regions of bone (osteons) and subsequent replacement with new bone formation, is due to degradation of bone by osteoclasts and the subsequent infiltration of osteoblasts that synthesise new collagen and allow remineralisation of replacement osteons (Aurbach 1988). The inital effect of PTH on bone is increased resorption. This is reflected in reduced osteoblast function and enhanced osteoclast activity. Later, new bone formation is enhanced. Bone growth factors are likely to be involved in mediating the new bone formation which occurs in response to the earlier phases of enhanced bone resorption (Tam et al, 1982).

Action of PTH on bone cells

PTH added to bone fragments causes enhanced bone resorption by increasing the activity of osteoclasts and initially by inhibition of osteoblast activity. PTH stimulates RNA synthesis in osteoclasts, increases the number of nuclei per osteoclast and increases the number of osteoclasts. These changes are accompanied by increases in the release of lysosomal enzymes which are dependent on new protein synthesis. Lysosomal enzymes are released rapidly from bone activated by PTH. Other effects of PTH on bone include enhanced synthesis of hyaluronate, inhibition of citrate decarboxylation, inhibition of collagen synthesis and changes in alkaline phosphatase activity (Aurbach 1988). Despite the importance of the increase in osteoclastic resorption by PTH studies *in vitro* indicate that PTH does not interact directly with the osteoclast. The primary target of PTH appears to be a mononuclear bone cell, thought to be the osteoblast. Osteoblasts in turn release factors which activate the osteoclast (Cohn and Wong 1978).

3.3 Action of PTH on gastrointestinal calcium absorption

The gastrointestinal tract responds indirectly to PTH as a consequence of the activation of the 1a hydroxylase system for vitamin D metabolites in the kidney. The enhanced gastrointestinal absorption of calcium induced by PTH is secondary to the action of 1,25-dihydroxyvitamin D formed in the kidney and acting on cells in the gut (Aurbach 1988).

3.4 Role of PTH in calcium homeostasis

The most significant physiological role of PTH is to control the concentration of calcium in the extracellular fluid (ECF) (Talmage and Meyer 1976). Calcium is the most abundant mineral in the human body. The average adult body contains 25,000 mmol (1 kg) of which 99% is in bones and teeth with less than 0.1% in the ECF. Numerous cell and organ functions are dependent on the maintenence of calcium

concentration in the ECF within narrow limits. These include the excitabilty of nerve function and neural transmission; proteolysis; exocytosis; blood coagulation; muscle contraction; membrane stability; enzyme regulation and mineralisation of bone (Aurbach et al, 1985).

Plasma calcium after correction for variations in plasma proteins shows little variation throughout the day, although the changes in protein levels induced by postural changes in blood volume are sufficiently large that correction for them may introduce errors that would obscure significant changes in the concentration of free calcium ions. Small changes have been observed in normal subjects in the concentration of adjusted clacium (Markowitz et al, 1988, Mallette 1989, Logue et al, 1990c) and ionised calcium (LoCascio et al, 1982) although the significance of these changes is not clear.

The concentration of calcium in the plasma is controlled by the coordination of the fluxes of calcium which occur between the plasma and the gut, bone and kidney. Figure 1.4 shows in diagramatic form the average fluxes in calcium per day in a normal adult. Bone is not metabolically inert, some of its calcium is rapidly exchangeable with the ECF, the turnover between bone and ECF being approximately 500 mmol/day. In the kidneys ionised calcium is filtered by the glomeruli and almost totally reabsorped in the tubules. Gastrointestinal secretions contain calcium some of which is reabsorbed together with dietary calcium. Since calcium in the ECF pool is exchanged through the kidneys, gut and bone about thirty-three times every 24 h a small change in any of these fluxes will have a profound effect on the concentration of calcium in the ECF and, therefore, in plasma. These fluxes in calcium are controlled by the co-ordinated action of the three major calcitropic hormones - PTH, 1,25-dihydroxyvitamin D and calcitonin.

The hormonal control of the fluxes between plasma and gut, kidney and bone is shown in Figure 1.5. At the gut level 1,25-dihydroxyvitamin D acts to increase

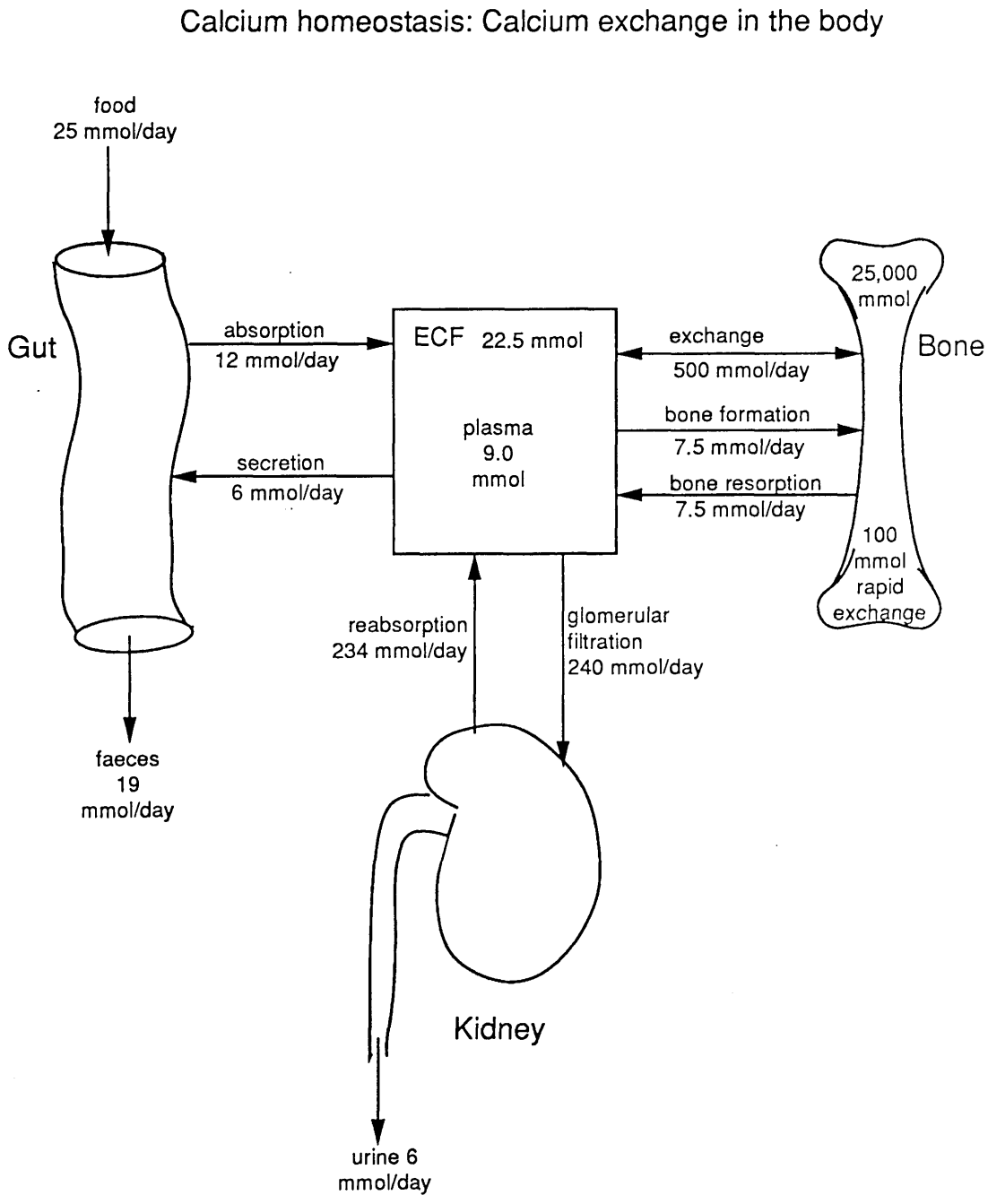

Figure 1.4

Scheme depicting the calcium fluxes between kidney, bone and gut over a 24 h period. Figures shown are the approximate average values for normal adults.

Hormonal control of calcium homeostasis: Role of PTH

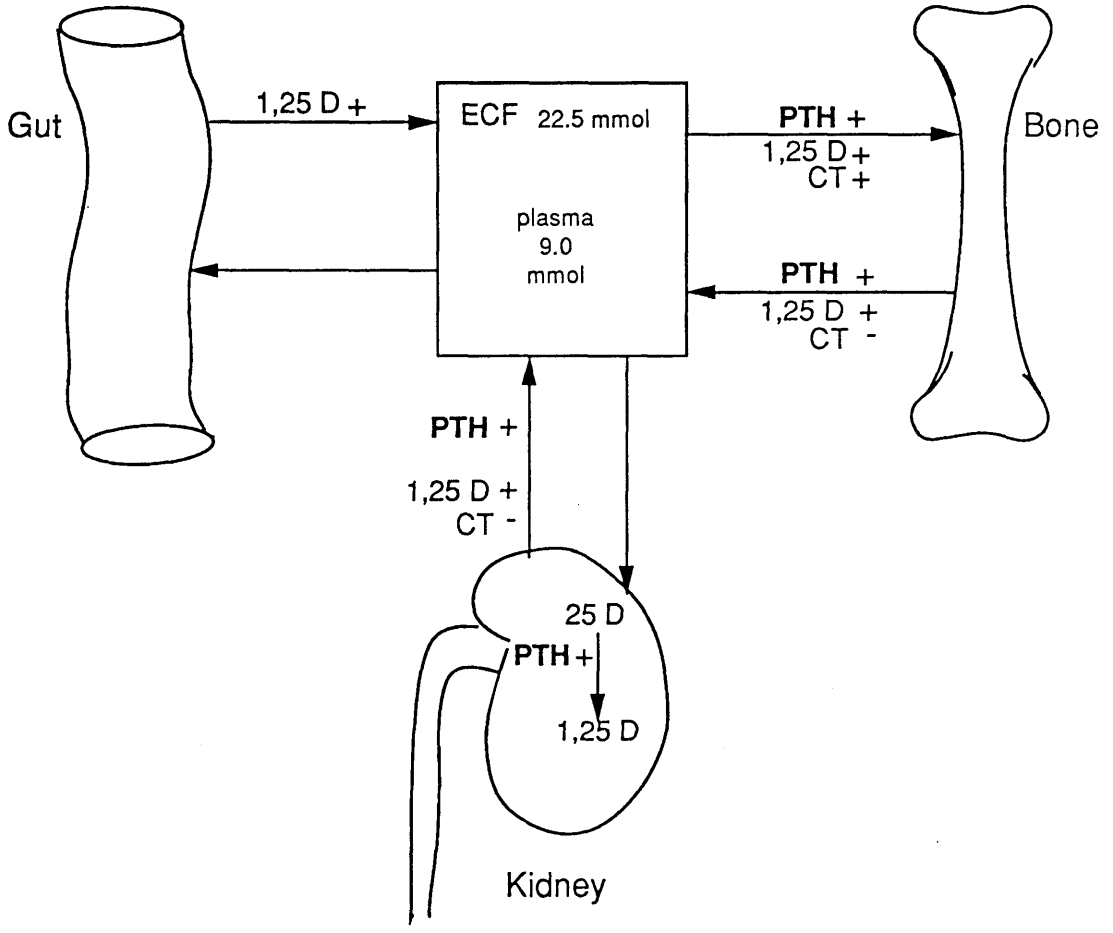

Figure 1.5

Scheme depicting the hormonal control of calcium metabolism. The sites of action of PTH, calcitonin (CT) and 1,25 dihydroxyvitamin D (1,25D) are shown with stimulatory action represented as (+) and inhibitory action as (-).

calcium absorption. PTH and calcitonin have no recognised effects on gut absorption by the intestine.

Bone remodelling is influenced by each of the three main calcitropic hormones. The effects of PTH are complex involving a balance between an anabolic effect (on the osteoblast) and a catabolic effect (on the osteoclast). *In vivo*, the balance between the anabolic and catabolic effects of PTH may be critically determined by dose and timing, with continuous high levels of PTH enhancing bone resorption and intermittent exposure enhancing the osteoblast-stimulating effects (Tam et al, 1982). The major effect of calcitonin is to inhibit osteoclastic bone resorption. The acute inhibition of osteoclastic activity allows the continuing osteoblastic activity to increase net bone uptake of calcium into bone (Talmage et al, 1983). 1,25-dihydroxyvitamin D also has dual effects on bone, it increases bone resorption by increasing the metabolic activity of the osteoclast especially at high concentrations (Malluche et al, 1986). 1,25-dihydroxyvitamin D also plays major role in facilitating bone formation but this is probably mediated by its maintenance of a normal calcium phosphate product (Underwood and De Luca 1984).

At the kidney PTH and 1,25-dihydroxyvitamin D promote tubular reabsorption of calcium: calcitonin tends to inhibit this process although probably to a minor degree in humans (Mallette 1989).

Several hormones other than those directly involved in calcium homeostasis, PTH, vitamin D and calcitonin, exert effects on bone and calcium metabolism. Growth hormone, adrenal glucocorticoids, thyroid hormones and the sex hormones are necessary for the normal growth and maintenance of bone balance. The precise cellular events which underly these effects are not fully understood; thyroid hormones, glucocorticoids and insulin appear to act directly on bone cells; growth hormone exerts its effects through growth hormone dependent intermediary compounds, somatomedins; and sex steroids appear to act indirectly but the

mechanisms involved have not been identified. For a review see Adams (1989).

Therefore, the physiological actions of PTH are in general directed to raise the concentration of calcium in the ECF. The actions of PTH on the kidney and bone in effecting calcium transfer are direct whereas the transfer of calcium from the gut to the ECF is indirect and secondary to the influence of PTH in enhancing the formation of 1,25-dihydroxyvitamin D from 25 hydroxyvitamin D in the kidney. In addition however, since activation of osteoclastic bone resorption is thought to be the primary step in the initiation of bone remodelling at each site, increasing the number of osteoclasts serves to maintain bone turnover at a higher rate. Thus, PTH under normal conditions plays a major role in the maintenance of bone turnover.

4 MEASUREMENT OF PTH

4.1 Assay of function

Bioassay

It is in the nature of the scientific investigation of endocrine systems that hormones are first discovered and initially defined by their physiological function. PTH was first prepared as an active extract of parathyroid glands by Collip (1925). The original bioassay for PTH was based on the rise in serum calcium observed in dogs injected with gland extract (Collip 1925). Subsequent *in vivo* bioassays utilised, rats, chicks or quail (Aurbach 1985). In general *in vivo* bioassays based on the ability of PTH to induce hypercalcaemia or phosphaturia or to stimulate urinary cAMP have not been sufficiently sensitive to measure circulating concentrations of PTH (Zanelli and Parsons 1980) and have largely been replaced by *in vitro* bioassay methods. A number of more sensitive *in vitro* systems based on measuring the cAMP response of the adenylate cyclase systems of the renal membrane (Nissenson et al 1981, Niepel et al, 1983) or the rat osteosarcoma cell (Klee et al, 1988) have been described. While these methods can be adapted to measure PTH concentrations in the

circulation, by concentration of the sample, they are not sufficiently sensitive to differentiate low, normal and elevated concentrations in sera (Armitage 1986).

Ultrasensitive cytochemical bioassays have been developed which allow the determination of PTH bioactivity in the circulation (Chambers et al, 1978, Goltzman et al, 1980). The cytochemical bioassay method is based on the PTH-mediated stimulation of glucose-6-phosphate dehydrogenase activity in guinea pig renal capsule segments. The end product, reduced neotetrazolium, is quantitated in sections by microdensitometry (Chambers et al, 1978). This assay has been shown to allow discrimination of normal subjects from patients with either hypoparathyroidism or hyperparathyroidism (Fenton et al, 1978, Allgrove et al, 1983).

Although bioassays, particularly the cytochemical bioassay, can provide clinically useful measurements they are cumbersome to perform and unsuited to processing large numbers of samples. Thus bioassays remain valuable research tools but are not practicable for routine use.

Indirect Measurements of PTH Function

Urinary cAMP

The discovery of the PTH-sensitive adenylate cyclase in renal cortex was followed by the demonstration that PTH rapidly increased the urinary excretion of cAMP (Chase and Aurbach 1967). This increase was due to enhanced renal production of cAMP rather than to a change in filtered load. The urinary excretion of cAMP was therefore used as an indirect assessment of circulating PTH activity. However, differences in methods of specimen collection and data expression limited the use of this test (Marcus 1989). Standardisation of the urinary cAMP measurement was achieved by Broadus et al, (1977) who normalised the excretion data for glomerular filtration rate and described a simple approach to estimate the renal contribution or

'nephrogenous' cAMP. The calculation is made by subtracting the plasma cAMP from the total urinary cAMP excretion:-

$$\text{NcAMP} = \text{Urinary cAMP} \times \frac{\text{serum creatinine}}{\text{urinary creatinine}} - \text{plasma cAMP}$$

The use of nephrogenous cAMP as an index of PTH function has centered on its ability to identify excess PTH function, as in primary hyperparathyroidism. While the calculation of NcAMP provides the best index of cAMP excretion, and there is a positive correlation between NcAMP and the degree of hypercalcaemia, the utility of this test in mild disease has been questionable (Marcus 1989).

Urinary Phosphate

The rapid increase in urinary phosphate excretion that follows PTH administration was one of the first described effects of PTH. The kidneys handle phosphate by a saturable reabsorption process with no apparent tubular secretion in humans. In considering the efficiency of phosphate reabsorption, in addition to the urinary phosphate excretion, factors that determine the load presented to the nephron should be taken into account - the plasma phosphate concentration and the glomerular filtration rate. Classical indices such as the tubular reabsorption of phosphate do not fully allow for these. The concept of a 'tubular maximum' for phosphate was defined, therefore, as the maximal tubular reabsorption of phosphate divided by the glomerular filtration rate (TmP/GFR) (Walton and Bijvoet 1975). This value estimates the set point for the serum phosphate concentration taking into account the serum phosphate concentration and the glomerular filtration rate (Broadus and Rasmussen 1981). Like NcAMP the sensitivity and specificity of this test, in the assessment of excess PTH function, has proved to be variable (Marcus 1989).

4.2 Assay of structure

Immunoassay

Immunoassay since its first description (Yalow and Berson 1960, Ekins 1960) has become the foremost method in the routine endocrine laboratory for the measurement of hormones. The technique is based on the inherent specificity and avidity of the binding of an antibody to its antigen. For the clinical laboratory the major advantages of immunoassay methods lie in the abilty to develop sensitive assays for a wide range of hormones and the applicabilty of the methods to the processing of large numbers of samples.

Radioimmunoassay

Radioimmunoassay (RIA) is the simplest example of saturation analysis (Ekins 1960). In RIA methods a fixed but limiting amount of antibody is reacted with a fixed amount of radioactively labelled antigen and a variable amount of antigen, either standard or unknown sample. At equilibrium the antibody binding sites are saturated with labelled antigen and antigen in proportion to their relative concentrations in the assay tube (Figure 1.6). Theoretically, from consideration of the Laws of Mass Action, it can be shown that for an RIA the maximal sensitivity is directly proportional to the antibody affinity, and that the optimal antibody concentration to maximise sensitivity approaches zero. In practice the optimal antibody concentration will dependent on the specific activity and level of non-specific binding of the labelled antigen. It is also demonstrable that incubation times must be extended in the direction of infinite time (Ekins 1981). Thus the development of sensitive RIA methods requires the production of high affinity antisera and high specific activity labelled antigen.

Since the first development of an RIA method (Berson et al, 1963), the PTH assay has become a commonly requested test in the Endocrine laboratory. However the

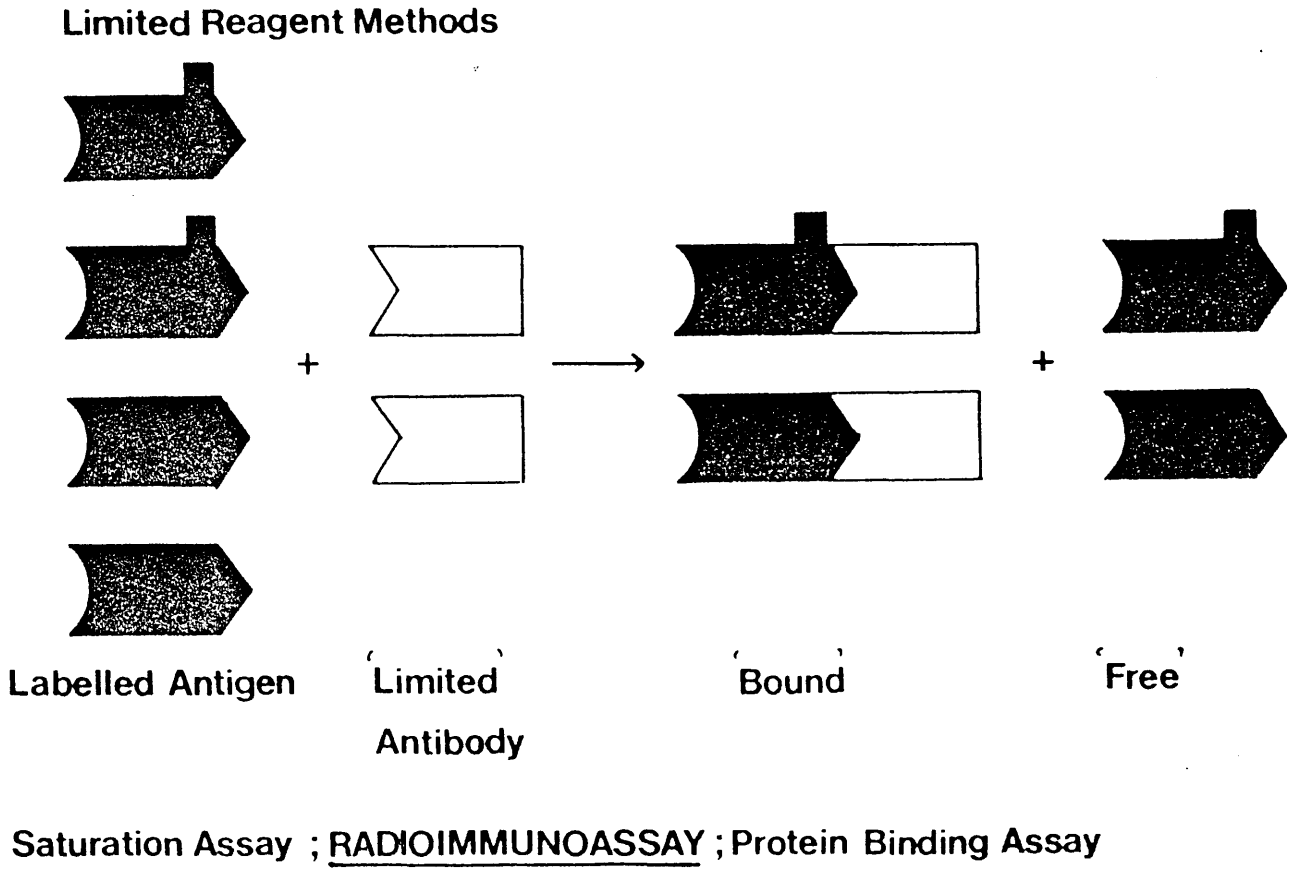

Figure 1.6

Principles of radioimmunoassay - a form of saturation analysis. A fixed (limited) amount of antibody is reacted with a relative excess of antigen. The antigen is distributed between the antibody bound and free fractions in proportion to its concentration. The reaction is monitored by the presence of a small amount of radiolabelled antigen. The bound and free fractions are separated prior to quantitation by precipitation (either immunological or physical) of the bound fraction or by centrifugation (antibody on solid-phased particle).

development of RIA methods for PTH has been problematic due the heterogeneity of circulating PTH, its poor immunogenicity and the scarcity of suitable human PTH for immunisation and use as radiolabel. Until recently most antisera were heterologous, utilising antisera raised to porcine or bovine PTH purified from parathyroid glands. The antisera were then selected for the ability to recognise human PTH, although in many cases this was with reduced sensitivity. In addition preparations of radiolabelled PTH were labile and had inherent high non-specific binding (Endres et al, 1989).

Later methods employed preparations of human PTH or synthetic fragments as immunogen thus producing homologous antisera (Fischer et al, 1974, Hendy et al, 1979). A further refinement was the use of fragments of PTH as radiolabel in conjunction with homologous polyclonal antisera to produce sequence specific assays (Marx et al, 1971, Mallette et al, 1982).

In general PTH RIA methods can be categorised in terms of their specificity into one of four groups:-

N-terminal assays: methods based on antisera to PTH (1-34), the biologically active region of PTH (Silverman et al, 1973, Arnaud et al, 1974, Woo and Singer 1974, Potts et al, 1983). As PTH (1-34) fragments, if present in the circulation, are thought to be in low concentration the N-terminal immunoreactivity measured in these assays is presumably predominantly intact PTH (1-84). It should be noted however, that many polyclonal antisera with predominantly N-terminal specificity, but raised to extracts of PTH, may well recognise additional C-terminal epitopes (Mallette 1980).

C-terminal assays: methods based on antisera to the C-terminal region PTH (53-84) (Silverman et al, 1973, DiBella et al, 1978b, Simon et al, 1980, Roos et al, 1981, D'Amour et al, 1984). These methods measure the intact PTH (1-84) and the biologically inactive C-terminal fragments. However the C-terminal fragments have a

longer half life than either intact or PTH (1-34) and are present in higher concentrations, therefore these assays measure mainly the C-terminal fragments.

Mid-molecule assays: The availability of synthetic fragments of PTH for immunisation and use as radiolabel has allowed the development of assays to the middle region (44-68) of the PTH molecule (Roos et al, 1981, Marx et al, 1981, Mallette et al, 1982). In general these assays detect fragments which contain the middle region and the intact hormone. In addition mid-molecule assays detect an additional fragment in the circulation which contains the mid-region but not the C-terminal region (Roos et al, 1981, Marx et al, 1981). This fragment is similar in size and immunochemical characteristics to the mid-region fragment found in parathyroid gland extracts. It has been proposed that this fragment is a specific marker for increased PTH secretion as it is absent from basal samples from normal subjects (Roos et al, 1981).

Intact PTH: At the time of considering this project there were two approaches to the measurement of intact PTH. The first was the production of antisera to the region in which PTH is proteolytically cleaved. Mallette (1983) raised antisera to PTH (28-48) and the resulting assay detected the intact hormone and the PTH (28-48) fragment but not hormone fragments thought to be formed *in vivo*. However the assay was not sufficiently sensitive to measure intact PTH concentrations in normal subjects. The second approach was to use sequential immunoextraction of N-terminal fragments followed by a mid-molecule assay (Lindall et al, 1983). Using this assay intact PTH was detectable in all normal subjects tested.

Immunoradiometric Assay

This technique, using an excess of labelled antibody (rather than a fixed amount of labelled antigen) was introduced by Miles and Hales (1968) (Figure 1.7). Theoretically, in comparison to the sensitivity in the limited reagent RIA method, the optimal concentration of antibody in this method approaches infinity and as a

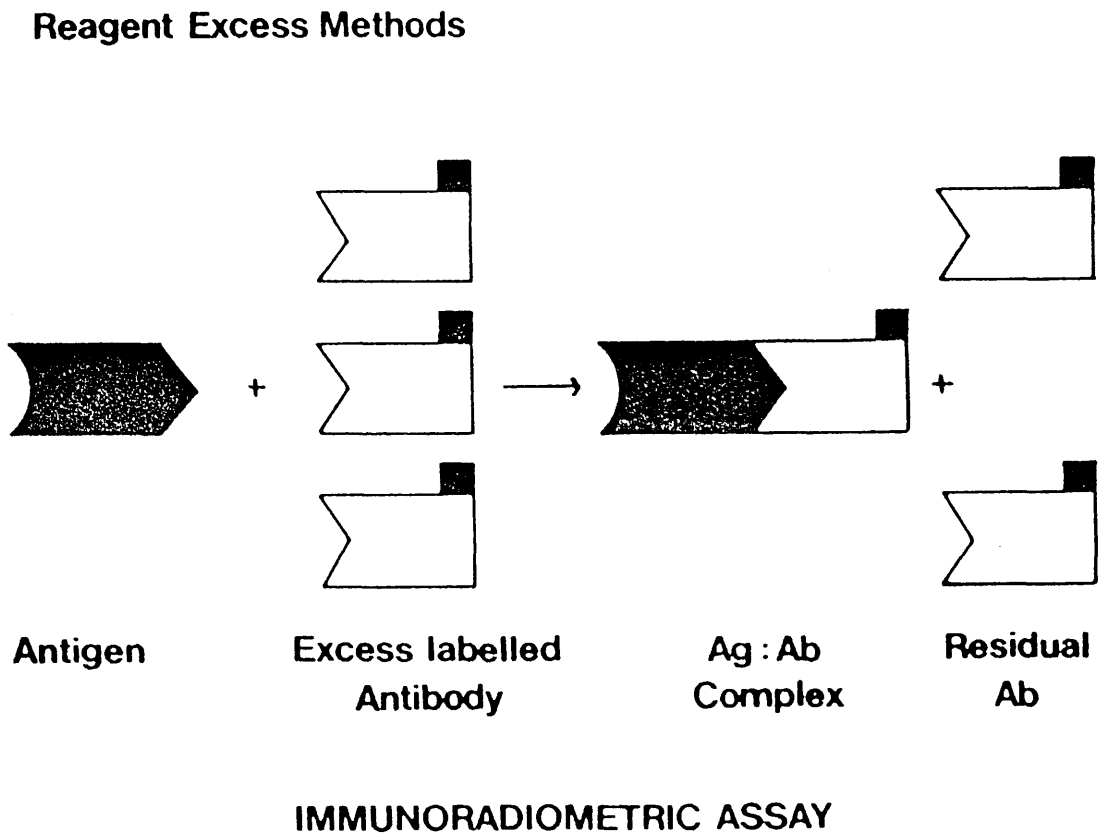

Figure 1.7

Principles of immunoradiometric assay. Antigen is reacted with an excess of radiolabelled antibody. The amount of antigen bound to the radiolabelled antibody is directly proportional to its concentration. Residual radiolabelled antibody is separated by the addition of antigen on a solid-phase support.

consequence incubation times tend towards zero. The limiting sensitivity in the labelled antibody technique is determined to a large extent by the non-specific binding of the labelled antibody (Ekins 1981). The two main advantages of the use of this technique in a PTH assay would therefore be a potential increase in sensitivity and, in view of the problems in obtaining suitable PTH material for iodination and the lability of iodinated PTH, the use of a labelled antibody.

Initially antisera were raised to extracts of bovine PTH and selected for recognition of human PTH. A bovine PTH immunoadsorbent was used used to remove the residual antibody (see Figure 1.6) producing an assay for total immunoreactive PTH in the circulation (Addison et al 1971). The assay was further refined by raising antisera to partially purified human PTH (1-84) and by the use of a PTH (1-34) or PTH (53-84) immunoadsorbent, homologous immunoradiometric assays were produced to N-terminal (Manning et al, 1980) and C-terminal (Manning et al, 1981) PTH respectively.

These assays while providing a rugged estimate of N-terminal or C-terminal PTH were not widely developed probably because of the difficulties in maintaining the large volumes of antisera required. In addition the N-terminal assay was not sufficiently sensitive to measure detectable PTH in sixty percent of normal subjects (Papapoulos et al, 1980).

4.3 Utility of PTH assays in the laboratory investigation of patients with disorders of calcium homeostasis

In the laboratory assessment of patients with disorders of calcium metabolism the major application of PTH assays is in the differential diagnosis of hypercalcaemia and in the monitoring of secondary hyperparathyroidism in renal failure. Hypercalcaemia and particularly primary hyperparathyroidism is a common differential diagnostic problem. Many patients are either asymptomatic or have only vague non-specific symptoms (Heath 1989). There are differences in the incidence

and causes of hypercalcaemia between the general population, or unselected outpatients, and hospital inpatients. The incidence of hypercalcaemia in the general population has been reported as 0.1% to 1.6%, while in hospital inpatients the incidence ranged from 0.5 to 3.6% (Fiskin et al, 1980). Primary hyperparathyroidism was the most common cause of hypercalcaemia in the general population while in the hospital inpatients hypercalcaemia was most commonly associated with malignancy. In primary hyperparathyroidism increased PTH secretion due to adenoma, hyperplasia or carcinoma of the parathyoid gland results in increased calcium in serum (Clark and Duh 1989). In hypercalcaemia associated with malignancy the release of humoral factors by the tumour such as parathyroid hormone related peptide, prostaglandins, transforming growth factors, cytokines or 1,25-dihydroxyvitamin D may cause an increase in osteoclastic bone resorption, increased renal tubular absorption of calcium or increased absorption of calcium from the gut leading to hypercalcaemia. (Mundy 1988). Secondary hyperparathyroidism is a consistent feature of advanced renal failure. The concentration of PTH in the circulation rises early in renal disease and often continues to increase in as renal function declines. Many patients develop progressive secondary hyperparathyroidism and eventually require subtotal parathyroidectomy (Arnaud 1973, Fuss et al, 1976)

In primary hyperparathyroidism, which is characterised by oversecretion of PTH, it would be expected that assays of PTH function if sufficiently sensitive, would correlate strongly with the clinical status of the patient. Only the most sensitive bioassay, the cytochemical bioassay (Chambers et al, 1978), has been shown to reliably discriminate between normals and either hypoparathyroid or hyperparathyroid patients (Allgrove et al, 1983). In hypercalcaemia of malignancy in which PTH secretion is suppressed, as a consequence of the humoral induced hypercalcaemia, the assay of PTH function (bioassays, NcAMP and TmP/GFR) provides a less specific assessment of circulating PTH than do immunoassay methods due to the ability of parathyroid-like peptides to interact with the PTH

receptor and initiate a biological response (Stewart et al, 1983). Indeed it has been as a consequence of careful consideration of the discrepancy between the estimates of immunoreactive PTH and the those of PTH function in hypercalcaemia of malignancy that the presence of parathyroid hormone-related peptide was identified (Stewart et al 1983), isolated and sequenced (Suva et al, 1987) and immunoassay methods for its measurement developed (Burtis et al, 1990, Henderson et al, 1990).

N-terminal immunoassays measure the biologically active region of PTH and therefore might be expected to be the most useful for clinical purposes. However the evaluation of early N-terminal immunoassays had shown variable discrimination between normal subjects and patients with primary hyperparathyroidism (Armitage 1986). This probably reflects the limited sensitivity of these methods in which most normal sera have undetectable PTH concentrations. Improved discrimination between these groups, with up to 90% of patients with primary hyperparathyroid patients having elevated PTH concentrations, has been obtained with more sensitive N-terminal assays (Manning et al, 1980, Potts et al, 1983, Hawker et al, 1984) although they are not sufficiently sensitive to measure circulating PTH in normal subjects. N-terminal assays are useful in the investigation of hypercalcaemia of malignancy as PTH concentrations are low or suppressed (Armitage 1986). The half-life of the immunoreactive N-terminal PTH is similar to that of intact PTH and therefore these assays can be applied to the study of acute changes in PTH secretion. As N-terminal immunoreactive PTH is less affected than C-terminal fragments by diminished renal clearance these assays are also useful in monitoring patients with renal failure.

Paradoxically C-terminal and mid-molecule assays which measure predominantly biologically inactive fragments assays have provided clearer discrimination between normal and abnormal parathyroid function (Armitage 1986). It has been suggested that the results of C-terminal PTH assays correlate well with parathyroid gland activity because, with the longer half-lives of the C-terminal fragments, these assays

provided an indication of the integrated secretory activity of the parathyroid glands (Arnaud et al, 1974). The finding that the small mid-region fragment reflects parathyroid activity has given the importance to mid-molecule assays (Roos et al, 1981). An assay has been developed with an antiserum to mixed C-terminal and mid-molecule activity (Hawker et al, 1984). Although mid-molecule and C-terminal assay have been shown to provide good discrimination of normal from hyperparathyroid patients these assays are markedly affected by renal function and give consistently detectable values in patients with hypercalcaemia of malignancy (Armitage 1986).

At the time of considering this project the procedure for intact PTH (1-84) had recently been developed and data available on the clinical validation of this method was limited. Initial studies suggested that the method could measure circulating PTH (1-84) in normal subjects and gave good discrimination between the normals and patients with primary hyperparathyroidism (Lindall et al, 1983). However, this method requires an immunoextraction step and may not be suited to processing large numbers of specimens.

In summary, therefore, no one method for the assay of circulating PTH is optimal for the clinical laboratory. The cytochemical bioassay while providing valuable clinical information is technically complex and therefore the method remains a reference method rather than a routine procedure. Immunoassay methods are ideally suited to the processing of large numbers of samples and will remain the method of choice for the clinical laboratory. At this time, the mid-molecule and C-terminal assays provide the best discrimination between normal and hyperparathyroid patients. However, it is probable the development of more sensitive assays for the biologically active N-terminal PTH and more particularly specific assays for intact PTH (1-84) will produce methods with the discrimination required for the laboratory investigation of patients with disorders of calcium metabolism and which will be applicable to all clinical samples. In addition the ability to measure directly changes in circulating

PTH (1-84) would allow the study of the physiology of PTH (1-84) secretion.

5 STRATEGY FOR THE MEASUREMENT OF PTH (1-84)

5.1 Objectives

From consideration of the review of the PTH assay methods and their clinical utility I decided that the proposed assay should be specific for the major circulating bioactive form of PTH - intact PTH (1-84). The assay should not measure and should be free of interference from N-terminal or C-terminal fragments and therefore be applicable to samples from patients with renal failure. In the initial assay design parameters I included the following general characteristics:-

a) The assay should be capable of measuring circulating PTH in all normal subjects. Given this requirement then, from the estimates available (Lindall et al, 1983), the assay will be required to measure low picomolar concentrations of PTH (1-84).

b) The estimates of circulating PTH concentrations obtained from the assay should allow good discrimination between normal subjects and patients with either primary hyperparathyroidism or hypercalcaemia of malignancy i.e. the assay should be of use in the differential diagnosis of hypercalcaemia.

c) The assay should be suitable for use in the routine clinical biochemistry laboratory in that it should being capable of processing large numbers of specimens and be applicable to samples from all patients groups including patients with advanced renal failure.

5.2 Two-site Immunometric Assay

As the proposed assay was to be applicable to the clinical laboratory I judged immunoassay to be the method of choice. Further, given the aims for specificity and sensitivity, I proposed to develop a two-site immunometric assay for PTH (1-84).

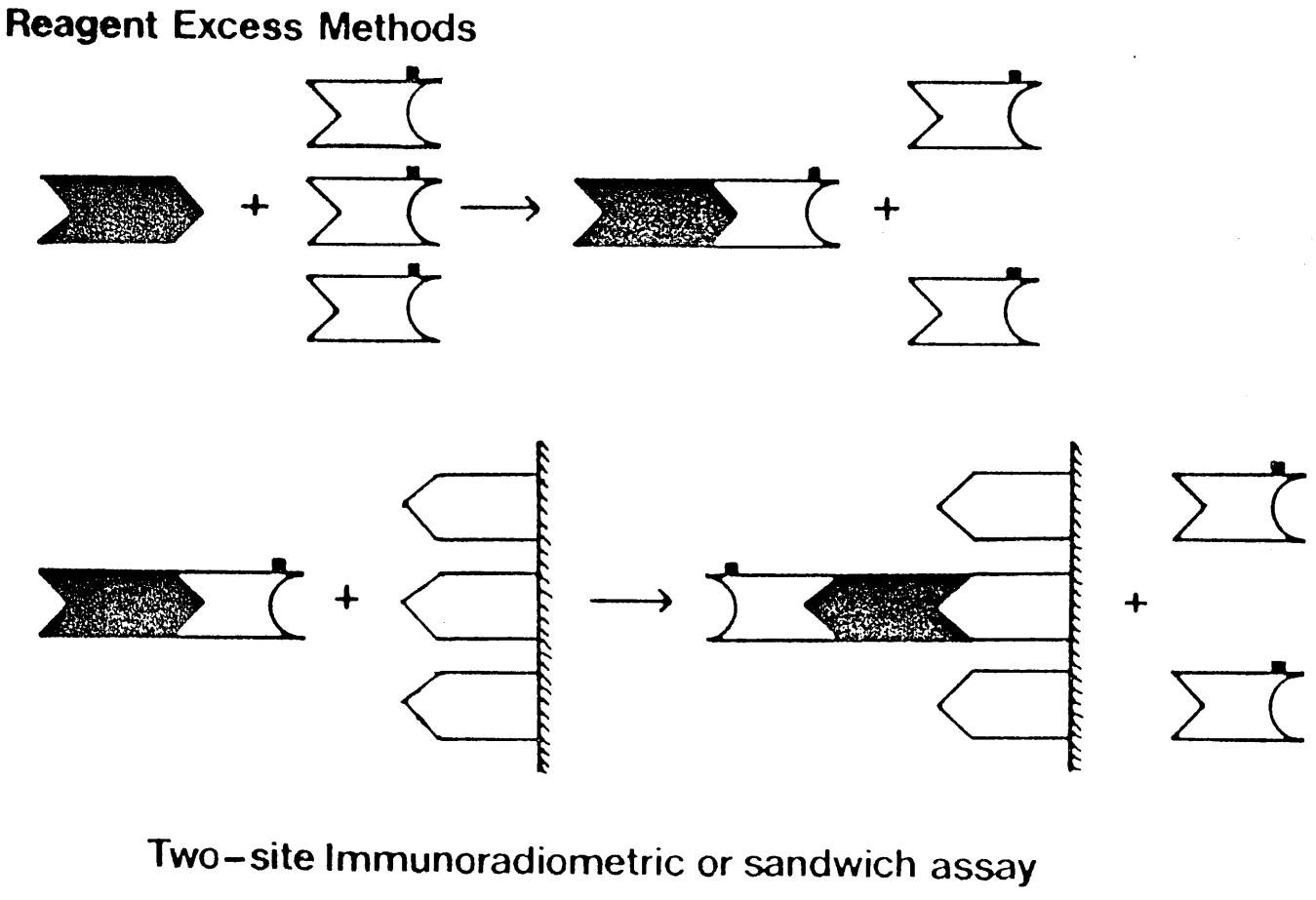

Figure 1.8

The two-site immunoradiometric assay. Antigen is reacted with an excess of radiolabelled antibody and a second antibody (on a solid-phase support) raised to a different epitope on the antigen. The antigen sandwich is separated from the unbound radiolabelled antibody by centrifugation and washing.

This technique is a modification of the immunometric, or labelled antibody, assay in which two antibodies are combined to provide the signal (Figure 1.8). This technique combines the advantages of the excess labelled antibody methods, (increased sensitivity, precision and working range), with the defined specificity inherent in employing two antibodies, raised to different but complementary epitopes on the molecule to be measured. The use of labelled antibody also avoids the problems associated with the production and storage of radiolabelled PTH.

5.3 Monoclonal antibody production

The development of a two-site immunometric assay (IRMA) specific for for PTH (1-84) would require two antibodies, one with absolute specificity for N-terminal PTH, the other with absolute specificity for C-terminal PTH. The antibodies would be either coupled to a solid-phase support or radiolabelled and therefore would be required in large quantities and in purified form.

The development of the techniques to produce monoclonal antibodies (Kohler and Milstein 1975) has had a major impact on the development of two-site IRMA methods. The classical disadvantage of having to maintain large volumes of antisera and the problems inherent in radiolabelling polyclonal antisera have been to a large extent circumvented as monoclonal antibody (Mab) technology enables the production of gram quantities of purified antibodies of defined specificity.

The principle underlying the successful production of monoclonal antibodies is outlined in Figure 1.9. Lymphocytes from immunised animals will not themselves grow *in vitro* but they may be immortalised by fusion with myeloma cell lines. The resulting hybrid myeloma (hybridoma) cells retain the important properties of both cell types involved in the fusion. Thus they not only grow indefinitely as tumour cells like the myeloma but also continue to secrete the antibody characteristic of the lymphocyte. The key to this success was the careful choice of fusion partners, these being not only from the same species (originally from mouse) but also of the same

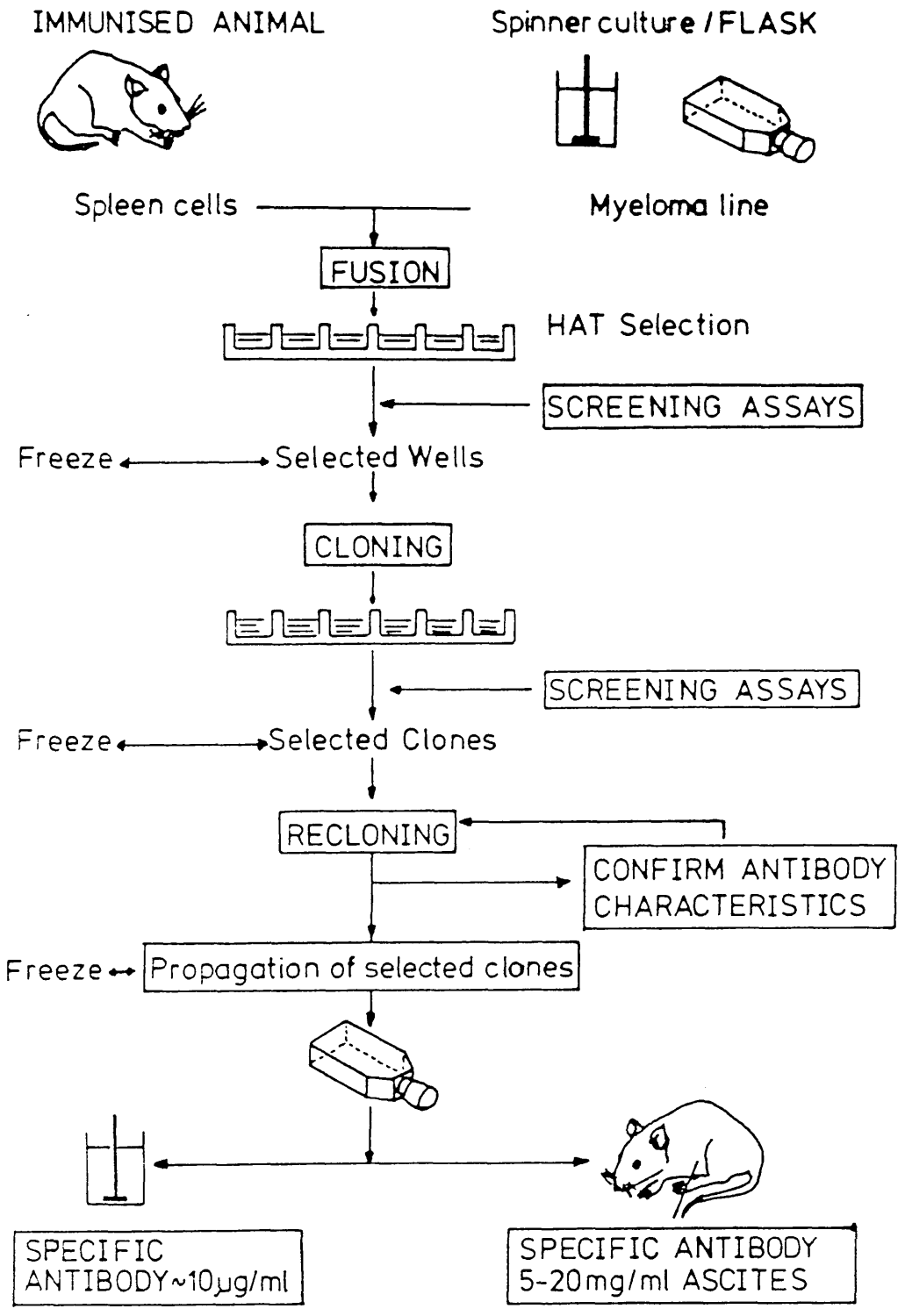

Figure 1.9

Outline of the steps involved in the production of monoclonal antibodies.

basic cell type. As an essential refinement the myeloma cells were engineered to have an enzyme (hypoxanthine-guanine phosphoribosyltransferase) deficiency. Thus, after cell fusion, hybrids but not parental cells survive to grow in selective medium containing hypoxanthine, aminopterin and thymidine (HAT). This is important as the efficiency of fusion is low (of the order of 1 in 10,000 cells) so that hybrids are initially vastly outnumbered by myeloma cells. Hybrid cells may be cloned and individual colonies, each secreting a different homogeneous, defined antibody, can be grown in tissue culture or as tumours in animals, for antibody production. Cells can be maintained indefinitely in culture or stored frozen until required.

Thus I proposed that the production of large quantities of purified antibodies of defined specificity by monoclonal antibody technology was to be central to the strategy for the development of the two-site IRMA for PTH (1-84).

5.4 Chemically synthesised PTH peptides as immunogen

At the time of initiating this work the above approach of two-site immunometric assay based on monoclonal antibodies was begining to be applied to the measurement of hormones. Two-site IRMA's had been successfully developed for human chorionic gonadotrophin (HCG), alphafoetoprotein (AFP) and luteinising hormone (LH) (Shimizu et al, 1982, Hunter et al, 1982, Hunter et al, 1983) These assays had confirmed the theoretical predictions of improved sensitivity, precision and working range over the corresponding RIA methods. However these molecules are highly immunogenic and material for immunisation was readily available.

For monoclonal antibody production it is possible to use a crude extract of an antigen, which is immunogenic, and then select for the hydridoma clones with the appropriate specificity - the approach successfully adopted for HCG, AFP and LH. Nevertheless it would also be possible to use defined preparations of the antigen and therefore ensure antisera of defined specificity. PTH is a poor immunogen and human material for immunisation was not available at the outset of this project. I

proposed therefore to utilise the available chemically synthesised peptide sequences of PTH which had been used to raise polyclonal antisera but not monoclonal antibodies, useful in diagnostic systems.

The use of synthetic human PTH peptide sequences offered several potential advantages - a) the synthetic peptide sequences were readily available in sufficient quantities; b) by immunising with only either the N-terminal or C-terminal sequence it would be improbable that antibodies raised would recognise both regions, thus ensuring totally N or C-terminal specific antisera; and importantly, c) as PTH is a poor immunogen it was hoped that immunisation with parts of the PTH (1-84) molecule would raise antisera to epitopes not normally immunogenic as part of the whole molecule (Lerner 1982).

6 AIMS FOR THE WORK OF THE THESIS

The primary aim at the outset of this project was to develop an assay for circulating PTH (1-84) which would supersede- the existing RIA method which was based on the predominantly N-terminal specific antibody AS 211/32. The secondary aim of the work of this thesis was the application of the assay to a re-examination of the physiology and pathophysiology of PTH secretion. It was was hoped the assay , by virtue of increased specificity and sensitivity, would be able to measure changes within the reference range for normal subjects with confidence and thus provide a clearer insight into the control of PTH secretion particularly with regard to dynamic changes in circulating PTH concentrations.

Thus the aims and proposed strategy for the work of this thesis can be summarised as follows:-

1) The production of monoclonal antibodies of defined specificity using chemically synthesised peptide sequences of PTH.

2) The development and validation of a two-site IRMA specific for intact PTH (1-84).

3) The application of the assay developed to the physiology and pathophysiology of PTH (1-84) secretion.

CHAPTER 2

MATERIALS AND METHODS

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1 MATERIALS

	Supplier
BioGel P10	Biorad Ltd
Bovine serum albumin (BSA)	Sigma Chemical Co.
Carbonyldiimidazole	Sigma Chemical Co.
Cell culture plastics	Northumbria Biologicals UK
Cell culture reagents	Flow Laboratories UK.
Cysteine hydrochloride	Sigma Chemical Co.
Dimethyl Sulphoxide (DMSO)	Sigma Chemical Co.
Donkey anti-sheep gamma globulin	SAPU
ECDI (ethyl-dimethyl-aminopropyl carbodiimide)	Sigma Chemical Co.
Freund's adjuvant	Difco Laboratories
Glutaraldehyde (electron microscopy grade)	Sigma Chemical Co.
Mouse/Rat strains	Bantam and Kingman UK
Non-immune bovine and ovine serum	SAPU
Non-immune equine serum	Wellcome Laboratories UK
Non-immune rat serum	Serotec UK

Nude mice	Olac Ltd UK
Pristane (2,6,10,14-tetramethylpentadecane)	Sigma Chemical Co.
PTH (1-34)	Universal Biologicals
PTH (53-84)	Universal Biologicals
PTH (1-84)	Peninsula Laboratories
PTH (1-10)	Cambridge Research Biochemicals
PTH (1-10)-KLH	Cambridge Research Biochemicals
PTH (74-84)	Cambridge Research Biochemicals
PTH (74-84)-KLH	Cambridge Research Biochemicals
Sepharose 6B	Pharmacia
Sepharose CL-4B	Pharmacia
Sheep anti-mouse gamma globulin	SAPU
Sheep anti-rat gamma globulin	SAPU

All other reagents and chemicals were Analar grade from BDH.

2 IMMUNISATIONS AND SERUM ANTIBODY EVALUATION

2.1 N-terminal PTH Immunogens

(a) Synthetic human PTH (1-34) peptide.

(b) Synthetic human PTH (1-34) conjugated to albumin. PTH (1-34) was coupled to bovine serum albumin in the initial experiments by the carbodiimide method and latterly by the glutaraldehyde method both as described by Orth (1979) except that the quantities were scaled down to allow coupling of 400 μg peptide batches:-

Carbodiimide: Reagents were dissolved in 0.04 mol/l potassium phosphate buffer pH 4.5. ECDI (0.1 ml; 3.7 mg/ml) was added to a solution of bovine serum albumin (0.4 ml; 3.6 mg/ml) in a polystyrene test tube (55 x 75 mm) and incubated for 60 minutes at room temperature with mixing. The PTH peptide (0.4 ml; 400 μg) was added to the reaction mixture in 4 x 0.1 ml aliquots over the next 60 minutes with continued mixing. This is equivalent to a molar ratio of peptide to albumin to ECDI of 1: 0.1: 100. The conjugate was purified by by dialysis against 0.05 mol/l sodium phosphate buffer pH 7.4 in a microdialysis system (Bethesda Research Laboratories). Incorporation of peptide was monitored by the inclusion of radiolabelled peptide (10,000 cpm/tube).

Glutaraldehyde: Reagents were dissolved in 0.05 mol/l sodium phosphate buffer pH 7.4. PTH peptide (0.4 ml; 400 μg) was added to a solution of bovine serum albumin (0.4ml; 3.6 mg/ml). Glutaraldehyde (0.025 % v/v) was then added to the mixture in 4 x 0.05 ml aliquots over 60 minutes with stirring. The conjugate was purified by dialysis against 0.05 mol/l sodium phosphate buffer pH 7.4 in a microdialysis system (Bethesda Research Laboratories). Incorporation of peptide was monitored by the

inclusion of radiolabelled peptide (10,000 cpm/tube).

(c) Synthetic human PTH (1-10) conjugated to keyhole limpet haemocyanin (KLH). PTH (1-10) was custom synthesised and conjugated to KLH, using a bis-diazotised tolidine linkage that produced a C-terminally bound peptide, by Cambridge Research Biochemicals.

2.2 C-terminal PTH Immunogens

(a) Synthetic human PTH (53-84).

(b) Synthetic human PTH (53-84) conjugated to albumin. PTH (53-84) was coupled to bovine serum albumin in the initial experiments by the carbodiimide method and latterly by the glutaraldehyde method both described by Orth (1979) as in Section 2.1 above.

(c) Synthetic human PTH (74-84) conjugated to keyhole limpet haemocyanin. PTH (74-84) was custom synthesised and conjugated to KLH, using a bis-tolidine linkage that produced an N-terminally bound peptide, by Cambridge Research Biochemicals.

2.3 Preparation of Immunogens

Immunogens were prepared as water-in-oil emulsions. One volume of immunogen in buffer and three volumes of adjuvant, in separate glass syringes, were emulsified by repeated passage through a 19 g needle fitted with twin Luer hubs.

2.4 Immunisation Schedules

Mice: Groups of six female mice (six to eight weeks old) were given primary immunisations of peptide or conjugate administered intraperitoneally (ip) followed by up to four secondary immunisations (also ip) at intervals of two

weeks.

Rats: Groups of six female rats (150 to 200 g) were given primary immunisations of peptide or conjugate followed by up to five secondary immunisations at intervals of two weeks. Immunisations were administered subcutaneously using the multi-site method (Vaitukaitus et al 1971).

Sheep: Groups of two Suffolk wethers were given a primary immunisation with peptide or conjugate followed by secondary immunisations at monthly intervals. Immunisations were administered intramuscularly and subcutaneously using the multi-site method (Vaitukaitus et al 1971).

2.5 Blood Spot Sampling

Mice and rats were anaesthetised with ether, the ventral vein punctured with a sterile 23 g needle and the exuding blood absorbed onto filter paper card to form a discrete spot. The blood spot was allowed to dry and a 6 mm disc punched out into 1.0 ml 0.9% saline.

2.6 Iodination of PTH Peptides

PTH peptides were iodinated by the solid-phase lactoperoxidase method of Karonen et al (1975).

To PTH peptide (2 µg) the following additions were made:-

10 µl 0.5 mol/l sodium phosphate pH 7.4

10 µl solid-phase lactoperoxidase (prepared locally)

10 µl ^{125}I, carrier-free (1 mCi).

10 µl hydrogen peroxide (30% w/v stock diluted 1:20,000 in distilled water).

The reaction was terminated by the addition of 200 µl of column buffer,

0.1 mol/l sodium acetate buffer pH 4.0 containing 0.1% BSA. The solution was then applied to a column (30 x 1 cm) of Biogel P10 and eluted in the column buffer. The iodinated peptide peak was then pooled and aliquots stored at -20°C.

2.7 Preparation of Sepharose Linked Second Antibodies

Sheep anti-mouse, sheep anti-rat and donkey anti-sheep gamma globulin antisera were coupled to Sepharose CL-4B by the carbonyldiimidazole method of Chapman and Ratcliffe (1982).

A calibrated solution of Sepharose Cl-4B in distilled water (200 ml; equivalent to 100 ml settled gel) was transferred to a sintered glass funnel (porosity 3) and dehydrated by washing the gel successively with 500 ml aliquots of distilled water, 30%, 50%, 70% and 100% acetone. The dehydrated gel slurry was resuspended in acetone to a volume of 200 ml to which was added 4.87 g of carbonyldiimidazole (0.15 mol/l). The solution was incubated for 60 minutes with stirring, then transferred to the sintered glass funnel and rehydrated by washing with 500 ml aliquots of acetone, 30%, 50%, 70% and 100% distilled water. The gel was then washed with 500 ml coupling buffer (0.1 mol/l EPPS pH 8.0 containing 0.05% sodium azide). The gel was transferred to a polycarbonate bottle containing 25 ml of the appropriate antiserum to be coupled, the volume adjusted to 200 ml by the addition of coupling buffer, and the solution incubated for 18-24 h with mixing.

The gel solution was then centrifuged, the supernatant containing uncoupled protein retained for further use and the gel washed, recovered by centrifugation (5 minutes at 1,200 g), according to the procedure of Wide (1969).

The gel was washed with 100 ml aliquots of :

(a) 0.5 mol/l sodium bicarbonate buffer pH 8.0, mix for 20 minutes

(b) repeat sodium bicarbonate wash as in (a)

(c) 0.1 mol/l sodium acetate buffer pH 4.0, mix for 60 minutes

(d) 0.1 mol/l sodium acetate buffer pH 4.0. mix for 18-24 hours

(e) 0.9% saline, mix for 20 minutes

(f) repeat saline wash as in (e).

The gel was then stored in isotonic saline at 4°C until required.

2.8 Antibody Screening Assay

The screening assay diluent was 0.05 mol/l sodium barbitone buffer pH 8.6, containing 0.1% BSA.

Dilutions of serum or blood spot eluate collected from immunised animals were incubated (0.1 ml) with iodinated PTH peptide(10,000 cpm/tube) overnight at room temperature. Sheep anti-mouse/anti-rat or donkey anti sheep gamma globulin coupled to Sepharose CL-4B was then added (1 mg/tube) and the assay incubated with shaking for 2 h at room temperature. The assay was then washed (x3) with 2 ml 0.9% saline/Tween 20 (0.2% v/v) by centrifugation and aspiration.

3 PRODUCTION OF MONOCLONAL ANTIBODIES

3.1 Myeloma Cell Medium

To 100 ml RPMI 1640 media containing 20 mmol/l HEPES the following were added:

10 ml foetal bovine serum (heat inactivated)

1 ml glutamine (200 mmol/l)

2 ml Penicillin/Streptomycin (100 U/ml, 100 μg/ ml respectively)

1 ml Fungizone (250 g/ ml)

3.2 Hybridoma Cell Medium

To 100 ml RPMI 1640 media containing 20 mmol/l HEPES the following were added:

20 ml foetal bovine serum (heat inactivated)

1 ml glutamine (200 mmol/l)

2 ml Penicillin/Streptomycin (100 U/ml, 100 μg/ ml respectively)

1 ml Fungizone (250 μg/ ml)

4 ml Hypoxanthine, Aminopterin, Thymidine (HAT) solution (100, 0.4 and 16 μmol/l respectively)

3.3 Maintenance of Myeloma Cell Lines

Aliquots (10 x 10^6 cells/ml) of the myeloma cell lines X63.Ag8.653 (mouse) and Y3 Ag 1.2.3 (rat) were stored in liquid nitrogen (-196^{o}C) until required. In preparation for hybridisation experiments an aliquot of myeloma cell line was thawed rapidly by immersion in a water bath at 37 ^{o}C and the cells transferred aseptically to a sterile universal container (20 ml). The cells were then washed (x3) in prewarmed myeloma cell medium.

The concentration of cells was then adjusted to 200,000 cells/ml with further myeloma cell medium and the cells incubated in a stoppered flask at 37 ^{o}C. The myeloma cells were maintained in logarithmic growth (minimum and maximum densities of 2.5 x 10^5 and 1.5 x 10^6 cells/ml; doubling time 16-24 h). Cells were counted and their viability assessed using a haemocytometer and the

trypan blue dye exclusion method.

3.4 Myeloma Cells

The volume of medium required to provide 150 x 10^6 myeloma cells was centrifuged at 800 rpm for 5 minutes and the supernatant discarded. The cell pellet was washed and resuspended in 15 ml RPMI. As each hybridisation experiment consisted of three separate fusion events the cells were split into 3 x 5 ml aliquots of 50 x 10^6 myeloma cells.

3.5 Spleen Cells

The spleen from an immunised mouse was removed under sterile conditions and placed in a petri dish (5.5 cm diameter) containing 10 ml RPMI. The spleen was dissected free of fatty tissue and the spleen cells carefully teased from the capsule with scalpel blades. The cell suspension was transferred to a universal container and allowed to settle. The supernatant cells were removed, centrifuged at 800 rpm for 10 minutes and the cells resuspended in 15 ml RPMI. The cells were counted and split into three aliquots of 50 x 10^6 cells.

3.6 Polyethylene Glycol (PEG) 1500

PEG (10 g) was weighed into a 20 ml measuring cylinder and 5 ml RPMI added. The PEG was dissolved by heating in water bath at 50 ^oC. The pH was then adjusted to 7.0 with NaOH (0.1 mol/l). The volume was then made to 20 ml with pre-warmed RPMI. The PEG solution was then sterilised by membrane filtration (0.22 μm).

3.7 Peritoneal Macrophages (feeder cells)

Balb/c mice (3) were killed and the peritoneal cavity washed with 5 ml RPMI using a syringe with a 19 g needle. The three peritoneal washings were pooled into a universal container, centrifuged at 800 rpm for 5 minutes, resuspended

and counted. The cells were then diluted in hybridisation medium to a density of 3×10^4 cells/ml.

3.8 Hybridisation Protocol

The hybridisation protocol employed is based on that of Fazekas De Groth and Scheidegger (1980).

Each hybridisation experiment consisted of three fusion events.The 3 x 5 ml aliquots of spleen and myeloma cells were mixed and centrifuged for 10 minutes at 800 rpm. The supernatants were then decanted and the containers with the cell pellets placed in a beaker of water at 37 ^{o}C. The PEG solution (0.8 ml) was added dropwise over 60 seconds, after a further 60 seconds 10 ml warmed RPMI was added over 5 minutes. The mixture of cells was centrifuged for 10 minutes at 800 rpm, washed in RPMI and suspended in 60 ml pre-warmed hybridoma cell medium.

3.9 HAT Selection

The hybridisation products were plated out into 5 x 96 well microtitre plates (0.2 ml/well) containing peritoneal macrophages. Plates were sealed with sellotape and incubated at 37^{o}C for 10-14 days before examination for growing hybrids and subsequent screening for antibody production.

3.10 Cloning of Hybridoma Lines

Hybridoma cells were cloned by limiting dilution. A dilution was made in hybridoma cell medium to a concentration 800 cells/ml. Doubling dilutions were then performed across the 12 columns of a 96 well plate containing peritoneal macrophages. The plates were sealed with sellotape and incubated at 37^{o}C for 10-14 days before examination for hybridoma growth and screening for antibody production. The cells growing from the highest dilution (lowest

starting cell numbers) and identified as producing antibody were selected for further cloning. Cell lines were cloned at least twice before expansion and ascitic fluid production.

3.11 Screening for PTH Antibody

All wells with growing hybrids were screened for antibody production. Cell culture supernatant (0.1 ml) was incubated with iodinated PTH peptide (10,000 cpm/tube) diluted in 0.05 mol/l barbitone buffer pH 8.6 containing 0.5% BSA, overnight at room temperature. Sheep anti-mouse, or anti-rat gamma globulin coupled to Sepharose CL-4B was then added (1 mg/tube) and the assay incubated with shaking for 2 h at room temperature. The assay was then washed (x3) with 2 ml 0.9% saline/Tween 20 (0.2% v/v) by centrifugation and aspiration.

3.12 Assessment of Immunoglobulin Class of Antibodies

Rat monoclonal antibody typing kits (Serotec) were used to assess the immunoglobulins produced by the hybridoma lines. The test entails diffusing six antisera, directed against rat IgG1, IgG2a, IgG2b, IgG2c, IgA and IgM, against neat culture supernatants in immunodiffusion plates.

3.13 Expansion of Hybridoma Lines

Selected hybridoma lines were transferred from the 0.2 ml wells of the clone plates to 2 ml wells and allowed to expand before seeding in 25 ml and 100 ml flasks. Feeder cells were present at each stage and cell densities in flasks were maintained at 10^5-10^6 cells/ml.

3.14 Cell Freezing

All cells were stored frozen in liquid nitrogen. Cells were centrifuged and resuspended at 1-5 x 10^6 cells/ml by the dropwise addition of ice cold foetal

bovine serum containing 10% DMSO. Aliquots (1ml) containing 5-10 x 10^6 cells were dispensed into cryovials and frozen slowly overnight in a polystyrene box at -70 °C . The cells were then transferred to liquid nitrogen for long term storage.

3.15 Bulk Production of Monoclonal Antibody

Congenitally athymic (Nu/Nu; nude) mice were used for the production of ascitic fluid and were maintained under sterile conditions in a laminar flow hood. The nude mice were primed by the intraperitoneal injection of 0.5 ml pristane 7 days before the injection of 5 x10^6 hybridoma cells, which had been washed and resuspended in 5 ml RPMI. Ascitic fluid was tapped at intervals of 2-3 days from anaesthetised nude mice using a 19 g needle allowing the fluid to to drain from the abdomen under gravity.

IgG was purified from ascitic fluid using the method of Steinbuch and Audran (1969). Ascitic fluids of the same tap number were pooled to a volume of 20 ml and adjusted to pH 5.0 with acetic acid (0.1 mol/l). N-octanoic was added dropwise with stirring (1.76 ml/20 ml ascitic fluid). After stirring for 30 minutes the solution was centrifuged at 1500 g for 20 minutes and the supernatant containing the IgG retained. The pellet was washed (x2) with 20 ml 0.1 mol/l sodium bicarbonate pH 8.0. The supernatants were pooled and dialysed against 0.01 mol/l sodium bicarbonate pH 8.0 and stored at -50 °C .

3.16 Protein Estimation

Protein estimations were carried according to the method of Schacterle and Pollack (1973), based on the original work of Lowry et al (1951).

Reagents:

(a) Alkaline copper reagent: 20 g Na_2CO_3, 4 g NaOH, 200 mg sodium tartrate and 100 mg copper sulphate ($5H_2O$) were dissolved and made up

to a final volume of 200 ml with distilled water.

(b) Folin-Ciocalteau reagent: a 1:25 dilution of the pre-prepared stock solution in distilled water.

(c) Protein standards: a stock solution of 50 mg BSA in 50 ml distilled water was double diluted (x4) prior to use.

Protocol:

Standard, buffer blank or test solution (0.2 ml) was mixed with reagent (a), vortexed and allowed to stand for 10 minutes at room temperature. Reagent (b) (1.0 ml) was then added, the solution vortexed, incubated at 55°C for 5 minutes, cooled and the absorbance measured at 650 nm.

4 DEVELOPMENT OF PTH (1-84) IRMA

4.1 Iodination of Monoclonal Antibodies

PTH Mab's were iodinated by the solid-phase lactoperoxidase method of Karonen et al (1975).

To PTH Mab (25 µg) the following additions were made:

10 µl 0.5 mol/l sodium phosphate pH 7.4

10 µl solid-phase lactoperoxidase (prepared locally)

10 µl ^{125}I, carrier-free (1 mCi).

10 µl hydrogen peroxide (30% w/v stock diluted 1:20,000 in distilled water).

The reaction was terminated by the addition of 200 µl of column buffer, 0.1 mol/l sodium phosphate buffer pH 7.0 containing 0.1% BSA. The solution was then applied to a column (30 x 1 cm) of Sepharose 6B and eluted in the column buffer. The iodinated antibody peak was then pooled and aliquots stored

at -20°C.

4.2 Preparation of Solid-phased Monoclonal Antibodies

Mab coupled to Sepharose CL-4B

Mab IgG was coupled to Sepharose CL-4B by the carbonyldiimidazole method of Chapman and Ratcliffe (1981) as described for second antibodies. Mab IgG (70 mg) was added to the activated Sepharose CL-4B gel and the procedure followed as in methods Section 1.7 above.

Mab coated to Microtitre Plates

Mab IgG was coated to microtitre plates using the following procedure: the Mab IgG (0.2 ml) diluted in coating buffer (0.05 mol/l TAPS buffer pH 8.6) to a concentration of 100 µg/ ml was incubated overnight at 37°C in 96 well microtitre plates (0.2 ml wells). The IgG solution was aspirated and the wells washed (x4) with coating buffer containing 0.5 % BSA (0.2 ml) . The plates were stored at 4°C in the presence of coating buffer containing 0.5% BSA until required.

4.3 Oxidation/reduction of PTH Peptides

PTH peptides were oxidised/reduced by the method of Tashijian et al (1964).

Oxidation: stock aliquots (2 µg) of PTH peptide were diluted with 1 ml sodium acetate buffer pH 3.8 (0.1 mol/l) and incubated with hydrogen peroxide (0.1 mol/l) for 3 hours at room temperature.

Reduction: stock aliquots (2 µg) were diluted with 1 ml sodium acetate buffer pH 3.8 (0.1 mol/l) and incubated with cysteine hydrochloride (0.12 mol/l) for 3 hours at 80°C.

5 COMPARISON OF PTH (1-84) METHODS

5.1 Protocol for Allegro^{R} Method

The Allegro^{R} method (Nichols Institute Diagnostics, San Juan Capistrano, CA 92675) for intact PTH method is a two-site immunoradiometric assay for the measurement of PTH (1-84). It is based on two goat polyclonal antibodies to human PTH which have been affinity purified to be specific for mid-region and C-terminal PTH (39-84) respectively. The C-terminal specific antibody is immobilized onto plastic beads and the N-terminal antibody radiolabelled.

Sample, standard or control (0.2 ml) is incubated simultaneously with radiolabelled antibody (0.1 ml; 200,000 cpm/tube) and antibody coated bead for 22 hours at room temperature. The tubes are washed (x2) with 2.0 ml of the wash solution provided and the radioactivity counted for one minute.

5.2 Protocol for Magic-Lite^{R} Method

The Magic-Lite^{R} method (Ciba Corning Diagnostics Limited, Essex, UK CO9 2DX) is a two-site chemiluminometric assay employing an acridinium ester labelled sheep anti-human PTH (1-34) and a mouse monoclonal anti-human PTH (44-68) covalently coupled to paramagnetic particles.

Sample, standard or control (0.2 ml) is incubated with acridinium ester labelled N-terminal antibody, vortexed and incubated for 18 hours at room temperature. The solid-phased C-terminal monoclonal antibody is added (0.5 ml) and incubated for 1 hour at room temperature. The assay is washed with 1.0 ml distilled water. Separation is achieved using magnetic separation racks and decantation of the supernatants. Distilled water (0.1 ml) is added and tubes vortexed prior to counting for 2 seconds in the Magic-Lite^{R} Analyzer.

CHAPTER 3

PRODUCTION OF MONOCLONAL ANTIBODIES

CHAPTER 3 : PRODUCTION OF MONOCLONAL ANTIBODIES

1 INTRODUCTION

Since the first development of a radioimmunoassay (Berson and Yalow, 1963), the measurement of PTH has played a central role in the differential diagnosis of hypercalcaemia (Boyd and Ladenson 1984). However, the low picomolar concentrations and molecular heterogeneity of circulating PTH have remained significant obstacles for the analyst and compromised the clinical utility of PTH assay development (Armitage, 1986). Classically, these problems have been addressed by attempting to develop sensitive limited reagent assays of defined specificity for PTH fragments. This requires the production of high avidity antisera, of defined specificity for PTH fragments (Segre et al, 1975).

The source of immunogen for the production of antibodies to PTH has also caused difficulty. PTH is not stored in large quantities in the parathyroid glands (Cohn and Elting 1983). Therefore supplies of PTH, especially human, are limited. Bovine parathyroid glands have been used as a source of immunogen, either partially purified extracts or purified PTH (1-84) (Nussbaum et al, 1981). Similarly adenomatous and hyperplastic human parathyroid tissue have been used as a source of immunogen (Di Bella et al, 1978a).

In general, limited reagent assays based on antisera to the N-terminal region of PTH, classically raised to purified bovine PTH (1-84) preparations in guinea pigs, chicken and goats, have lacked the sensitivity to measure circulating concentrations in the plasma of normal subjects (Arnaud et al, 1974, Martin et al, 1980, Beastall et al, 1983). In addition, these assays could not be guaranteed to be totally N-terminal specific. For example, the N-terminal antiserum AS 211/32, commercially available, and one of the most widely used in human studies was found to react also with C-terminal epitopes (Mallette, 1980).

The limited reagent assays based on antisera specific for the biologically inactive mid or C-terminal regions raised to extracts of parathyroid tissue (Di Bella et al, 1978b, Simon and Cuan 1980) while able to measure circulating concentrations, present at up to tenfold greater concentrations than N-terminal fragments or intact PTH, were at best an index of chronic parathyroid hormone secretion and markedly influenced by renal function.

The advantages of excess reagent immunometric assays, have been extensively reviewed (Miles and Hales, 1968, Woodhead et al, 1974, Ekins, 1981). Theoretically the immunometric assay can achieve greater sensitivity, for an antibody of a given avidity than limited reagent systems. Further, by using the two-site or sandwich technique assays of defined specificity can be developed (Addison and Hales 1971, Miles et al, 1974). The principles of immunometric assays were first elucidated in 1968 (Miles and Hales, 1968). However, practical difficulties - the large volumes of antisera required, high blanks in the single antibody immunometric assay which limited the sensitivities realised and the requirement for affinity purification of antibodies for labelling in the two-site assay - restricted the general application of these methods. It was with the development of the techniques for the production of monoclonal antibodies (Mab) (Kohler and Milstein, 1975) that the immunometric assays came of age. The Mab technology made available for the first time milligram to gram quantities of purified antibody.

Generally, Mab's tend to be of lower avidity than polyclonal antisera and so unsuitable for sensitive limited reagent assays (Siddle, 1985). However, using excess reagent principles in the immunometric assays greater sensitivities can be achieved than using limited reagent systems even with Mab of lower avidity, in the range 10^9 - 10^{11} (Ekins, 1981). Thus the combination of Mab technology and immunometric two-site assay principles offered the potential for the development of sensitive and specific assays for hormones circulating low concentrations. This

approach has been applied to a wide range of hormones and proteins, eg, TSH, LH, FSH and HCG - for a review see Siddle (1985).

The aim of this project was to raise Mab's of defined specificity to N-terminal and to C-terminal PTH which could be combined in an immunometric two-site assay system with the sensitivity and specificity to measure intact PTH (1-84).

The production of Mab's of defined specificity posed the combined problems as to the source of material for the immunogen and the poor immunogenecity of PTH. The availability of synthetic peptide sequences of PTH offered a ready source of milligram quantities of immunogen and importantly of guaranteed purity. The studies on the generation of an antibody response to an immunogen (Young et al, 1983) and on the 'tapping of the immune repertoire' (Lerner, 1984) had shown that small synthetic peptide sequences from a larger molecule could generate an antibody response. Further, it was shown that antibodies could be generated in response to the small sequence epitopes that would not generate a response as part of the intact molecule (Schmitz et al, 1983). Thus the aims of this project were fully defined as: the production of monoclonal antibodies of defined specificity to PTH, using synthetic peptide sequences as immunogen, which would form the basis of a two-site immunometric assay for intact PTH (1-84).

2 IMMUNOGEN AND IMMUNISATION SCHEDULES

2.1 N-terminal PTH Peptide Immunogens

Three preparations of N-terminal immunogens were employed:

(i) Synthetic human PTH (1-34) peptide

(ii) Synthetic human PTH (1-34) peptide conjugated to bovine serum albumin

(iii) Synthetic human PTH (1-10) conjugated to keyhole limpet haemocyanin (KLH)

The source of peptides and preparation of the peptide-protein conjugates is described in Methods (Chapter 2; Section 2.1).

2.2 C-terminal PTH Peptide Immunogens

Three preparations of C-terminal immunogen were employed:

(i) Synthetic human PTH (53-84)

(ii) Synthetic human PTH (53-84) conjugated to bovine serum albumin

(iii) Synthetic human PTH (74-84) conjugated to keyhole limpet haemocyanin (KLH)

The source of peptide and preparation of the peptide-protein conjugates is described in Methods (Chapter 2; Section 2.2).

2.3 Immunisation Schedules

For all primary immunisations a solution of peptide/conjugate was emulsified in Complete Freund's adjuvant. For secondary immunisations Incomplete Freund's adjuvant was employed, (see Methods, Chapter 2; Section 2.3).

(a) Mice: Groups of six female mice of six different strains, were given a primary immunisation of peptide/conjugate administered intra-peritoneally (i.p.) followed by up to four secondary immunisations (also i.p.) at intervals of two weeks.

(b) Rats: Groups of three female rats were given a primary immunisation of peptide conjugate followed by up to five secondary immunisations at intervals of two weeks. Immunisations were administered subcutaneously using the multi-site method (Vaitukaitus et al, 1971).

(c) Sheep: Groups of two sheep were given a primary immunisation followed by up to six secondary immunisations at monthly intervals. Immunisations were administered subcutaneously and intra-muscularly using the multi-site method (Vaitukaitus et al, 1971).

3 RESPONSE TO IMMUNISATION SCHEDULES

A summary of the responses to the PTH peptide immunogens obtained in sheep, mice and rats is shown in Table 3.1.

3.1 Antibody Screening Procedure

Blood samples taken by venesection were collected either in glass tubes (sheep) or absorbed on to filter paper (mice, rats). Serum or blood-spot eluate was diluted in barbitone buffer pH 8.6 (0.05 mol/l, 0.5% BSA) and incubated with radiolabelled peptide (either PTH (1-34) or PTH (53-84)) overnight at 4°C. Second antibody (either anti-sheep or anti-rat/mouse IgG) coupled to Sepharose Cl-4B was added, the assay shaken for 2 h at room temperature, then washed (x2) in Saline/Tween 20 (0.2% v/v) and counted.

3.2 Response in Sheep

There was a brisk response in Suffolk wethers to immunisation with unconjugated PTH (1-34) and PTH (53-84) peptides (50 μg primary and secondary immunisations). Antibody titres were detected at one month post primary immunisation in 2/2 and 1/2 sheep immunised with PTH (1-34) and PTH (53-84) peptides, respectively. Similar results were obtained in Suffolk wethers using PTH (1-34) and PTH (53-84) conjugated to albumin as immunogens (100 μg conjugate; equivalent to 11 μg peptide for primary and secondary immunisations). Maximum titres of 1:64,000 and 1:18,000 for PTH (1-34) and PTH (53-84) were achieved (Figures 3.1 and 3.2).

3.3 Response in Mice

No antibody titres were detected in balb/c mice in response to immunisation with unconjugated PTH (1-34) and PTH (53-84) peptides at 10, 20 or 50 μg primary and secondary immunisations. Similarly, no response was detected using PTH (1-34)

Table 3.1

Summary of responses to PTH peptide immunogens

	N-Terminal Immunogens			C-Terminal Immunogens		
	PTH (1-34)	PTH (1-34)-Alb	PTH (1-10)-KLH	PTH (53-84)	PTH (53-84)-Alb	PTH (74-84)-KLH
Sheep						
Suffolk Wether	++	++	N/A	+	+	N/A
Mice						
balb/c	-	-	-	-	-	-
NZB	-	-	-	-	-	-
balb/c/NZB	-	-	-	-	-	-
SWR	-	-	-	-	-	-
SJL	-	-	-	-	-	-
CBA	-	-	-	-	-	-
Rat						
DA	++	N/A	N/A	-	-	N/A
Lou	-	-	N/A	-	-	N/A

++ strong response detected
+ response detected
- no response detected
N/A experiment not attempted

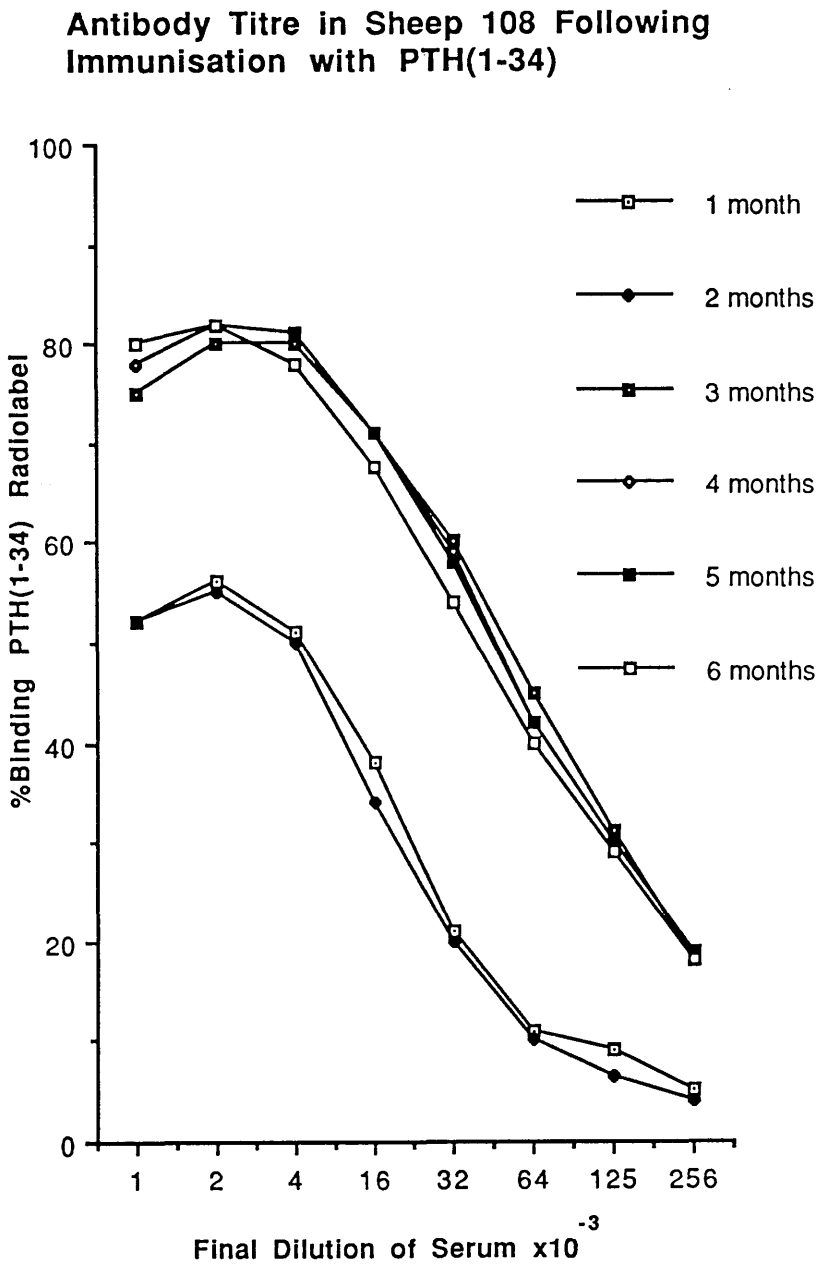


Figure 3.1

Serum titre of PTH (1-34) antibodies in sheep 108 following immunisation with PTH (1-34) peptide.

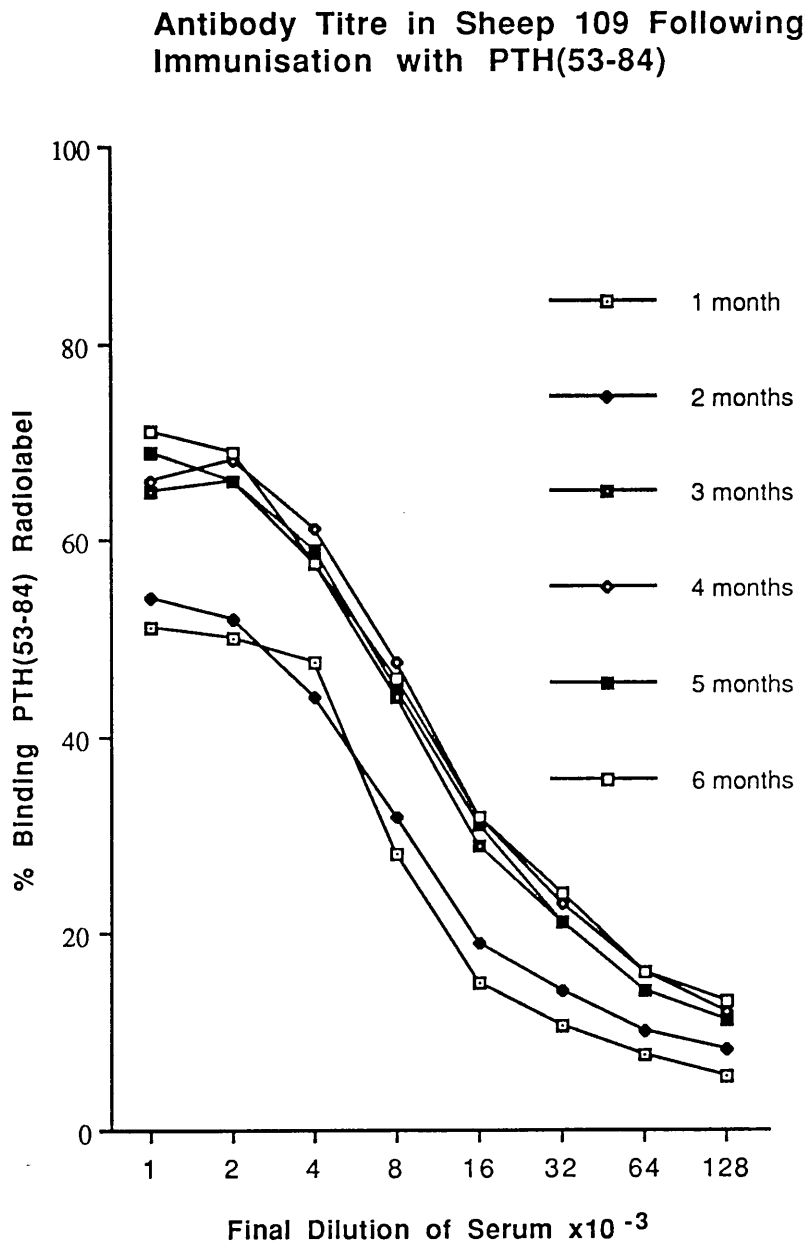


Figure 3.2

Serum titre of PTH (53-84) antibodies in sheep 109 following immunisation with PTH (53-84) peptide.

and PTH (53-84) conjugated to albumin at 100 μg conjugated (equivalent to 11 μg peptide) or 200 μg primary and secondary immunisations.

Immunisation with PTH (1-10) and PTH (74-84) conjugated to KLH (100 μg conjugate; equivalent to 30 μg peptide, primary and secondary immunisations) also failed to produce a detectable antibody titre.

The above immunisation experiments were also carried out in the following strains of mice considered likely to be higher antibody responders than the balb/c strain - NZB, balb/c NZB F1 hybrids, SWR and SJL, however no antibody titre was detected.

Following publication of the work of Nussbaum et al, (1985), on the genetic control of the generation of an immune response to PTH, the CBA strain of mouse was immunised as above. No antibody titre was detected.

3.4 Response in Rats

DA and Lou strains of rats were immunised with PTH (1-34) and PTH (53-84) peptides (50 μg primary and secondary immunisations). No antibody titres were detected in the Lou rats or in DA rats immunised with PTH (53-84) peptide. In contrast a consistent response was detected in DA rats immunised with PTH (1-34) (50 μg primary and secondary immunisations) with 6/6 DA rats responding with serum titres of 1:3,000 - 1:5,000 at one month post primary immunisation. Maximum titres of 1:50,000 were detected after a primary and five secondary immunisations (Figure 3.3).

4 PRODUCTION OF HYBRIDOMA LINES

4.1 Mouse Spleen Cell/Mouse Myeloma Cell Fusions

Despite the failure to detect an antibody titre three fusion experiments were undertaken using the spleen cells from balb/c mice immunised with either PTH (1-34) or PTH (53-84) peptides.

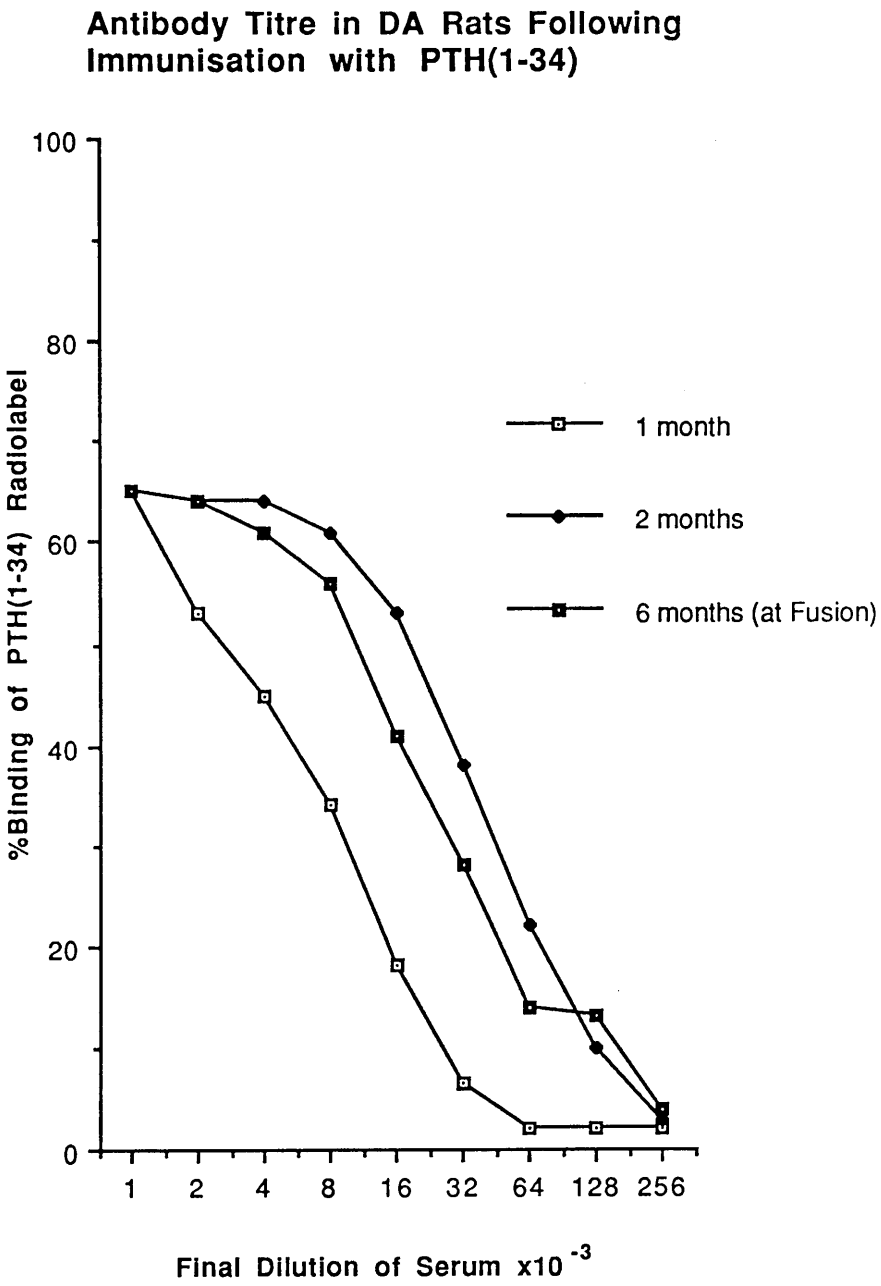


Figure 3.3

Serum titre of PTH (1-34) antibodies in a DA rat following immunisation with PTH (1-34) peptide.

The mouse myeloma line X63 Ag 8.653 was chosen for the fusion experiments. The fusion protocol was based on that of Fazekas de Groth and Scheidegger, (1980), using 50% polyethylene glycol 1500 and a myeloma cell to spleen cell ratio of 1:1. Each fusion experiment consisted of three separate fusion events with the spleen cells recovered being split into three aliquots of approximately 50 x 10^6 cells. Each of the fusion events was plated-out into five microtitre plates (96 x 0.2 ml wells) (Methods, Chapter 2; Section 3.9). In addition to the immunisation schedule detailed above an intravenous dose of 10 μg peptide was administered via the tail vein three days prior to fusion.

In each of the experiments growing hybridoma lines were seen in greater than 70% of wells. However, no cell lines were detected to be secreting anti-PTH antibody.

4.2 Rat Spleen Cell/Rat Myeloma Cell Fusions

Three fusion experiments were undertaken with the spleen cells from the DA rats found to be positive for serum antibody following immunisation with PTH (1-34). In addition to the immunisation schedule described above, an intravenous dose of 50 μg PTH (1-34) was administered intrajugularly via the pectoral muscle three days prior to fusion. The Y3 Ag 1.2.3 rat myeloma cell line (Galfre et al, 1979) was obtained through Dr A Campbell, Department of Biochemistry, University of Glasgow. Fusion experiments were carried out using the same protocol as for the mouse myeloma system (Section 4.1 above; Methods , Chapter 2; Section 3.8).

No growing hybrids survived for longer than seven days in the fusion experiments using the rat myeloma line.

4.3 Rat Spleen Cell/Mouse Myeloma Cell Fusions

Using the same protocol as the mouse spleen/mouse myeloma experiments (Section 4.1; Methods, Chapter 2; Section 3.8). Spleen cells from the DA rats found to have

anti-PTH (1-34) serum titres were fused with the myeloma cell line x63 Ag 8.653. The DA rats were given a 50 μg dose of PTH (1-34) administered intrajugularly via the pectoral muscle four days prior to fusion.

In three fusion experiments growing hybrids were seen in greater than 70% of wells. A total of 39 wells were identified as positive for anti-PTH (1-34) antibodies. The distribution of binding in the positive wells is shown in Figure 3.4. Five hybridoma lines were stabilised and remained secreting anti-PTH (1-34) antibodies through cloning (x2) by limiting dilution. The initial binding in the wells from which the five lines 1D1, 3B3, 4G3, 6E3 and 9E3 were stabilised, is also shown in Figure 3.4.

4.4 Initial Assessment of Hybridoma Lines

Culture fluid from the five hybridoma lines was used for isotype analysis and initial displacement studies using PTH (1-34) peptide and a PTH (1-84) preparation from NIBSC (75/549). Of the five lines, four producing IgG and one IgM Mab, the best displacement with PTH (1-34) was observed with 9E3, then 4G3, 6E3, 3B3 and 1D1. However, 1D1 and 9E3 appear specific for PTH (1-34) in that only 3B3, 6E3 and 4G3 show displacement with PTH (1-84) (MRC 75/549), having displacements of 0.96, 0.90 and 0.43 for PTH (1-84) relative to that obtained with PTH (1-34) respectively (Table 3.2).

5 BULK PRODUCTION OF MAB

On the basis of the displacement experiments (Table 3.2) the three cell lines which recognised PTH (1-84), 3B3, 4G3 and 6E3, were chosen for bulk production of Mab. As the hybridoma lines are mouse/rat hybrids, and not histocompatible with balb/c mice, congenitally athymic (Nu/Nu; nude) mice were used for the production of ascitic fluid. For each hybridoma line six nude mice were pristane primed and inoculated with 5×10^6 cells intra-peritoneally. All nude mice responded with ascites production. Taps of ascitic fluid showed anti PTH (1-34) antibody titres of $1:10^5$ at

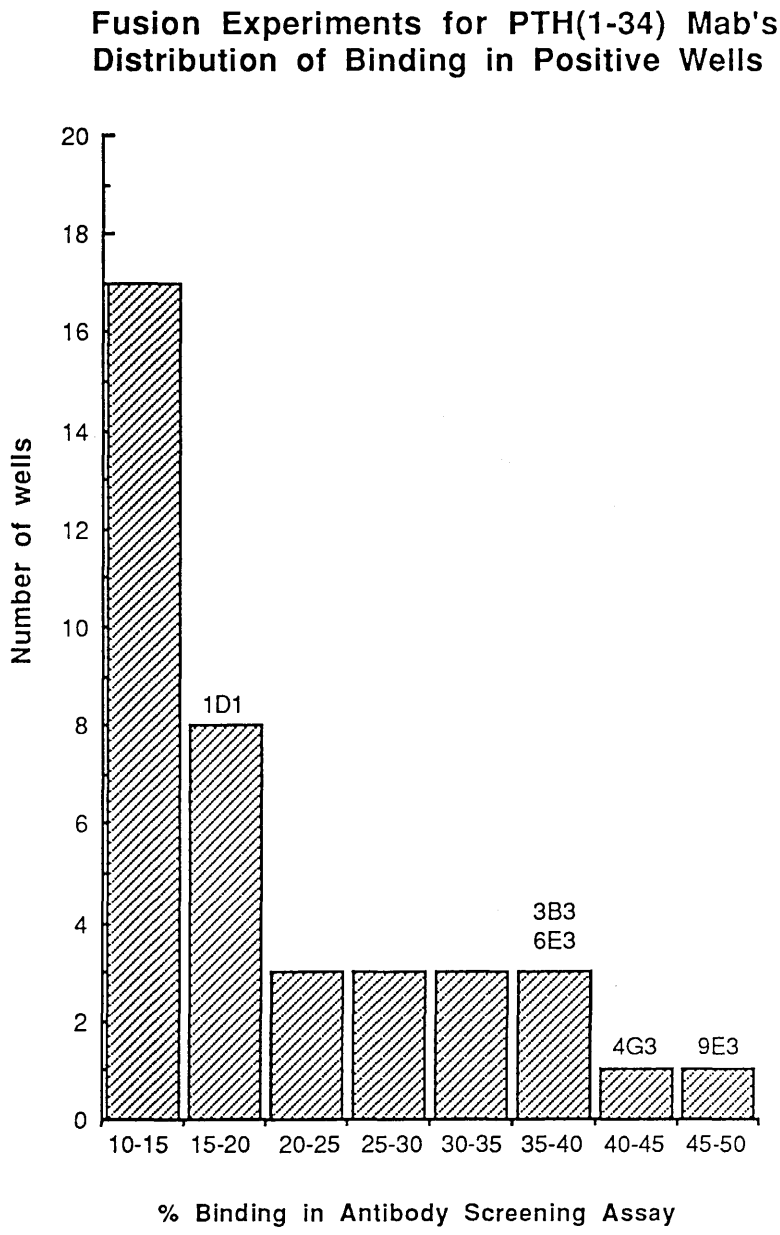


Figure 3.4

Distribution of binding in wells found to be positive for PTH (1-34) antibodies. Also shown are the binding levels in the wells from which the Mab lines 1D1, 3B3, 4G3, 6E3 and 9E3 were isolated.

Table 3.2

Isotope analysis and initial displacement studies on culture fluid from the mouse/rat hybridoma lines

Hybridoma Line	Isotype of Mab	Concentration of PTH (1-34) at 50% Displacement (nM)	Relative Displacement with Human PTH (1-84)
1D1	IgG 2b	>77.0	-
3B3	IgG 2b	38.0	0.96
4G3	IgG 2a	1.2	0.43
6E3	IgG 2a	9.7	0.90
9E3	IgM	0.6	-

20 days post inoculation ranging to >1:10^{10} at 40 days (Figure 3.5).

Mab IgG was purified from ascitic fluid by n-octanoic acid precipitation (Methods, Chapter 2; Section 3.15). Typical recoveries of IgG from ascitic fluid are shown in Table 3.3. Total protein concentrations for the ascitic fluids were in the range 64.3 - 75.2 mg/ml with 20.4% to 31.1% of the protein being recovered as IgG.

6 ASSESSMENT OF ANTIBODIES TO PTH

6.1 Sheep Polyclonal Antisera

(i) Sheep 108 anti-PTH (1-34).

The displacement with PTH (1-34) and the Scatchard analysis of the data are shown in Figure 3.6. The avidity constant estimated for the highest avidity component of the antiserum is 1.6 x 10^{10} L/M.

(ii) Sheep 109 anti-PTH (53-84)

Despite reaching a titre of 1:18,000 the antiserum showed no significant displacement even at nanomolar concentration of PTH (53-84) (data not shown).

6.2 Mab's to PTH (1-34)

(i) Displacement with PTH (1-34)

The displacement obtained with PTH (1-34) and the Scatchard analysis of the data for the Mab's 3B3, 4G3 and 6E3 are shown in Figure 3.7, 3.8 and 3.9. The avidity constants were estimated as 5.9 x 10^{9}, 3.0 x 10^{10} and 1.1 x 10^{9} L/M for 3B3, 4G3 and 6E3 respectively.

(ii) Displacement with PTH (1-10)

In an attempt to localise the epitope in PTH (1-34), the Mab's were tested for displacement with PTH (1-10). None of the Mab's showed significant displacement with PTH (1-10) at up to nanomolar concentrations.

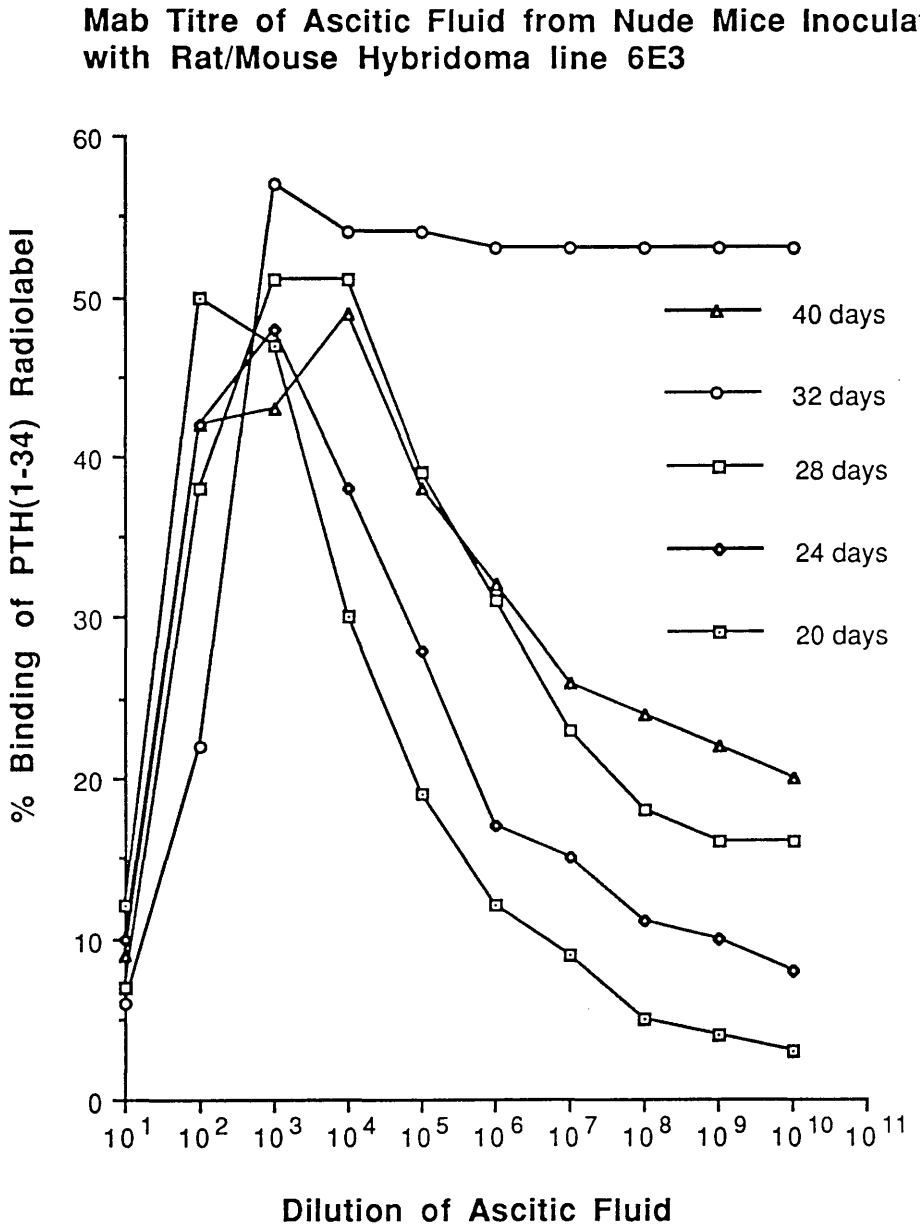


Figure 3.5

Titre of PTH (1-34) antibodies in ascitic fluid from pristane-primed congenitally athymic mice inoculated with 5 x 10^6 cells of the hybridoma line 6E3.

Table 3.3

Purification of Mab IgG (3B3) from ascitic fluid

	TAP: Aspiration Interval		
	28 days	32 days	40 days
Total Protein (mg/ml)	64.3	70.1	75.2
Protein Recovered as IgG (mg/ml)	20.7	20.0	15.4
Total IgG Recovered/5 ml Ascitic Fluid (mg)	103.5	100	77.0

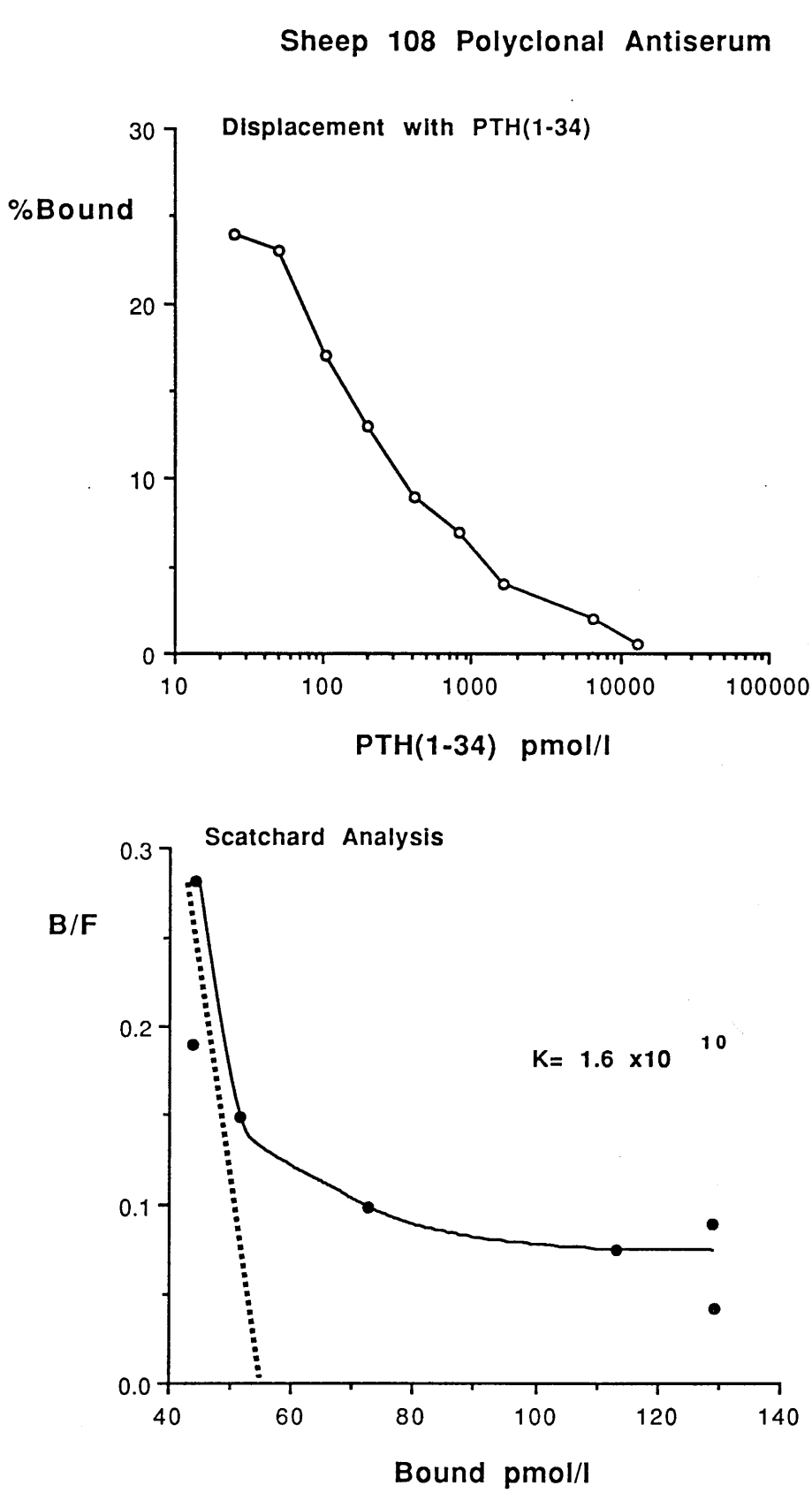


Figure 3.6

PTH (1-34) standard curve obtained with sheep 108 antiserum (top). The Scatchard analysis of the data is also shown (bottom).

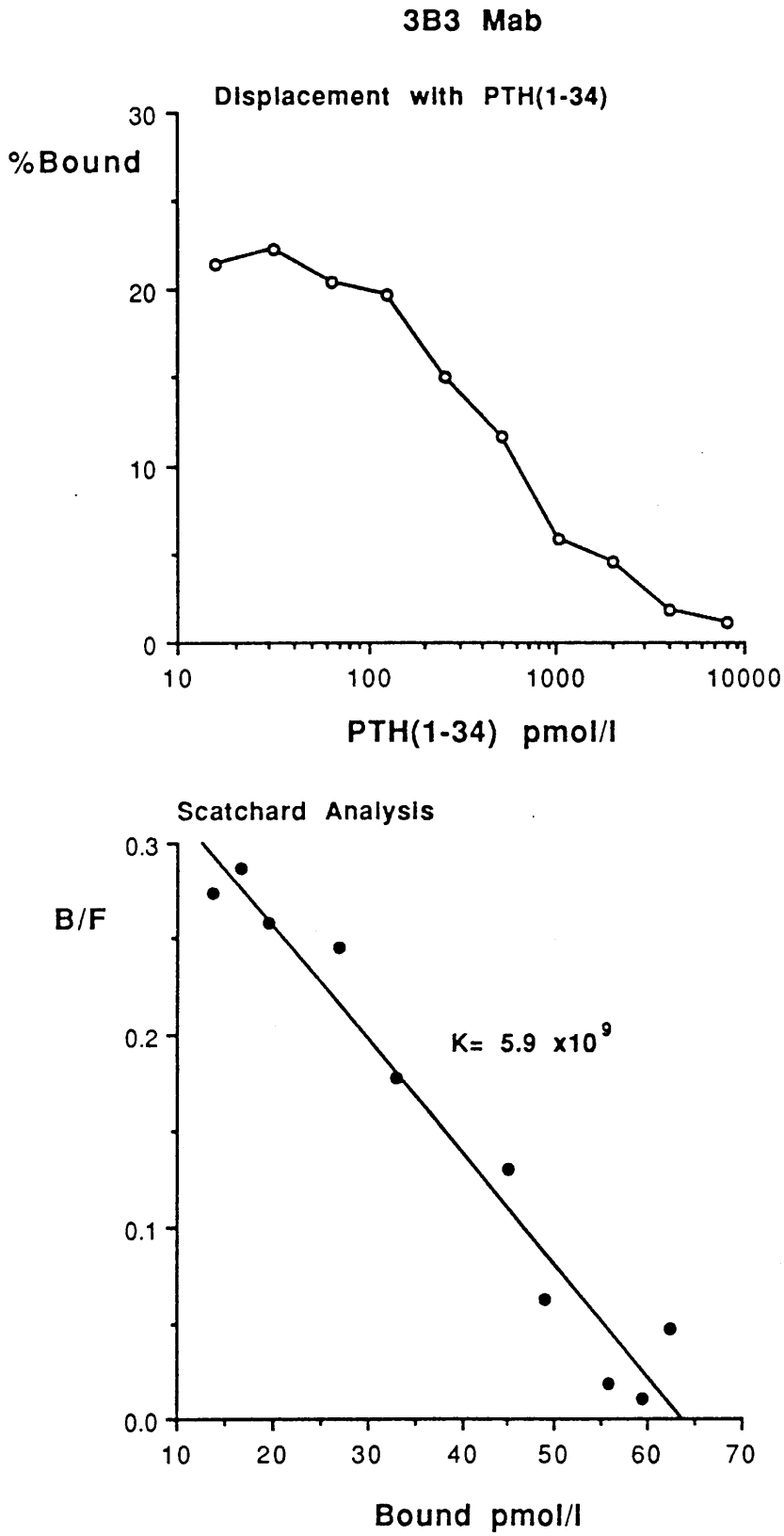


Figure 3.7

PTH (1-34) standard curve obtained with Mab 3B3 (top). The Scatchard analysis of the data is also shown (bottom).

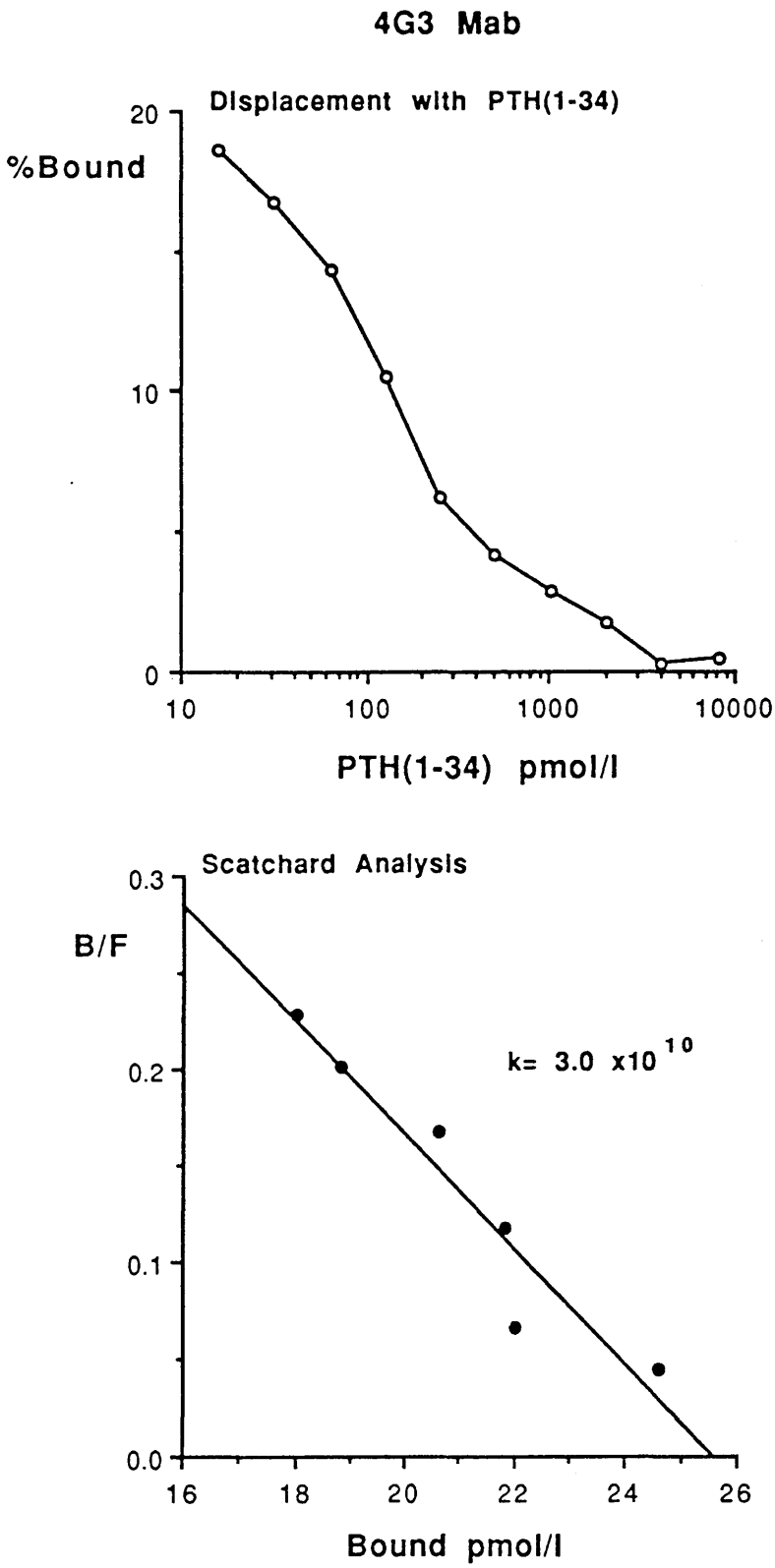


Figure 3.8

PTH (1-34) standard curve obtained with Mab 4G3 (top). The Scatchard analysis of the data is also shown (bottom).

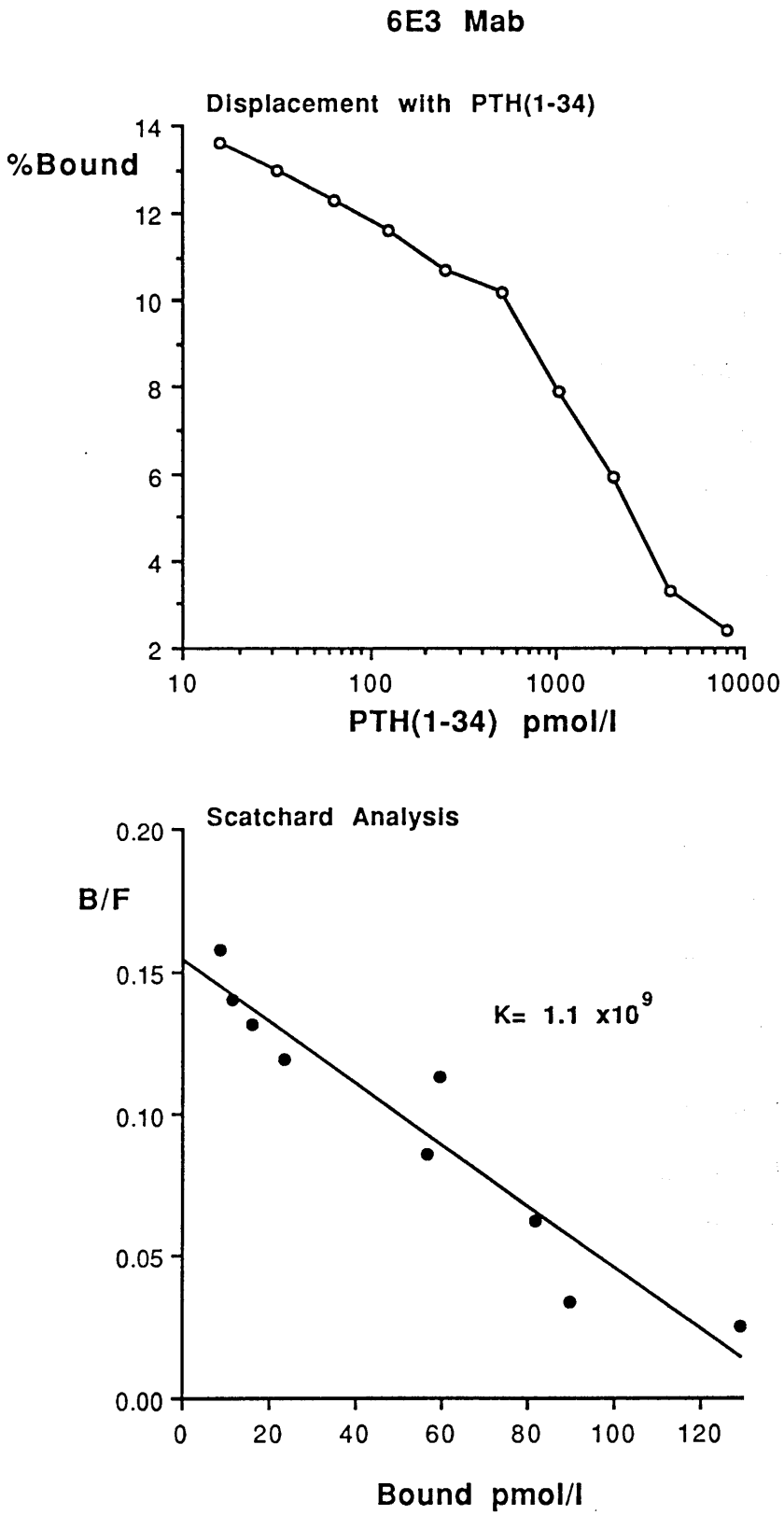


Figure 3.9

PTH (1-34) standard curve obtained with Mab 6E3 (top). The Scatchard analysis of the data (bottom).

(iii) Displacement with PTH (1-84)

The displacements with PTH (1-84) for the Mab's 3B3, 4G3 and 6E3 are shown in Figure 3.10. The Mab's 3B3 and 6E3 show essentially equipotent displacement with PTH (1-34) and PTH (1-84) consistent with the initial displacement studies (Table 3.2). 4G3 however shows little displacement even at high nanomolar concentrations of synthetic PTH (1-84). This is contrary to the initial displacement studies (Table 3.2) which indicated that 4G3 recognised PTH (1-84) with approximately 50% potency of PTH (1-34). It is probable that the impure standard preparation used in the initial displacement studies contained significant amounts of PTH (1-34).

7 DISCUSSION

Studies on the immunogenicity of peptide sequences have varied in their conclusions. Some reports have indicated that an antibody response can be obtained to peptides of 6 to 35 residues even without conjugation to a protein carrier (Lerner, 1984, Schmidtz et al, 1983, Young et al, 1983). Others have maintained that only hydrophilic peptide sequences of at least 15 residues conjugated to protein carriers are consistently immunogenic (Hopp and Woods, 1981, Delmas et al, 1985).

Palfreyman et al (1984) reviewed the literature on 103 peptide sequences, up to 20 residues long, which had been used as immunogen to generate antisera specific for the intact proteins of which they represented small fragments. The guidelines from this study were that for successful antibody production peptide sequences should be a minimum of 10 residues and coupled to a carrier protein. Highest success rates, approaching 100%, were achieved with sequences from the N- and C-terminal regions of proteins. This was ascribed to the suggestion (Walter, 1980) that the terminal sequences of polypeptides may be conformationally less constrained and more likely to be on the available surface of proteins than internal sequences. While the immunogens used in the present study conform to the above guidelines the success rate was less than predicted for N- and C-terminal peptide sequences. This

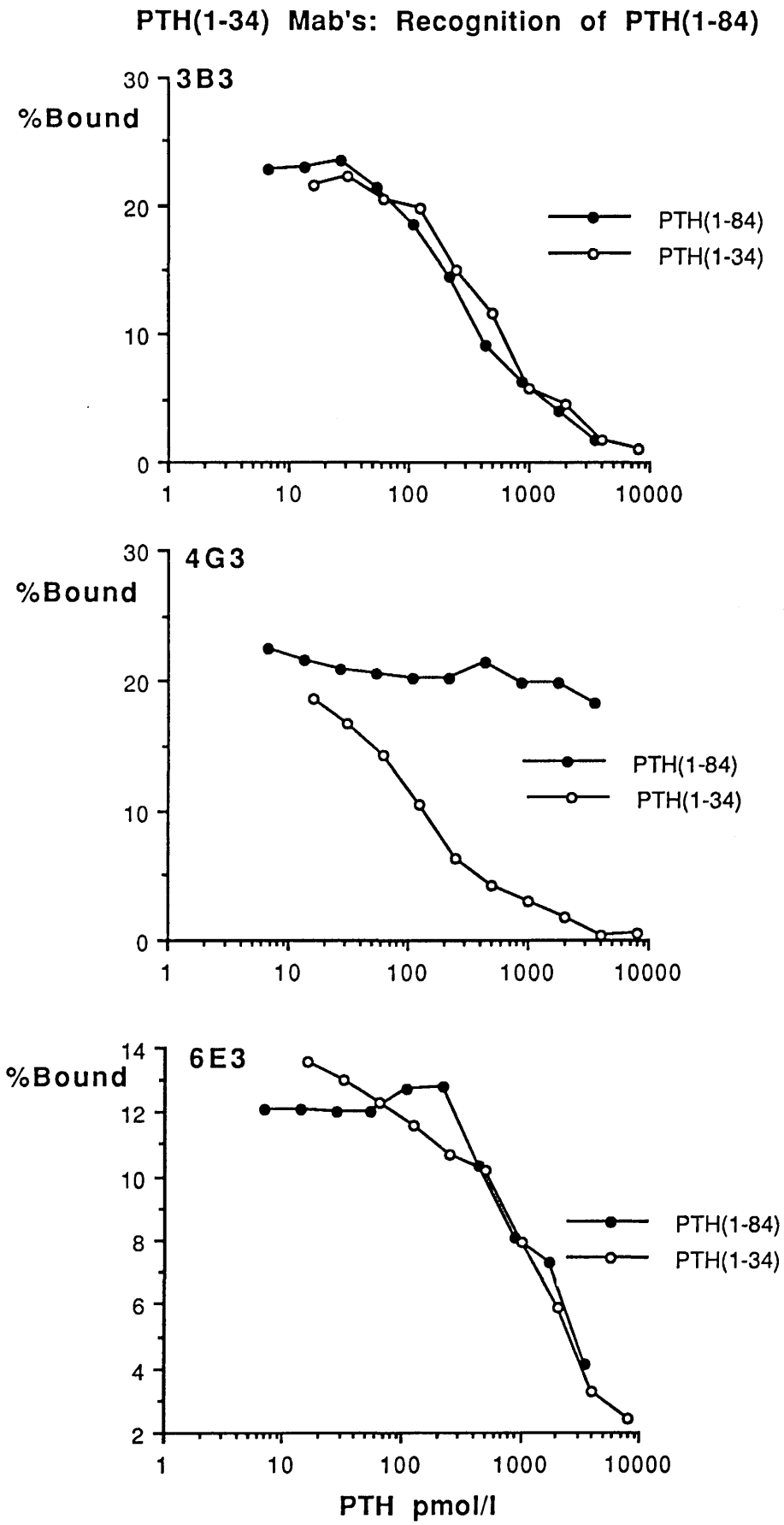


Figure 3.10

Comparison of the PTH (1-34) and PTH (1-84) standard curves obtained with the Mab's 3B3, 4G3 and 6E3.

undoubtedly reflects the choice of species for immunisation. The authors compiling the review were concerned with production of polyclonal antisera and therefore selected an animal for immunisation appropriate to the antigen of interest. The main aim of this project was the production of monoclonal antibodies and therefore it was essential to produce an antibody response in an animal compatible with the myeloma lines used for fusion experiments i.e. mice, ideally balb/c mice, failing this Lou or DA rats.

The pattern of responses obtained in this study - all mouse strains tested and Lou rats negative, 6/6 DA rats positive for PTH (1-34) antibodies, 2/2 sheep positive for PTH (1-34) and 1/2 sheep positive for PTH 53-84) antibodies - is consistent with a species and strain specific response. In previous attempts to produce monoclonal antibodies mice have been shown to be poor responders to bovine PTH (1-84) with only low avidity IgM Mab's obtained (Nussbaum et al, 1981, Van de Walle et al, 1983). Studies on the immunoresponsivity to human PTH (1-34) in inbred mice indicated that the response was under genetic control of Ir genes located in the IA and IE subregions of the major histocompatability complex (Nussbaum et al, 1985). Balb/c mice were also confirmed as genetic non-responders and the CBA mouse strain found to be high responders to PTH (1-34). Despite this no response was detected in the CBA mice immunised in this project.

The fusion experiments with the spleen cells from the DA rats, positive for PTH (1-34) antibodies, produced a total of 39 positive hybrids, representing 0.64% of the wells screened, of which 5 lines survived cloning. This is a low yield compared to the results obtained for highly immunogenic proteins eg, TSH, LH, FSH and AFP (Siddle and Soos 1981, Stevenson et al, 1987) where positive rates of 60-80% can be obtained. However, it is similar to the results obtained with the poorly immunogenic peptide hormone ACTH (White et al, 1985). The assessment experiments on the monoclonal antibodies produced indicate that 3B3 and 6E3 are of the required avidity and specificity to be of use in a two-site immunometric assay for

PTH (1-84). 4G3 having the highest avidity for PTH (1-34) but not recognising PTH (1-84) may well be of use in a two-site assay for PTH (1-34) perhaps in combination with either 3B3 or 6E3 Mab's.

The polyclonal antisera produced were found not to be of sufficiently high avidity to be of use in an assay system for PTH (1-84). Useful antisera to both N- and C-terminal PTH have been produced in sheep and goats (Brown et al, 1987, Nussbaum et al, 1987). This may indicate that the PTH peptide conjugates prepared for this project were not highly immunogenic and explain the failure to produce a response in CBA mice. Further work is required to produce a C-terminal Mab compatible with either 3B3 or 6E3. In a study on the importance of hydrophilic sequences in C-terminal PTH, the sequence 49-53 was identified as an important epitope for the production of antibodies in mice, sheep and goats (Delmas et al, 1985). Continued immunisations with peptides containing the PTH (49-53) sequence eg, PTH (39-84) or PTH (44-68) would seem to offer the best chance of success.

The immediate development of the assay for PTH (1-84) would continue by a search for C-terminal Mab's available either from other research groups or commercially which would be compatible with 3B3 or 6E3.

CHAPTER 4

DEVELOPMENT OF A TWO-SITE IMMUNOMETRIC ASSAY FOR PTH (1-84)

CHAPTER 4 : DEVELOPMENT OF A TWO-SITE IMMUNOMETRIC ASSAY FOR PTH (1-84)

1 INTRODUCTION

The strategy adopted for the production of Mab's to PTH peptides had yielded two Mab's (3B3, 6E3) directed to the N-terminal region of PTH which were of the requisite specificity and avidity to be of potential use in a two-site immunometric assay for PTH (1-84). As Mab's to the C-terminal region were not obtained it was decided to search for Mab's available from other research groups which would be compatible with 3B3 and 6E3. Two Mab's were located which were C-terminal specific and reacted with intact PTH (1-84).

(a) $E_{10}A_{10}$; specific for the 44-68 region (Ka 5.0 x 10^8 L/M). Produced by Dr J S Woodhead, Department of Medical Biochemistry, University of Wales College of Medicine.

(b) ESQ1; specific for the 74-84 region (Ka 1.1 x 10^{10} L/M). Produced by Dr K James, Department of Surgery, University of Edinburgh.

Samples of the Mab's were kindly supplied by the producers in order to assess their compatibility with 3B3 and 6E3 in a two-site immunometric assay system.

2 ASSESSMENT OF MAB COMPATIBILITY IN A TWO-SITE IRMA

2.1 Iodination of Mab's

IgG aliquots (25 μg) of each of the four Mab's 3B3, 6E3, and ESQ1 and $E_{10}A_{10}$ were iodinated by the lactoperoxidase method (Methods section 4.1). The iodination mixture was then applied to a Sepharose 6B column (1.6 x 70 cm) and run overnight at 10 ml/h 0.05 mol/l phosphate buffer pH 7.0, 0.5% BSA.

The column profiles of the iodinated Mab's are shown in Figures 4.1-4.4. The

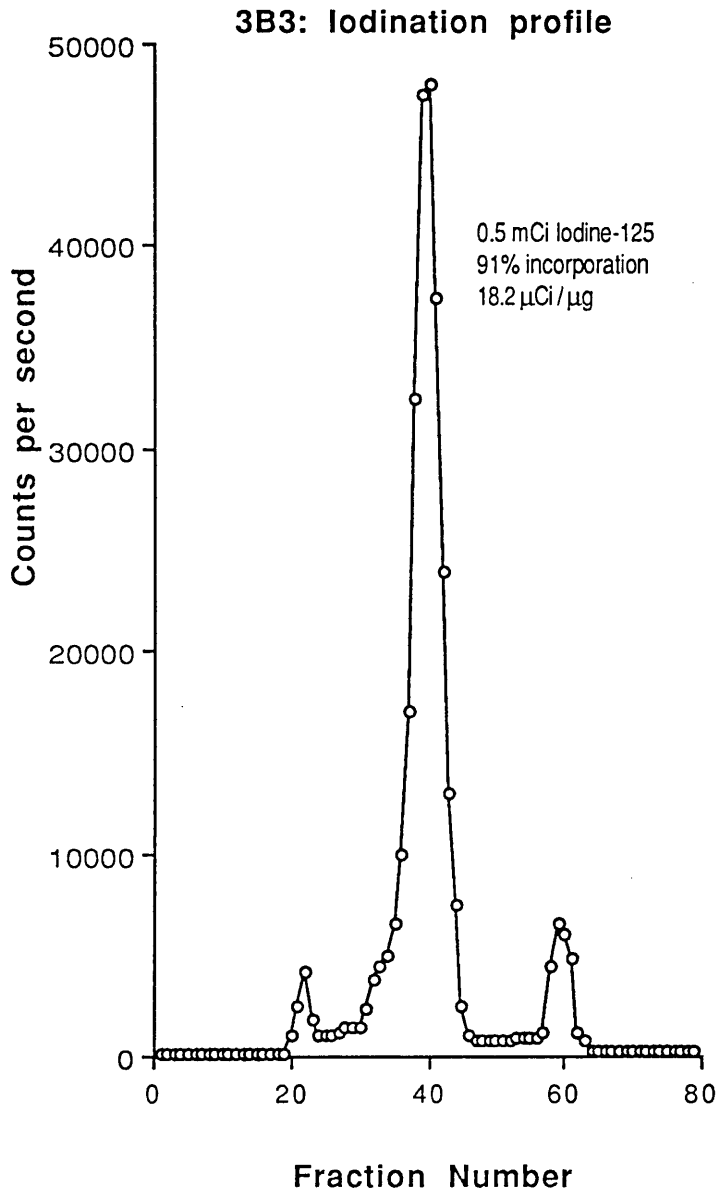


Figure 4.1

The profile for iodinated Mab 3B3 run on a Sepharose 6B column (1.6 x 70 cm) in 0.05 mol/l phosphate buffer pH 7.0, 0.5% BSA (10 ml/h).

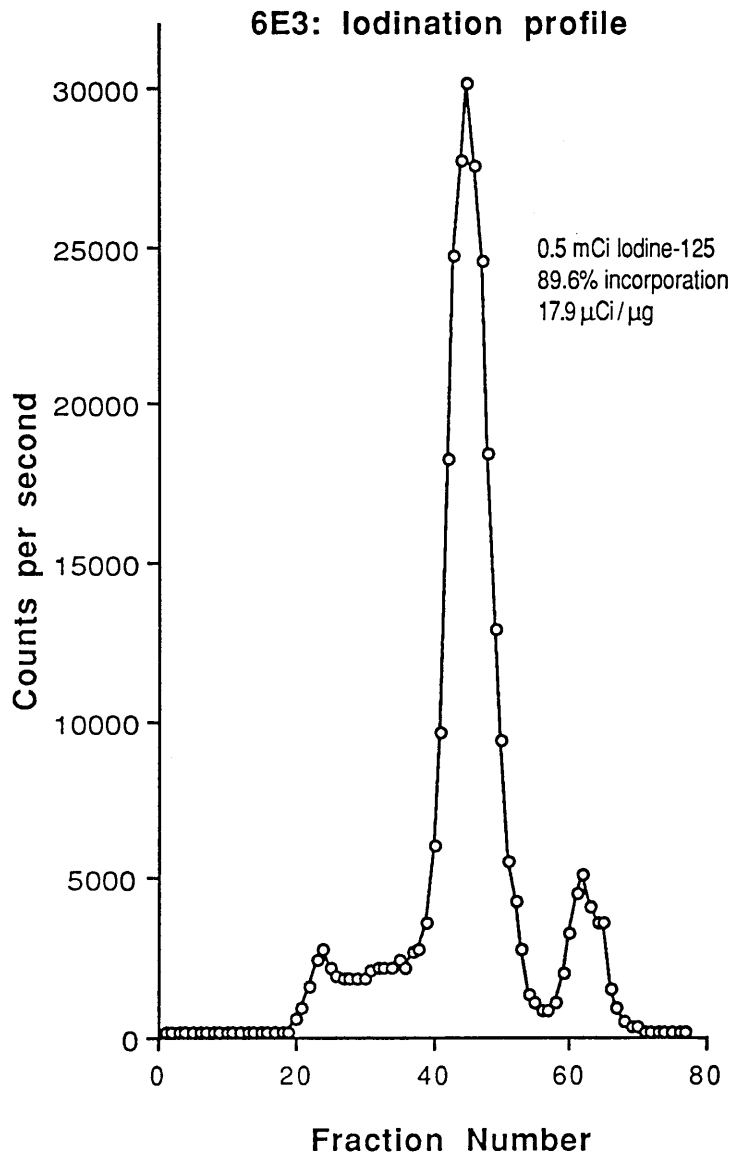


Figure 4.2

The profile for iodinated Mab 6E3 run on a Sepharose 6B column (1.6 x 70 cm) in 0.05 mol/l phosphate buffer pH 7.0, 0.5% BSA (10 ml/h).

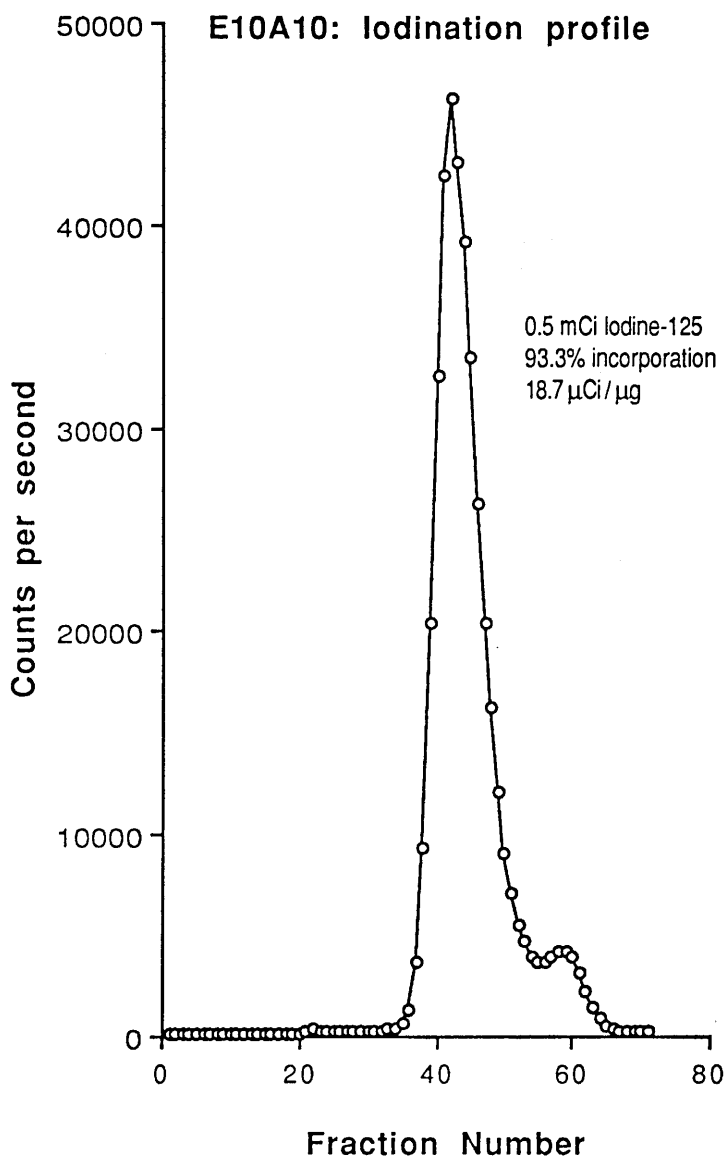


Figure 4.3

The profile for iodinated Mab $E_{10}A_{10}$ run on a Sepharose 6B column (1.6 x 70 cm) in 0.05 mol/l phosphate buffer pH 7.0, 0.5% BSA (10 ml/h).

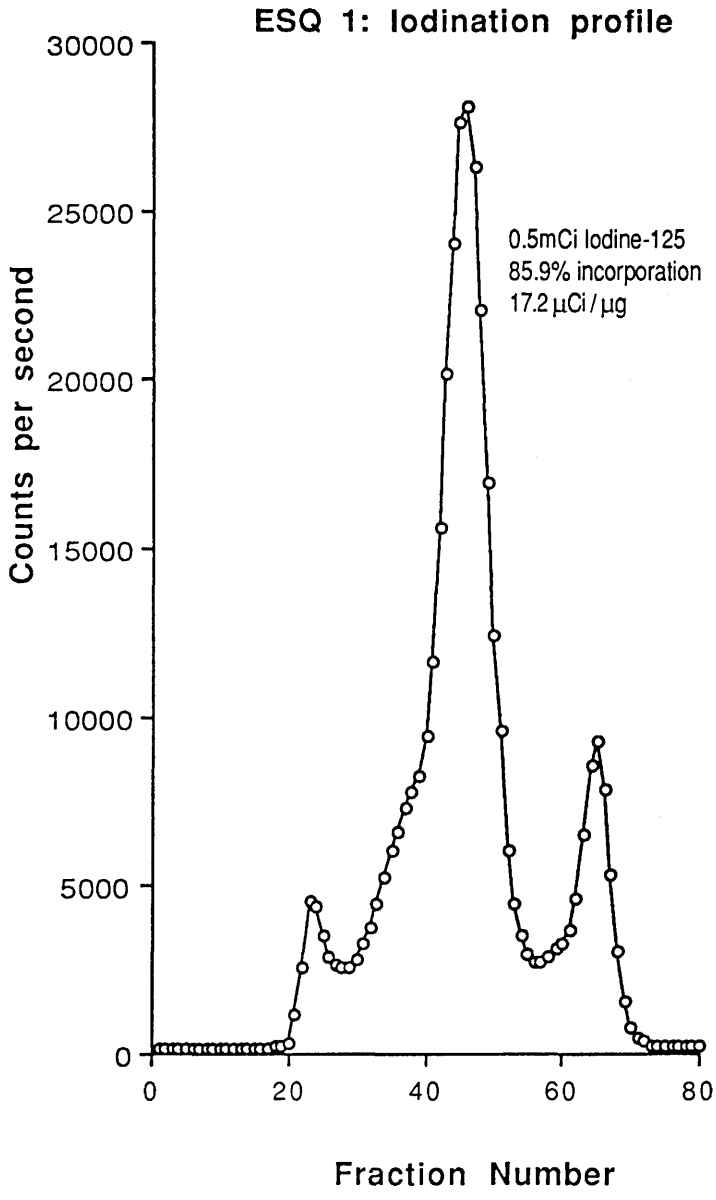


Figure 4.4

The profile for iodinated Mab ESQ1 run on a Sepharose 6B column (1.6 x 70 cm) in 0.05 mol/l phosphate buffer pH 7.0, 0.05% BSA (10 ml/h).

profiles for 3B3, 6E3 and $E_{10}A_{10}$ show clean protein peaks while the ESQ1 protein peak has a 'shoulder' of higher molecular-weight material. This probably reflects the IgG purification procedure; 3B3, 6E3 and $E_{10}A_{10}$ IgG fractions were prepared by the n-octanoic acid method (Methods section 3.15) while ESQ1 was received as an IgG prepared by a sodium sulphate precipitation method.

The specific radioactivities calculated from the percentage incorporation of iodine^{125} into the protein peaks were 18.2, 17.9, 17.2 and 18.7 μCi/μg IgG for 3B3, 6E3, $E_{10}A_{10}$ and ESQ1, respectively. These were close to the target value of 20 μCi/μg.

2.2 Assessment of Mab's in Microtitre Plate-Based IRMA

Preparation of Plates

IgG fractions from each of the Mab's 3B3, 6E3, $E_{10}A_{10}$ and ESQ1 were coated onto micro-titre plates (0.2 ml x 96 well, Removastrips, Dynatech). The IgG fractions were diluted in 50 mmol/l TAPS buffer pH 8.6 to a protein concentration of 100 μg/ml and a 200 μl aliquot incubated in the wells overnight at 37°. The wells were then washed (x4) in 50 mmol/l TAPS buffer pH 8.6 containing 0.5% BSA prior to use.

Assessment of Mab Combinations

(i) Mab to N-terminal PTH coated on plate.

Wells (x4) coated with either 3B3 or 6E3 were incubated with 200 μl PTH (1-84) standard (0, 500, 1,000 pmol/l) in 0.05 mol/l barbitone buffer pH 8.6, 0.5% BSA overnight at room temperature. The wells were then washed (x2) with barbitone buffer pH 8.6, 0.5% BSA and incubated with radiolabelled C-terminal Mab, either $E_{10}A_{10}$ or ESQ1 (100,000 cpm/well) overnight at room temperature. Wells were then washed (x4) in barbitone buffer pH 8.6, 0.5% BSA prior to counting.

The results obtained with the N-terminal Mab's coated on plates are shown in Figure

4.5. In the 3B3 coated plate responses were obtained with both ESQ1 and $E_{10}A_{10}$ radiolabelled C-terminal Mab's. The responses obtained at both PTH (1-84) concentrations were greater with ESQ1 than with $E_{10}A_{10}$. A similar trend is seen in the plate coated with 6E3 (Figure 4.5(b)). However, the responses obtained were in each case lower than those in the 3B3 coated plate.

The response levels even with the best combination of 3B3 coated and ESQ1 radiolabel were not particularly encouraging (8% counts added bound at 1,000 pmol/l and 0.8% bound at 500 pmol/l PTH (1-84)) although the assay system had not been optimised at this point.

(ii) Mab to C-terminal PTH coated on plate:

Wells coated with C-terminal Mab, either $E_{10}A_{10}$ ESQ1, were incubated with radiolabelled N-terminal Mab, either 3B3 or 6E3, according to the protocol in (i) above.

The results are shown in Figure 4.6. Responses were obtained in the ESQ1 coated plate with both radiolabelled N-terminal Mab's 3B3 and 6E3. The responses with 3B3 were greater than those with 6E3. Similar results were obtained in the $E_{10}A_{10}$ coated wells (Figure 4.6(b)) but the responses were lower than in the ESQ1 coated wells in each case.

The results confirm the previous experiments in concluding ESQ1/3B3 as the combination of Mab's giving best results. The responses with Mab's used in this orientation (ESQ1 coated, 3B3 radiolabelled) were greater than in the previous experiment (3B3 coated, ESQ1 radiolabelled) with 14.0 versus 8.2% of counts bound at 1,000 pmol/l and 8.0 versus 0.8% at 500 pmol/l PTH (1-84).

Dose response curves using ESQ1 and 3B3 in both orientations were set up using the above protocols. The results are in agreement with the above experiments (Figure 4.7). The combination of 3B3 coated with ESQ1 radiolabelled gives an insensitive

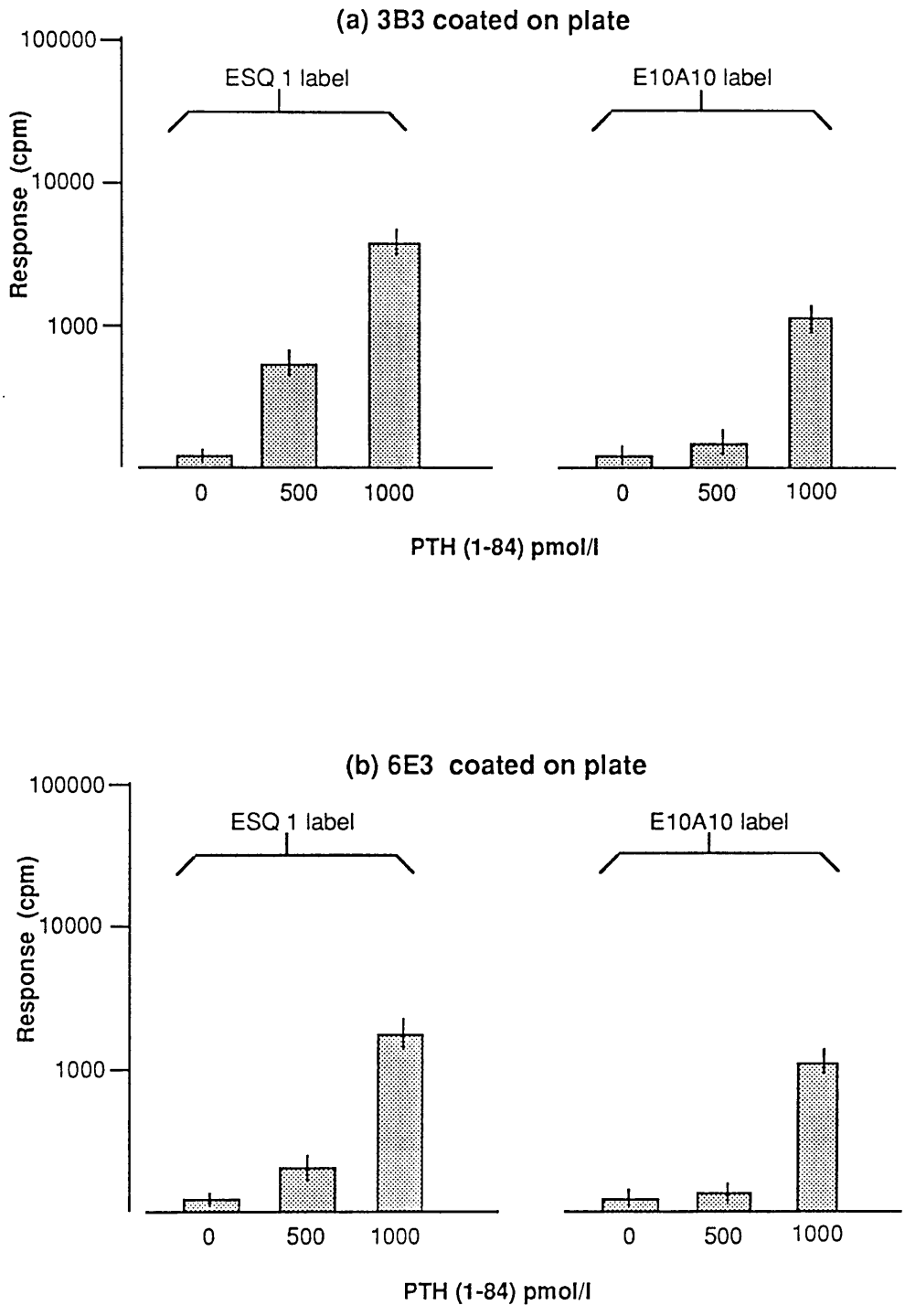


Figure 4.5

Responses obtained in the microtitre plate based IRMA with (a) 3B3 and (b) 6E3 Mab coated on the plate. Wells (x4) were incubated (18 h at room temperature) with PTH (1-84) standard, washed and then incubated (18 h at room temperature) with either ESQ1 or $E_{10}A_{10}$ radiolabelled Mab.

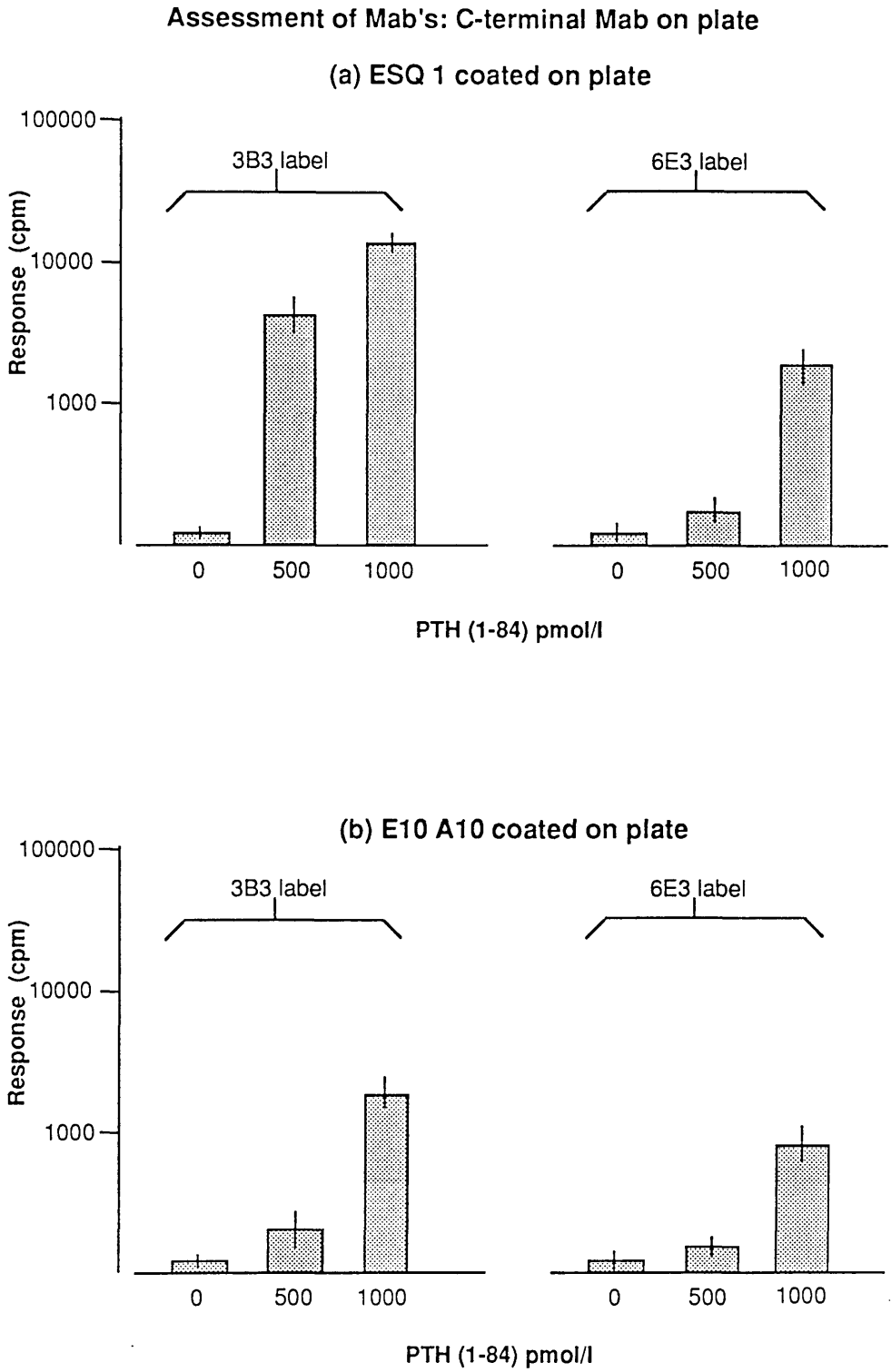


Figure 4.6

Responses obtained in the microtitre plate based IRMA with (a) ESQ1 and (b) $E_{10}A_{10}$ Mab coated on plate. Wells were incubated (18 h at room temperature) with PTH (1-84) standard, washed then incubated (18 h at room temperature) with either 3B3 or 6E3 radiolabelled Mab (100,000 cpm/well).

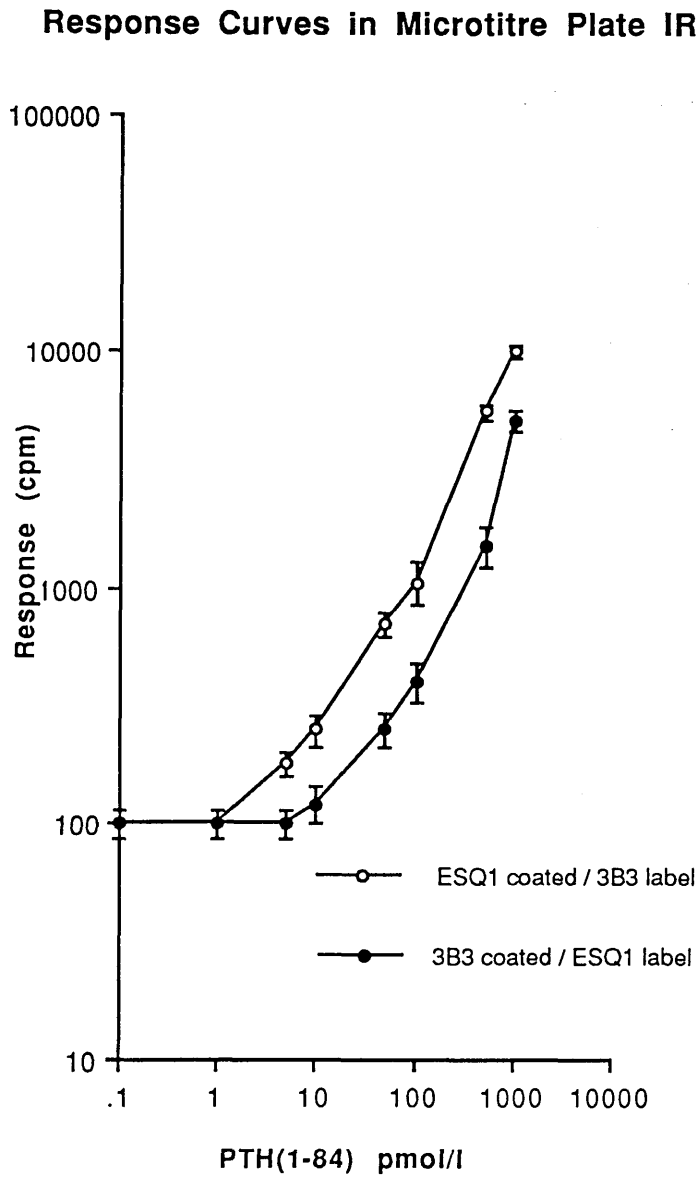


Figure 4.7

Dose response curves obtained for combinations of ESQ1 and 3B3 Mab's in the microtitre plate based IRMA. Wells coated with either ESQ1 or 3B3 Mab were incubated (18 h at room temperature) with PTH (1-84) standard, washed then incubated (18 h at room temperature) with either 3B3 and ESQ1 radiolabelled Mab (100,000 cpm/well).

response curve which even with extensive optimisation would probably not have been useful in measuring low picomolar concentrations of PTH (1-84). The combination of ESQ1 coated and 3B3 radiolabelled gave a substantially more sensitive response curve.

On the basis of these results it was decided to investigate the ESQ1/3B3 combination on the high capacity solid-phase support Sepharose CL-4B both in an attempt to increase the response levels obtained and also to develop an assay system with maximum capacity for C-terminal Mab in an attempt to minimise the influence of the high concentrations of C-terminal PTH fragments present in patient samples.

2.3 Assessment of Mab's in Sepharose CL-4B Based IRMA

Coupling of Mab's to Sepharose CL-4B

IgG fractions from Mab's ESQ1 and 3B3 were coupled to Sepharose CL-4B gel by the carbonyldiimidazole method of Chapman and Ratcliffe 1981 (Methods section 4.2). Using 12 mg of Mab IgG the estimated mean uptake of IgG onto 10 ml of Sepharose CL-4B gel was 46.6% (range 32.1-60.8%, n=5). This is equivalent to a mean ratio of 15 μg IgG/mg gel (range 14-18 μg/mg).

Assessment of ESQ1/3B3 Combinations

(i) Protocol 1

This protocol was devised to be equivalent to that employed in the plate based assay (Section 2.2). Mab, either 3B3 or ESQ1, coupled to Sepharose CL-4B (200 μl; 1 mg gel) was incubated with PTH (1-84) standard (200 μl; 1,000 pmol/l) in 0.05 mol/l barbitone buffer pH 8.6, 0.5% BSA overnight at room temperature with shaking. The tubes were then washed (x2) in barbitone buffer pH 8.6, 0.5% BSA and incubated with radiolabelled Mab either ESQ1 or 3B3 (200 μl, 100,000 cpm) overnight at room temperature. The tubes were then washed (x4) with barbitone buffer pH 8.6, 0.5% BSA prior to counting.

(ii) Protocol 2

Radiolabelled Mab either 3B3 or ESQ1 (100 µl, 100,000 cpm) was incubated overnight at room temperature with PTH (1-84) standard (200 µl, 1,000 pmol/l). Mab coupled to Sepharose CL-4B either ESQ1 or 3B3 (100 µl, 1 mg gel) was added and the mixture incubated overnight at room temperature with shaking. The tubes were then washed (x4) in 0.05 mol/l barbitone buffer pH 8.6, 0.5% BSA prior to counting.

The results obtained with 3B3 coupled to Sepharose CL-4B and radiolabelled ESQ1 are shown in Figure 4.8(a). The response obtained in both protocols (6.0-8.2% of counts bound) are equivalent to those achieved in the plate-based assay for these Mab's used in this orientation. The blanks (response at 0 pmol/l PTH (1-84)) are however significantly higher.

The results obtained with ESQ1 coupled to Sepharose CL-4B and 3B3 radiolabel are shown in Figure 4.8(b). The responses obtained were a significant improvement from the plate-based assay with 35.2% and 40.3% of counts bound at 1,000 pmol/l PTH (1-84) for protocols (i) and (ii) respectively.

A response curve using ESQ1 coupled to Sepharose CL-4B and radiolabelled 3B3 in protocol (ii) is shown in Figure 4.9. These results confirmed the choice of ESQ1 as the Mab on the Sepharose CL-4B solid-phase support and the use of 3B3 as the radiolabelled Mab. The response curve even though the assay conditions were not yet optimised appeared to have the potential to measure low picomolar concentrations of PTH (1-84).

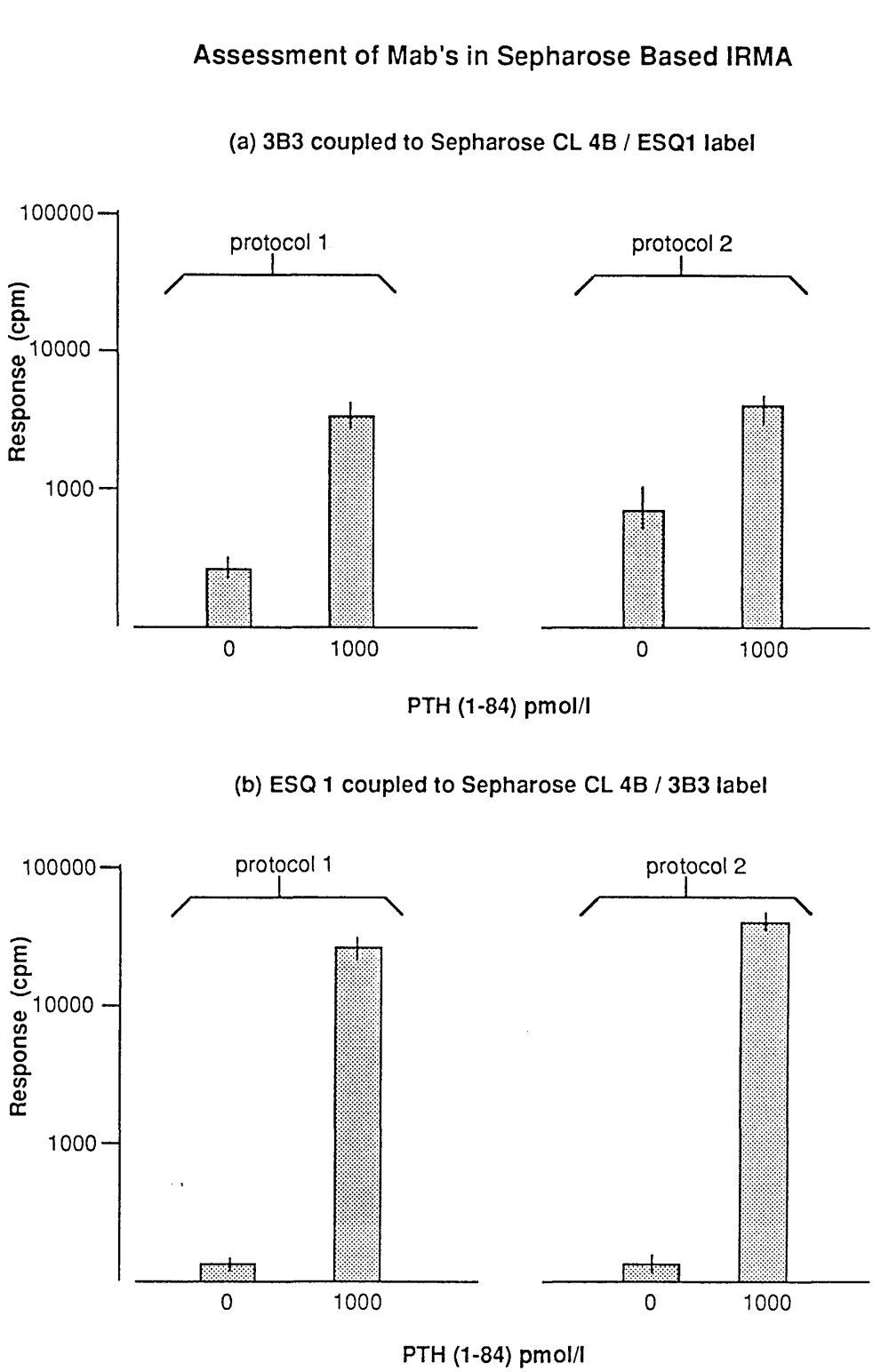


Figure 4.8

Responses obtained for combinations of 3B3 and ESQ1 Mab in Sepharose CL-4B based IRMA. PTH (1-84) standard was pre-incubated (18 h at room temperature) with either solid-phased Mab (protocol 1) or radiolabelled Mab (protocol 2).

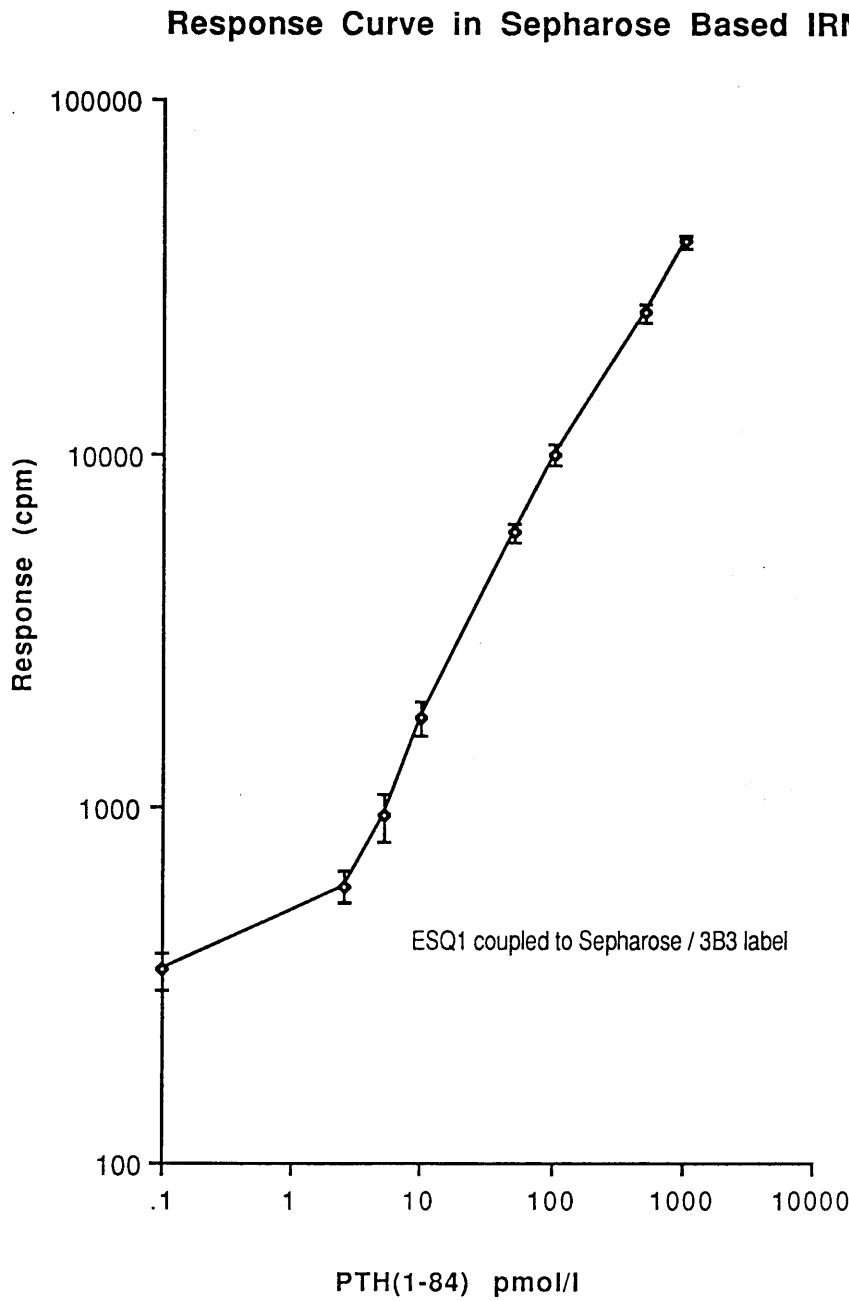


Figure 4.9

Response curve obtained using Mab's ESQ1 and 3B3 in Sepharose based IRMA. Radiolabelled 3B3 was pre-incubated with PTH (1-84) standard then ESQ1-Sepharose added and incubated for a further 18 h at room temperature.

3 OPTIMISATION OF PTH (1-84) IRMA SYSTEM

3.1 Introduction

The previous section on the assessment of the compatibility of the Mab's had shown clearly that the use of radiolabelled N-terminal Mab 3B3 with the C-terminal Mab ESQ1 on the Sepharose CL-4B support was the only combination of real potential in an assay system (Section 2). In the light of this information and previous discussion on the performance characteristics required of an assay capable of measuring circulating PTH (1-84) concentrations, the optimisation experiments were aimed primarily:

(i) to develop as sensitive a system as possible, ie. to maximise the response and precision at low picomolar concentrations.

(ii) to maximise the specificity of the system for PTH (1-84) by minimising any possible interference from C-terminal fragments and to a lesser extent N-terminal fragments.

3.2 Preliminary Optimisation Studies

The assay protocol shown in Table 4.1 was chosen as baseline to the preliminary optimisation studies.

Optimisation of ESQ1-Sepharose Incubation

(i) Time

Synthetic PTH (1-84) standard (0, 25, 250, 500 pmol/l) was incubated with radiolabelled 3B3 according to the initial protocol in Table 4.1. ESQ1-Sepharose was added (1 mg Sepharose/100 µl/tube) and the assay shaken for times varying between 0.5 and 4.0 h, prior to washing by centrifugation and aspiration using 4 x 2 ml 0.9% saline/Tween 20 (0.2% v/v). The response increased across the

Table 4.1

Initial protocol adopted as baseline to the preliminary assay optimisation studies

Assay Tubes	55 x 12 mm polystyrene (Sarstedt 55.484).
Assay Diluent	0.05 mol/l barbitone buffer pH 8.6 (0.1 mol/l NaCl, 0.5% BSA). Previously employed in the antibody screening and assessment studies.
PTH (1-84) Standard	Synthetic PTH (1-84) (Peninsula) diluted in assay diluent to concentrations covering the expected assay range (0 - 500 pmol/l).
Radiolabelled 3B3	N-terminal Mab 3B3 iodinated to a specific activity of approximately 20 μCi/μg by lactoperoxidase method. (Methods section 4.1).
1st incubation	200 μl PTH (1-84) standard 100 μl radiolabelled 3B3 (100,000 cpm) incubated at 22 h (overnight) at room temperature.
ESQ1-Sepharose	C-terminal Mab ESQ1 coupled to Sepharose CL-4B using carbonyldiimadazole (Methods section 4.2) approximately 12-17 μg IgG/mg gel.

concentration range with time as shown in Figure 4.10. The 2 h shaking time was selected as optimal as the increase in the blank level (the response at 0 pmol/l PTH (1-84)) seen at 4 h was considered potentially detrimental to the potential sensitivity of the final assay system.

(ii) Mass of ESQ1-Sepharose

ESQ1-Sepharose was added at 0.5, 1.0 or 2.0 mg per tube to a range of PTH (1-84) concentrations previously incubated overnight with radiolabelled 3B3 as above. The assay was shaken for 2 h prior to the 4 x 2 ml saline/Tween 20 (0.2% v/v) wash procedure.

The response curves at 0.5 and 1.0 mg ESQ1-Sepharose per tube were similar as shown in Figure 4.11. There was however, a significant increase in the blank level at 2.0 mg ESQ1-Sepharose per tube which was considered undesirable.

The addition of 1.0 mg ESQ1-Sepharose was adopted in order to minimise possible interference from C-terminal fragments by using as high a mass of ESQ1 Mab per tube as practicably possible. Given the coupling ratios achieved this approximates to 12-17 μg ESQ1-IgG per tube.

Optimum pH of Assay Diluent

(i) Buffer system alone

The effect of pH on the PTH (1-84) response curve was investigated by using a variety of buffer systems (acetate pH 6.0, phosphate pH 7.0 and pH 7.5, bicarbonate pH 8.0, barbitone pH 8.5 and pH 9.0). All buffers were 0.05 M and final assay diluents contained 0.1 M NaCl and 0.5% BSA. For each of the diluents PTH (1-84) standards were incubated overnight at room temperature with radiolabelled 3B3 (100,000 cpm). ESQ1-Sepharose at 1 mg per tube was added and the assay shaken for 2 h prior to washing with 4 x 2 ml saline/Tween 20 (0.2% v/v).

The results are shown in Figure 4.12. The response curves decreased across the

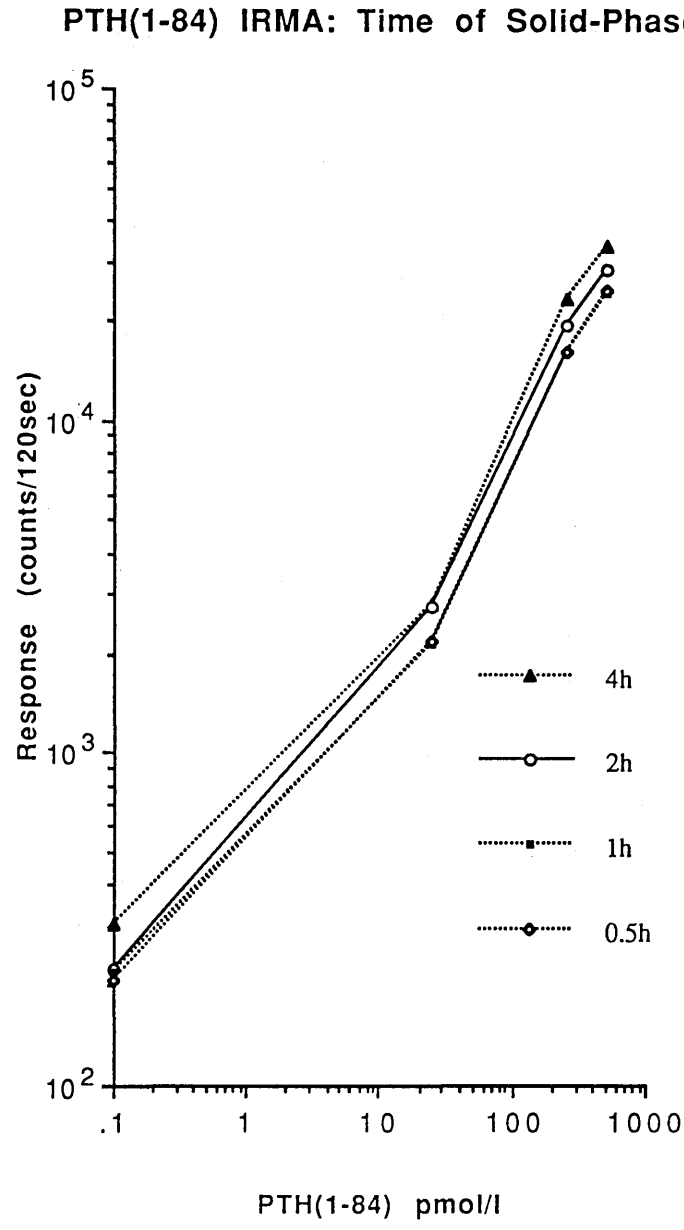


Figure 4.10

Optimisation of the time of incubation with ESQ1 coupled to Sepharose CL-4B. Radiolabelled 3B3 was pre-incubated with PTH (1-84) standard, ESQ1-Sepharose was then added and incubated at room temperature with shaking for periods of 0.5 - 4 h.

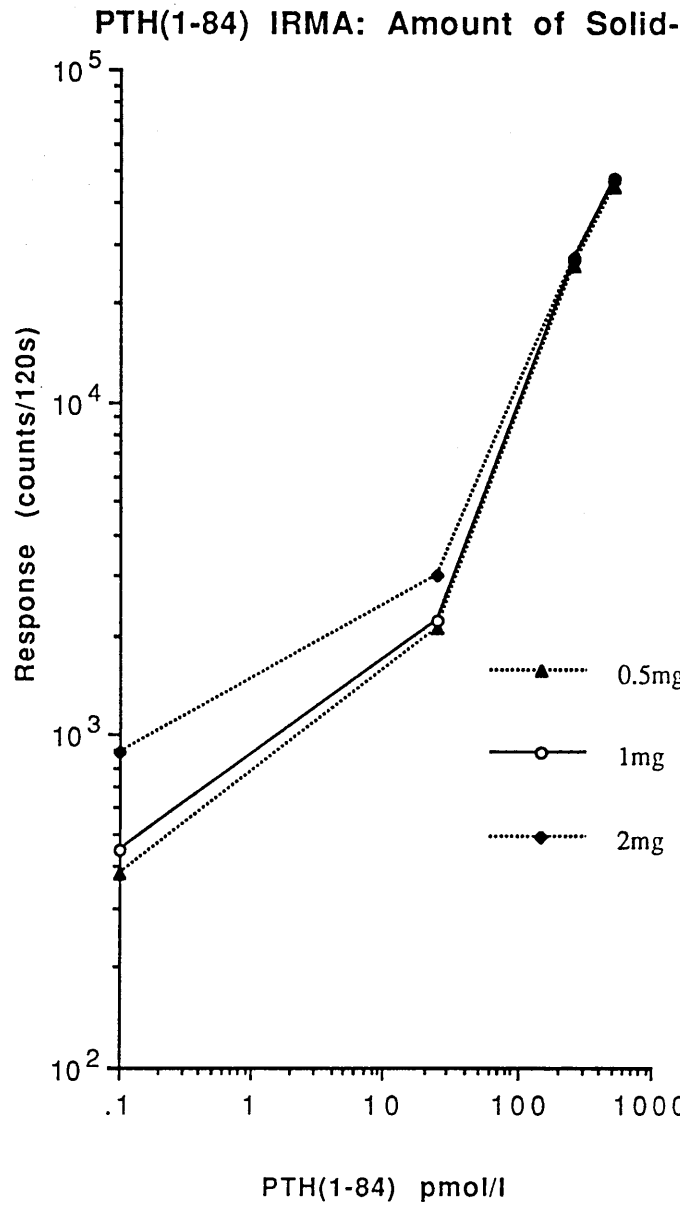


Figure 4.11

Optimisation of the amount of ESQ1 coupled to Sepharose CL-4B added per tube. Radiolabelled 3B3 was pre-incubated with PTH (1-84) standard and ESQ1-Sepharose corresponding to 0.5, 1.0 or 2.0 mg Sepharose per tube was added and the assay shaken for 2 h at room temperature.

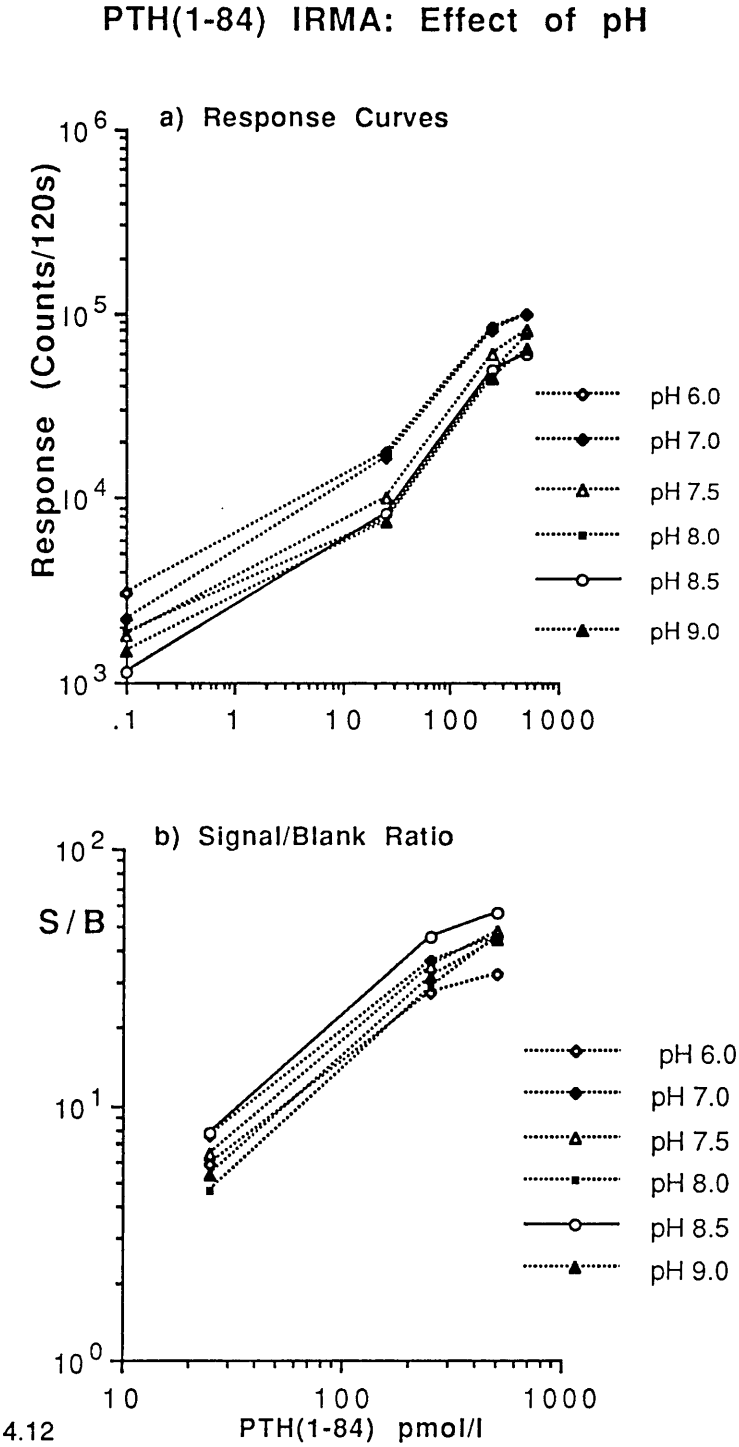


Figure 4.12

Investigation of the effect of assay buffer pH. PTH (1-84) response curves were set up in acetate (pH 6.0), phosphate (pH 7.0 & 7.5), bicarbonate (pH 8.0) and barbitone (pH 8.5, 9.0) assay buffers.

PTH (1-84) concentration range with increasing pH. The blank levels followed the same trend apart from the blank at pH 8.5, which was lower than would be expected from the pH trend. This is possibly because the isoelectric point of the Mab IgG is close to this pH.

Analysis of this data as a ratio of signal (response) to blank indicates pH 8.5 followed by pH 7.0 as the diluent systems giving maximum specific response. It was also noted that the actual blank levels in this experiment at 1,000-3,000 counts per 120 s were higher than previously encountered, perhaps due to ageing of the labelled 3B3 preparation in use at the time.

(ii) Buffer system with serum

The selection of the pH 8.5 diluent system as giving best specific response was mainly determined by the lower than expected blank at this pH. The final assay system would however contain 50% serum (assuming a 200 µl serum sample). The response curves in pH 8.5 and pH 7.0 diluents were repeated as per the protocol above but with the PTH (1-84) standard prepared and added in 200 µl hypoparathyroid serum. The results are shown in Figure 4.13. The blanks at pH 8.5 and pH 7.0 are reduced to the same level by the inclusion of serum. The greater response at higher PTH (1-84) concentrations at pH 7.0 is however maintained. The signal to blank ratio in the pH 7.0 diluent is therefore greater across the PTH (1-84) concentration range. The phosphate pH 7.0 diluent system with the inclusion of a 200 µl serum sample was adopted for further experiments.

Performance of Preliminary PTH (1-84) IRMA System

Figure 4.14 shows a typical response curve and associated precision profile. The error relationship was derived from the measurement of synthetic PTH (1-84) standard curves (140 duplicate pairs) in the preliminary optimised protocol (Table 4.2). The assay has a detection limit of 0.5 pmol/l estimated from the 22% CV on the precision profile (McConway et al, 1989). The intra-assay CV is less than 10%

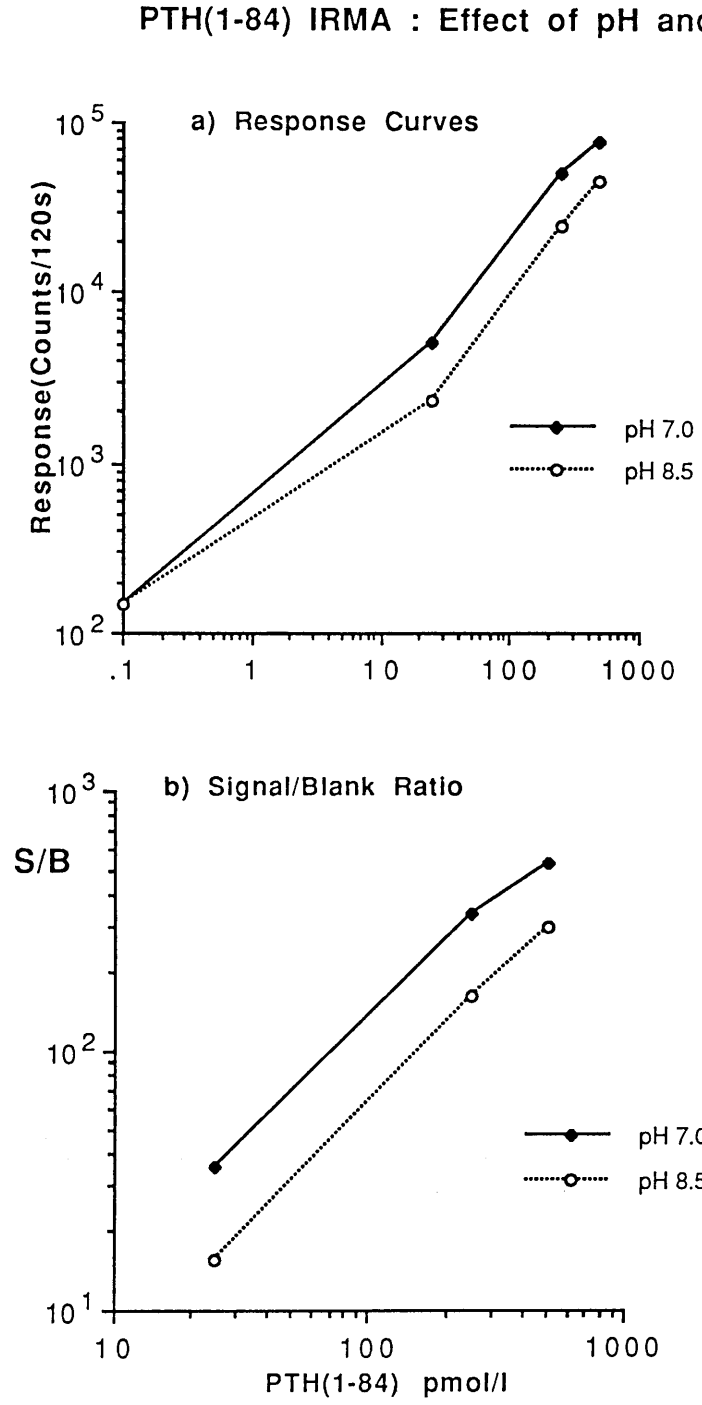


Figure 4.13

The effect of assay buffer pH in the presence of serum. PTH (1-84) response curves were set up in phosphate (pH 7.0) and barbitone (pH 8.5) assay buffers with added serum (50% v/v) corresponding to a 200 µl sample size.

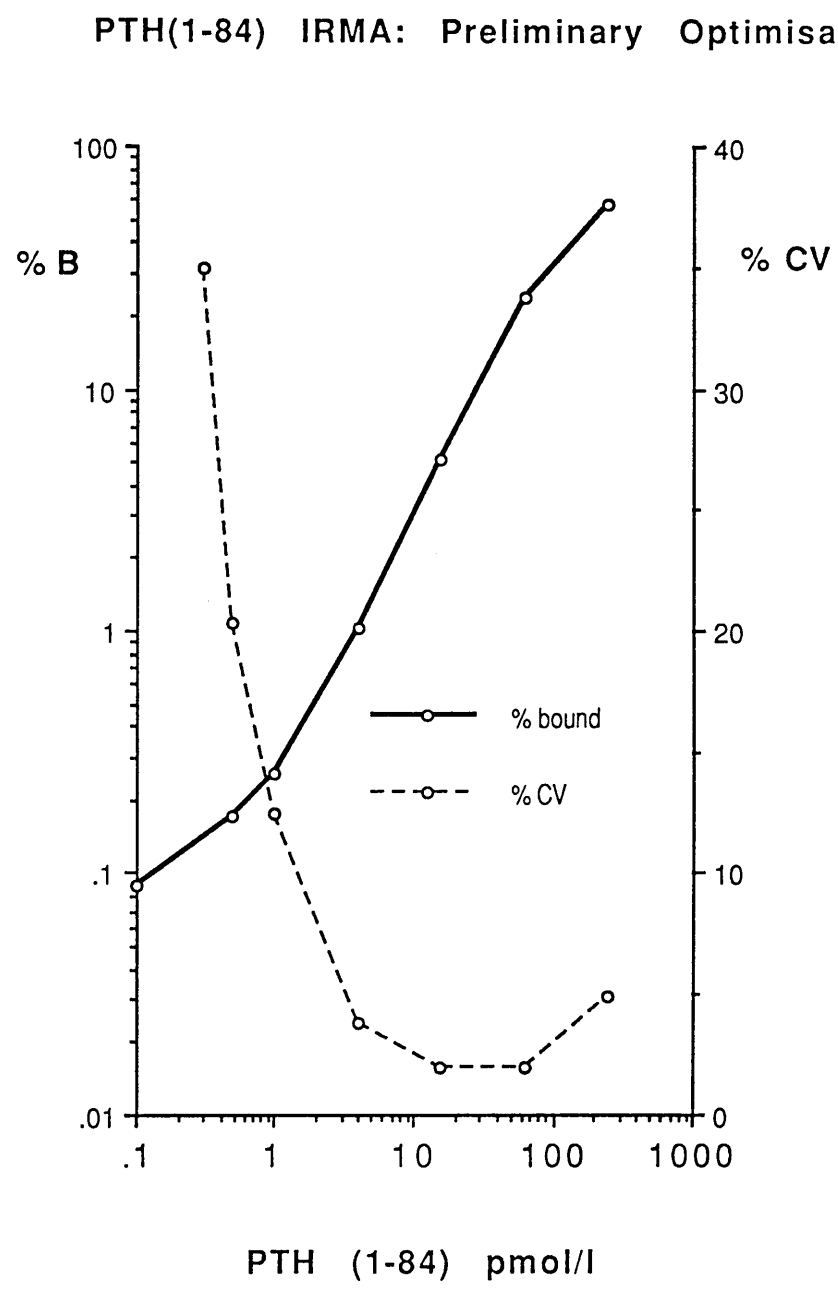


Figure 4.14

A typical PTH (1-84) response curve and associated precision profile. The error relationship was derived from the measurement of PTH (1-84) standard (140 duplicate pairs) in the preliminary optimised protocol.

Table 4.2

Preliminary optimised PTH (1-84) Assay Protocol

Assay Diluent	0.05 mol/l phosphate buffer pH 7.0 (0.1 mol/l NaCl, 0.5% BSA).
1st incubation	200 µl PTH (1-84) in hypoparathyroid serum 100 µl radiolabelled 3B3 (100,000 cpm) 22 h at room temperature.
2nd incubation	100 µl ESQ1-Sepharose (1 mg/tube) 2 h at room temperature with shaking.
Wash	4 x 2 ml 0.9% saline/Tween 0.2% (v/v).

between 1.5 and 250 pmol/l PTH (1-84) giving the assay an acceptably wide working range. Thus far, however, only synthetic PTH (1-84) standard had been measured in the assay system.

3.3 Measurement of Endogenous PTH (1-84) in Preliminary Assay System

Clinical Groups

Serum samples were taken, separated within 30 min of venesection, from a group of normal subjects (10) and patients with either primary hyperparathyroidism (10), hypercalcaemia of malignancy (10) or hypoparathyroidism (5). Samples were stored at -70°C until assay for PTH (1-84). Initially 2/10 samples from the group of normal subjects gave responses in the assay of 80% binding indicating PTH (1-84) concentrations well in excess of 500 pmol/l. As this seemed unlikely in normal subjects the possibility of the presence of heterophilic antibodies (Boscato and Stuart 1988) in these sera was suspected. The samples were re-assayed with the inclusion of 1% (v/v) rat serum in the assay diluent. The responses in the samples were then consistent with the rest of the samples in the normal subject group. Thereafter, rat serum at 1% (v/v) was incorporated into the assay diluent as routine.

The PTH (1-84) concentrations measured in the groups are shown in Figure 4.15. Also indicated are the detection limit of the assay (0.5 pmol/l) and a tentative reference range for PTH (1-84) in normal subjects (1.0-10 pmol/l) predicted from the literature (Papapoulos et al, 1980, Roos et al, 1981, Lindall et al, 1983). Eight of the ten samples from normal subjects had PTH (1-84) concentrations less than 0.5 pmol/l. The two measurable PTH (1-84) concentrations were both 0.6 pmol/l which is close to the assay detection limit.

The PTH (1-84) concentrations in the patients with primary hyperparathyroidism were as a group higher than those in normal subjects. The group mean was 0.8

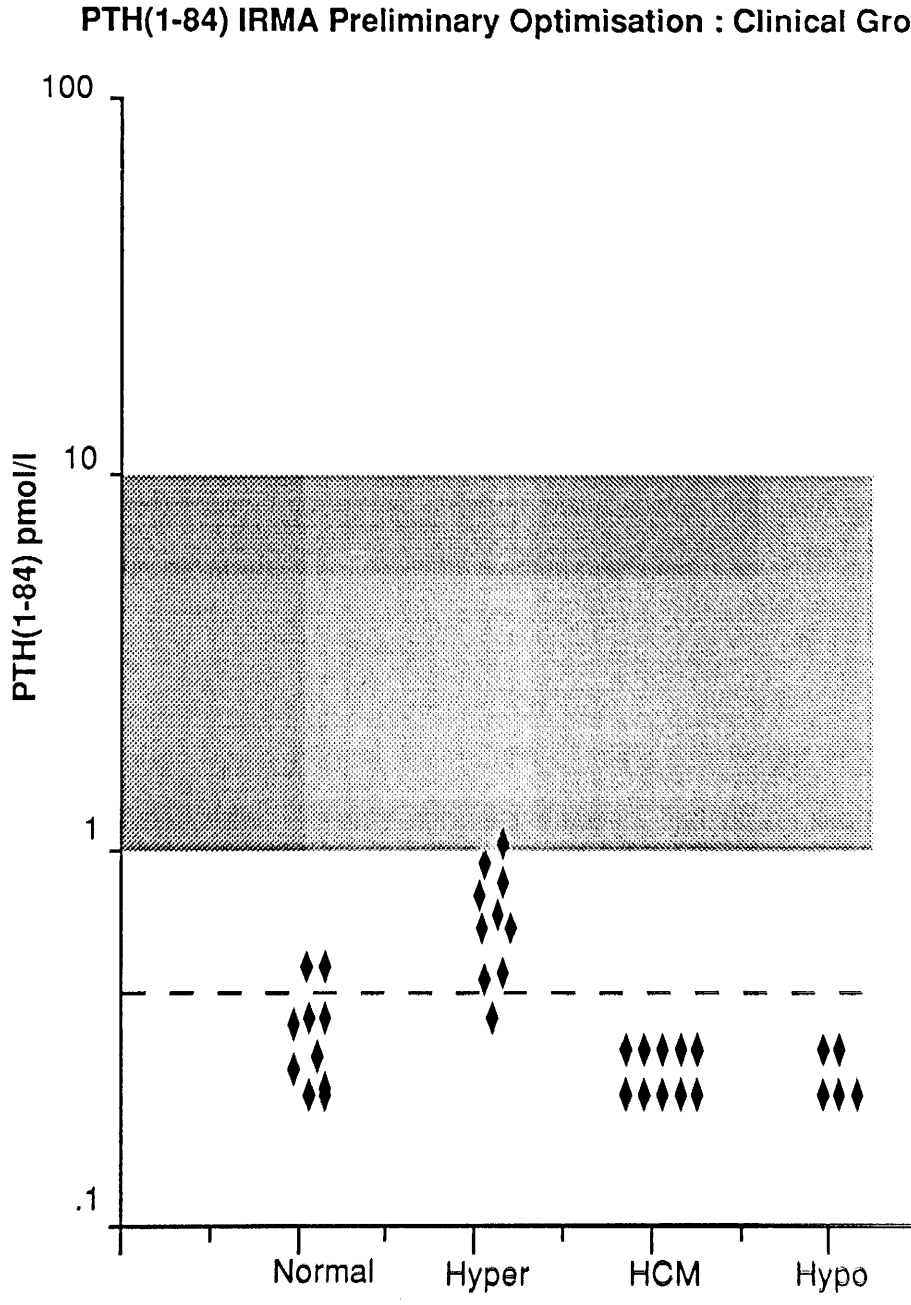


Figure 4.15

PTH (1-84) concentrations obtained in the preliminary optimised IRMA for serum samples from normal subjects (10) and patients with either primary hyperparathyroidism (10), hypercalcaemia of malignancy (HCM) (10) or hypoparathyroidism (5). Shaded area represents the predicted reference range for normal subjects.

pmol/l with a range 0.6-1.1 pmol/l (one result <0.5 pmol/l).

All patients in the hypercalcaemia of malignancy and hypoparathyroid groups had PTH (1-84) concentrations less than 0.5 pmol/l.

Therefore, although the ranking of the PTH (1-84) results appeared consistent with predicted concentration for these groups, i.e. hypoparathyroid and HCM patients < normal subjects < hyperparathyroid patients - the discrimination between the groups was very poor. In addition with eight out of ten normal subjects giving undetectable PTH (1-84) concentrations and only one result above 1.0 pmol/l in the hyperparathyroid group, all results were much lower than predicted.

Oral Calcium Load

This study was organised by Dr I Gunn, Consultant Clinical Biochemist at Law Hospital, Carluke, Lanarkshire. Twenty normal volunteers had serum samples withdrawn immediately prior to and 2 h post taking a 1 g oral calcium load. The samples were separated within 30 minutes of venesection and stored at -70° (prior to assay for PTH (1-84)). The results shown in Figure 4.16 were consistent with the previous experiment on the clinical groups. Most normal subjects had PTH (1-84) less than 0.5 pmol/l with no change seen post-calcium load.

Summary

The experiments on the measurement of endogenous PTH (1-84) in the preliminary assay system had indicated lower than predicted PTH (1-84) concentrations. Despite an apparent minimum detection limit of 0.5 pmol/l as assessed using synthetic PTH (1-84) (Figure 4.14), most normal subjects had undetectable PTH (1-84) concentrations. Discrimination between the clinical groups on the basis of the measured PTH (1-84) was also very poor.

It appeared therefore that if the PTH (1-84) concentrations predicted for the normal

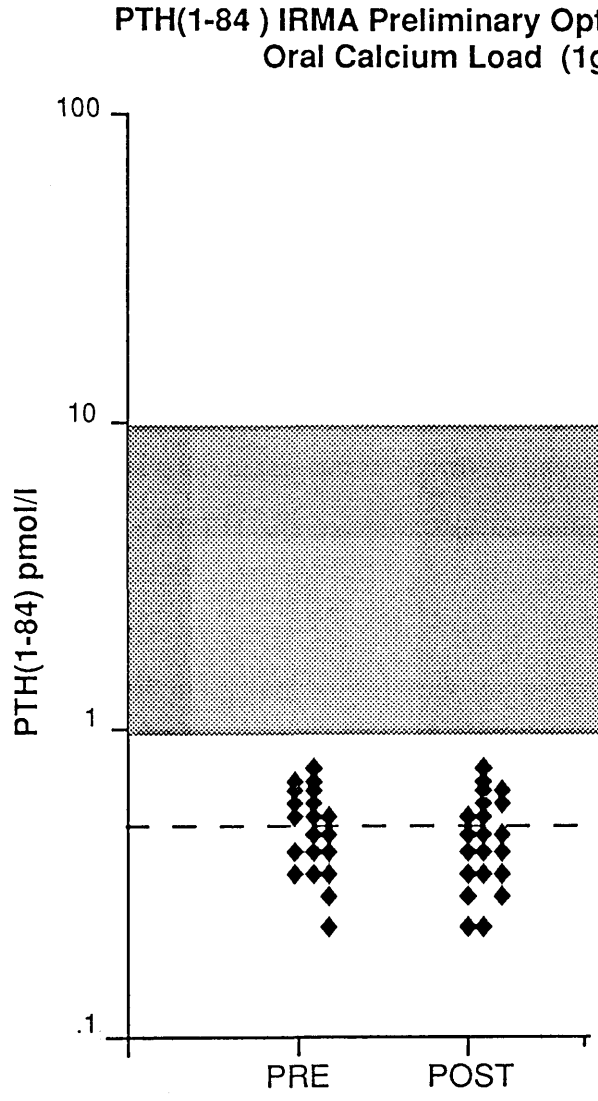


Figure 4.16

PTH (1-84) concentrations obtained with the preliminary optimised IRMA for samples from a group of normal subjects (20), pre- and post- oral calcium (1 g) load. Shaded area represents the predicted reference range for normal subjects.

subjects from the literature were in the right order of magnitude i.e. 1-10 pmol/l then the sensitivity of the assay system of 0.5 pmol/l as assessed using synthetic PTH (1-84) standards was not reflected in the measurement of endogenous PTH (1-84).

Two possible explanations for these results were considered for further investigation:

(i) The synthetic PTH (1-84) standards were calibrated incorrectly. If the actual PTH (1-84) concentrations in the standards were very much higher than the concentrations calculated from the dilution factor and the nominal weight of PTH (1-84) in the vial then the assay would appear to have a lower limit of detection for the synthetic PTH (1-84) standard than for endogenous PTH (1-84).

(ii) The assay system was able to distinguish between synthetic PTH (1-84) and endogenous PTH (1-84) and detected the latter much less effectively.

3.4 Investigation of PTH (1-84) Standard Preparations

Standard Preparations

Three preparations of PTH (1-84) standard were compared:

(1) Synthetic PTH (1-84) (Peninsula). This preparation had been used in the optimisation experiments thus far. The PTH (1-84) vial had been reconstituted in 2 ml acetic acid (0.1 mol/l) and stock aliquots of 2 µg stored at -70°C. Initial dilutions were made in 0.05 mol/l barbitone buffer pH 8.6, 0.5% BSA prior to dilution in hypoparathyroid serum.

(2) Synthetic PTH (1-84) (Peninsula). A new vial of PTH (1-84) was purchased and assayed immediately upon reconstitution (as above). Stock aliquots of 2 µg in acetic acid (0.1 mol/l) were also stored at -70°C.

(3) Human PTH (1-84) (NIBSC 79/500). The first international preparation (IRP) of human parathyroid hormone was reconstituted in 0.05 mol/l barbitone buffer

pH 8.6, 0.5% BSA according to the recommended procedure (Caygill 1977). The human PTH (1-84) was then diluted in hypoparathyroid serum prior to assay.

Standard Comparison

The comparison of standards is shown in Figure 4.17. There was a marked difference in potency between 'old' and 'new' synthetic PTH (1-84) preparations. The new synthetic PTH (1-84) having a potency of 10% relative to the old standard. The IRP of human PTH had a potency of 66% relative to the old standard.

These results appeared contradictory. The difference in potency between the old and new synthetic PTH (1-84) standard preparations could have been consistent with an error in the previous calibration and therefore account for the observed apparent difference in potency between synthetic and endogenous human PTH (1-84). However, the human PTH standards prepared from the IRP 79/500 although not equipotent with old synthetic PTH (1-84) standard had a relative potency of 66%. This was considered close to the expected value given that the IRP is calibrated by biological activity and the estimate of actual mass of PTH (1-84) in the IRP is approximate (Zanelli and Gaines-Das 1980). Other workers have since reported a potency by mass of 59% for the IRP 79/500 relative to synthetic PTH (1-84) (Brown et al, 1987).

Therefore although the PTH (1-84) assay system measured human PTH (1-84) in the IRP similar to the old synthetic PTH (1-84) standard, endogenous human PTH (1-84) when in serum appeared to have a low potency similar to that of the new synthetic PTH (1-84) standard. However, both the old and new synthetic PTH (1-84) standard preparations, although different batches, were from the same source. In addition, endogenous PTH (1-84) and the IRP material were of human origin although the latter had undergone extensive purification.

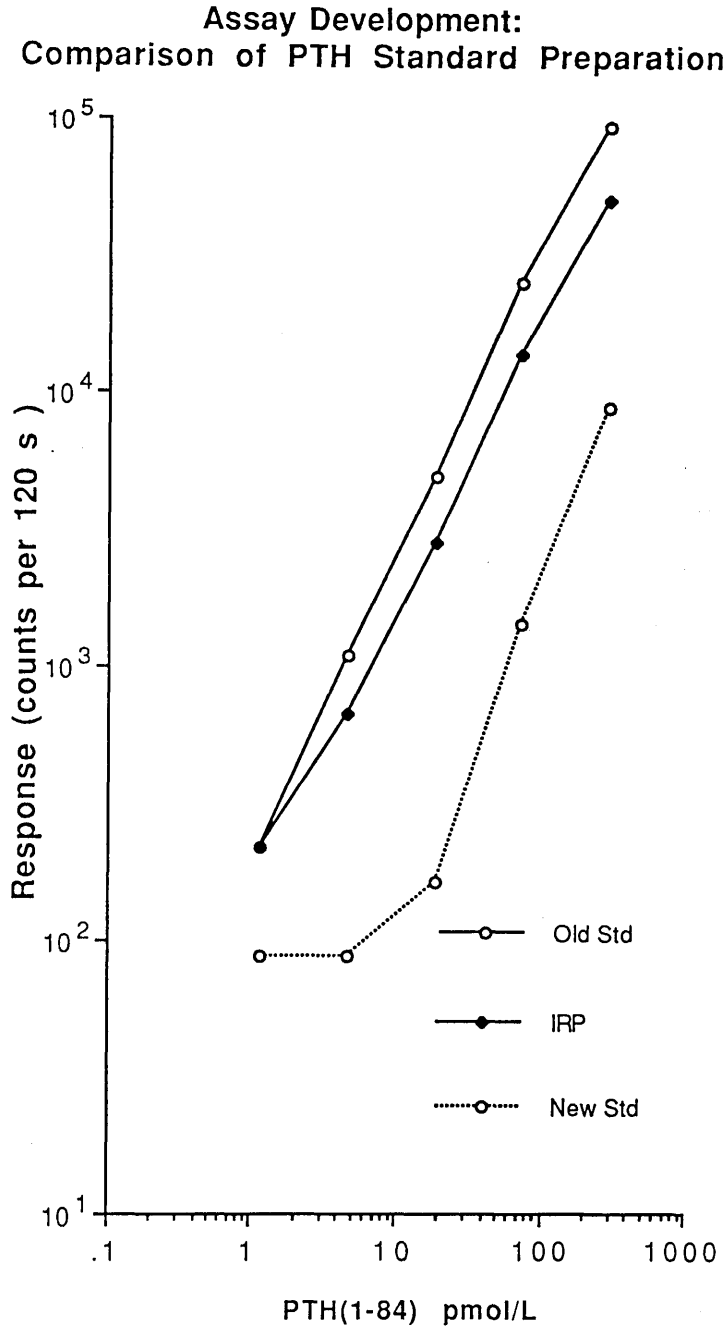


Figure 4.17

Comparison of PTH standard preparations in the preliminary optimised PTH (1-84) IRMA. Response curves were set up with the synthetic PTH (1-84) used for the optimisation experiments (old standard), a fresh batch of synthetic PTH (1-84) (new standard) and the 1st International Reference Preparation of Parathyroid Hormone 79/500 (IRP).

It was postulated that the PTH (1-84) assay system was sensitive to a conformational aspect of PTH (1-84) which had altered on reconstitution and storage of the old synthetic PTH (1-84) standard and also in the IRP material during the purification procedure.

Effect of Storage on Synthetic PTH (1-84) Standards

The postulate on the conformation of PTH (1-84) (above) was investigated. PTH is known to contain a greater degree of ordered helical structure at pH 7.5 than at pH 2.1 (Brewer et al, 1975). It has also be reported that the PTH molecule is more compact at alkaline pH (Cohn et al, 1974). A number of experiments were performed over a period of seven days in an attempt to affect the conformation of PTH (1-84) in solution and thereby alter the relative potencies of the three standard preparations in the PTH (1-84) assay system.

No change in the relative potencies of the standard preparations was achieved by a change of pH (4.0, 6.0, 7.0, 8.5 and 9.5) or by the addition of either EDTA (0.025, 0.05 mol/l) or mercaptoethanol (0.01, 0.1 mol/l) (data not shown in the interests of brevity). A small but significant increase in specific binding was observed, however, in all response curves on the addition of 0.05 mol/l EDTA which was subsequently incorporated into the PTH (1-84) assay diluent as routine.

While none of the above measures achieved an alteration from control curves within any one experiment the relative potencies of the old and new synthetic PTH (1-84) standards was observed to change over the seven day period. Figure 4.18 shows the control response curves for both preparations from the reconstitution of the new PTH (1-84) standard at day 0 through the seven day period of the experiment. The potency of the new PTH (1-84) standard was observed to rise steadily from the 10% estimate at day 0 to become indistinguishable from that of the old standard preparation after seven days storage.

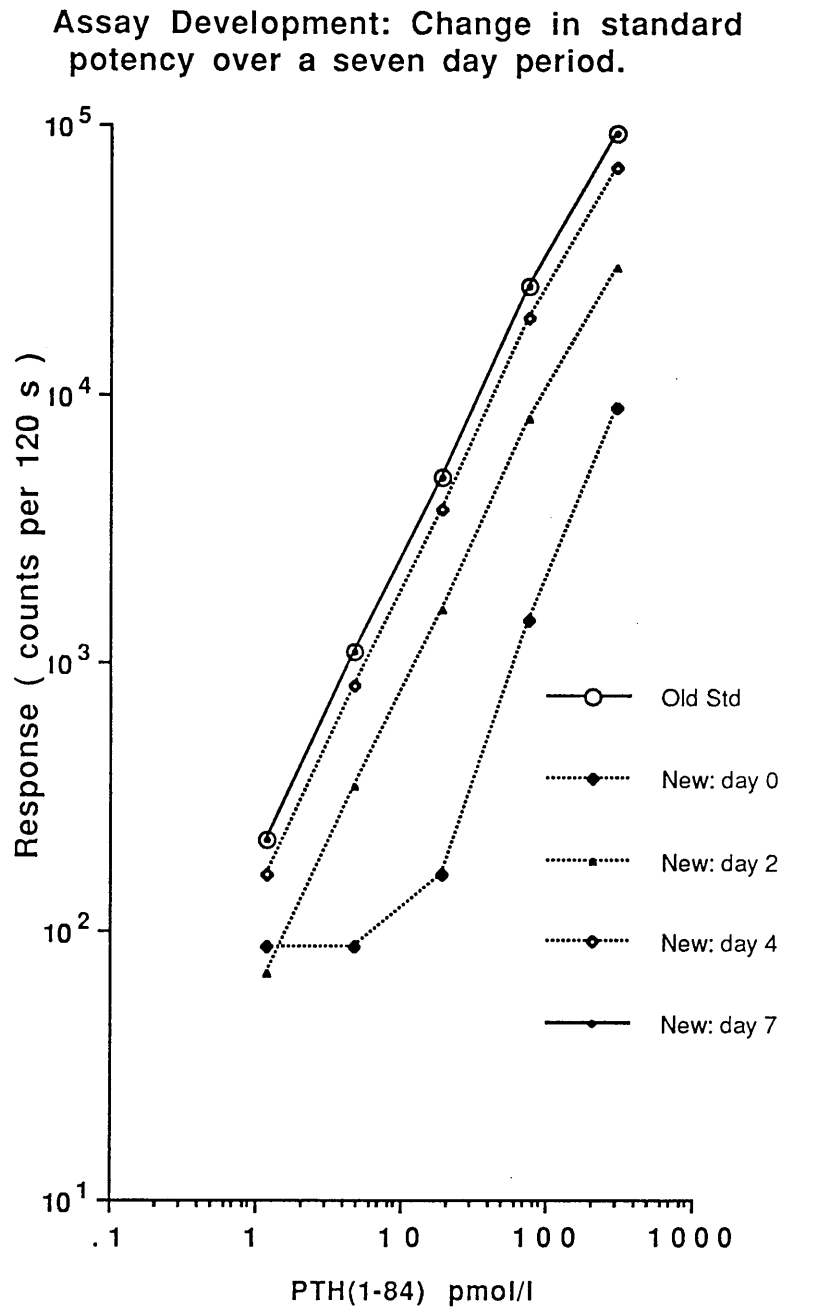


Figure 4.18

Comparison of the potency of the synthetic PTH (1-84) standard used in the initial optimisation experiments (old standard) and the new batch of synthetic PTH (1-84) immediately on reconstitution (New:day 0) and for seven days post reconstitution (days 2-7).

It was concluded that synthetic PTH (1-84) was altering on storage in acid at -70°C and that the PTH (1-84) assay system recognised the altered form preferentially.

3.5 Specificity of N-Terminal MAB's for Oxidised/Reduced PTH

Background

Early studies on the stability of bovine PTH indicated that the PTH molecule is susceptible to oxidation on storage. It was also shown that the PTH molecule lost biological activity on oxidation with hydrogen peroxide and that on subsequent reduction a complete restoration of biological potency occurred (Rasmussen 1959).

Further studies on the alkylation and oxidation of bovine PTH concluded that both alkylation with iodoacetate and oxidation with hydrogen peroxide involved only the methionine residues in PTH (Tashijian et al, 1964). Human PTH (1-84) has only two methionine residues both in the 1-34 N-terminal region of the molecule at positions 8 and 18 (Figure 4.19). The effect of oxidation and reduction on the ability of the N-terminal Mab's 3B3, 4G3 and 6E3 to recognise synthetic PTH (1-84) and PTH (1-34) was therefore investigated further.

Oxidation/Reduction Procedures

Preparations of oxidised PTH and reduced PTH were produced by the method of Tashijian et al, 1964:-

Oxidation: Stock aliquots (2 μg) of synthetic PTH (1-84) and PTH (1-34) were diluted with 1 ml acetate buffer pH 3.8 and incubated with hydrogen peroxide (0.1 mol/l) for 3 h at room temperature.

Reduction: Stock aliquots (2 μg) of synthetic PTH (1-84) and PTH (1-34) were diluted with 1 ml acetate buffer pH 3.8 and incubated with cysteine hydrochloride (0.12 mol/l) for 3 h at 80°C.

The Primary Structure of Preproparathyroid Hormone

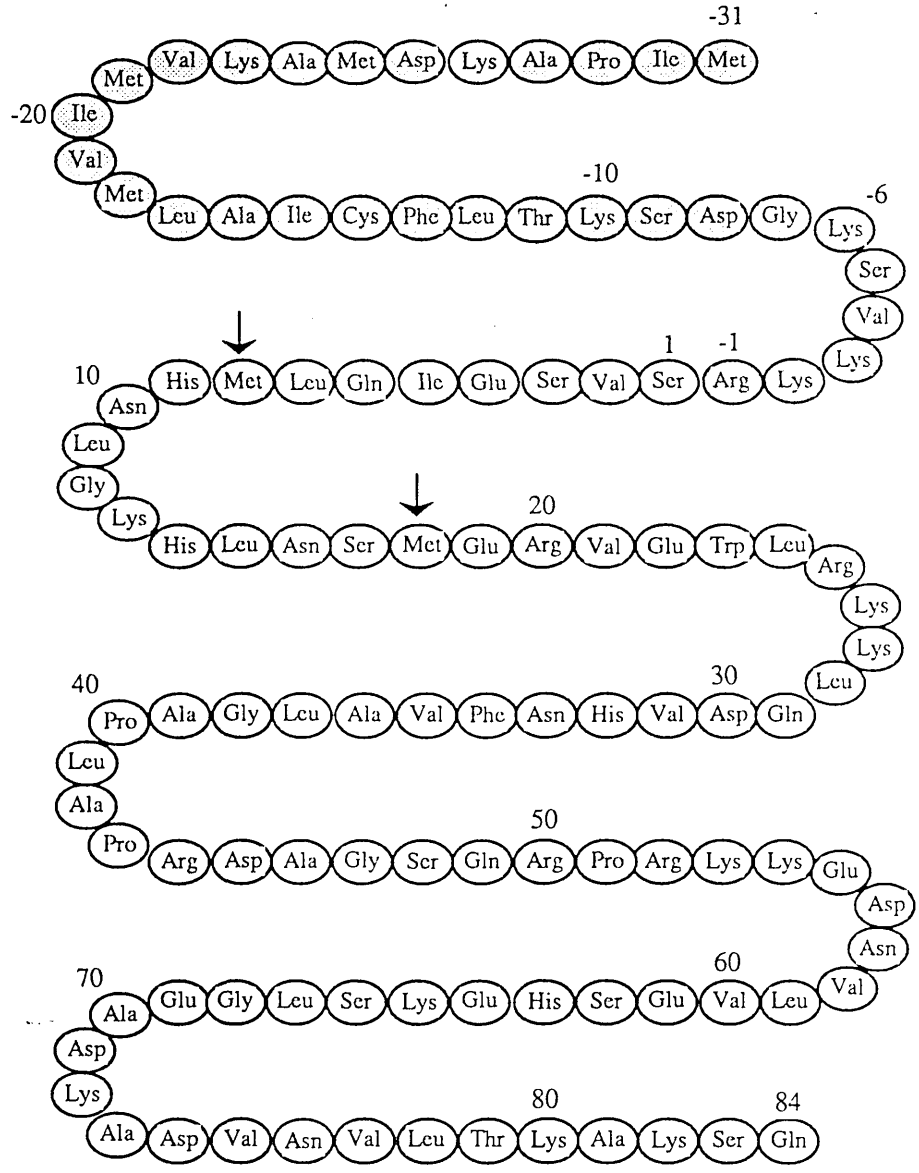


Figure 4.19

The primary structure of human pre- pro-parathyroid hormone (residues -31-84) incorporating the structure of PTH (1-84) (residues 1-84) and highlighting the positions of the methionine residues (8,18) the site of oxidation.

Displacement Studies

The specificity of the N-terminal Mab's for oxidised/reduced PTH was investigated using the conditions employed in the initial characterisation and specificity studies (Chapter 3, Section 6.2). Displacement curves for oxidised/reduced PTH (1-84) and PTH (1-34) were set up with limiting dilutions of 3B3 (1:4,000), 4G3 (1:4,000) and 6E3 (1:1,000) culture fluid in barbitone buffer using PTH (1-34) as radiolabel. The assay was incubated overnight at room temperature and separated with solid-phase sheep anti-rat IgG.

The displacement curve for 3B3 (Figure 4.20) confirms previous data that this Mab reacts with PTH (1-84) and PTH (1-34) on an essentially equimolar basis. The data also show that 3B3 recognises oxidised PTH peptides preferentially. The reduced PTH peptide having a potency of 10% relative to oxidised PTH in this system. Figure 4.21 shows the displacement curves for 4G3 which confirm that this Mab does not recognise PTH (1-84). 4G3 also recognises oxidised PTH (1-34) in preference to reduced PTH (1-34) which is twofold less potent with this Mab.

6E3 (Figure 4.22) reacts with PTH (1-84) and PTH (1-34) equally and also shows a preference for oxidised PTH by a factor of two.

In summary therefore, the N-terminal Mab's 3B3, 4G3 and 6E3 react preferentially with oxidised PTH. The Mab chosen for the development of the PTH (1-84) IRMA shows a tenfold preference for oxidised PTH (1-84).

The PTH molecule is known to oxidise on storage (Rasmussen 1959, Tashijian et al, 1964) and the synthetic PTH (1-84) has been demonstrated to increase in potency on storage (Section 3.4). It was concluded therefore that the difference in potency between synthetic PTH (1-84) and circulating endogenous PTH (1-84) observed in the PTH (1-84) IRMA reflected a difference in the oxidation state of the PTH molecule.

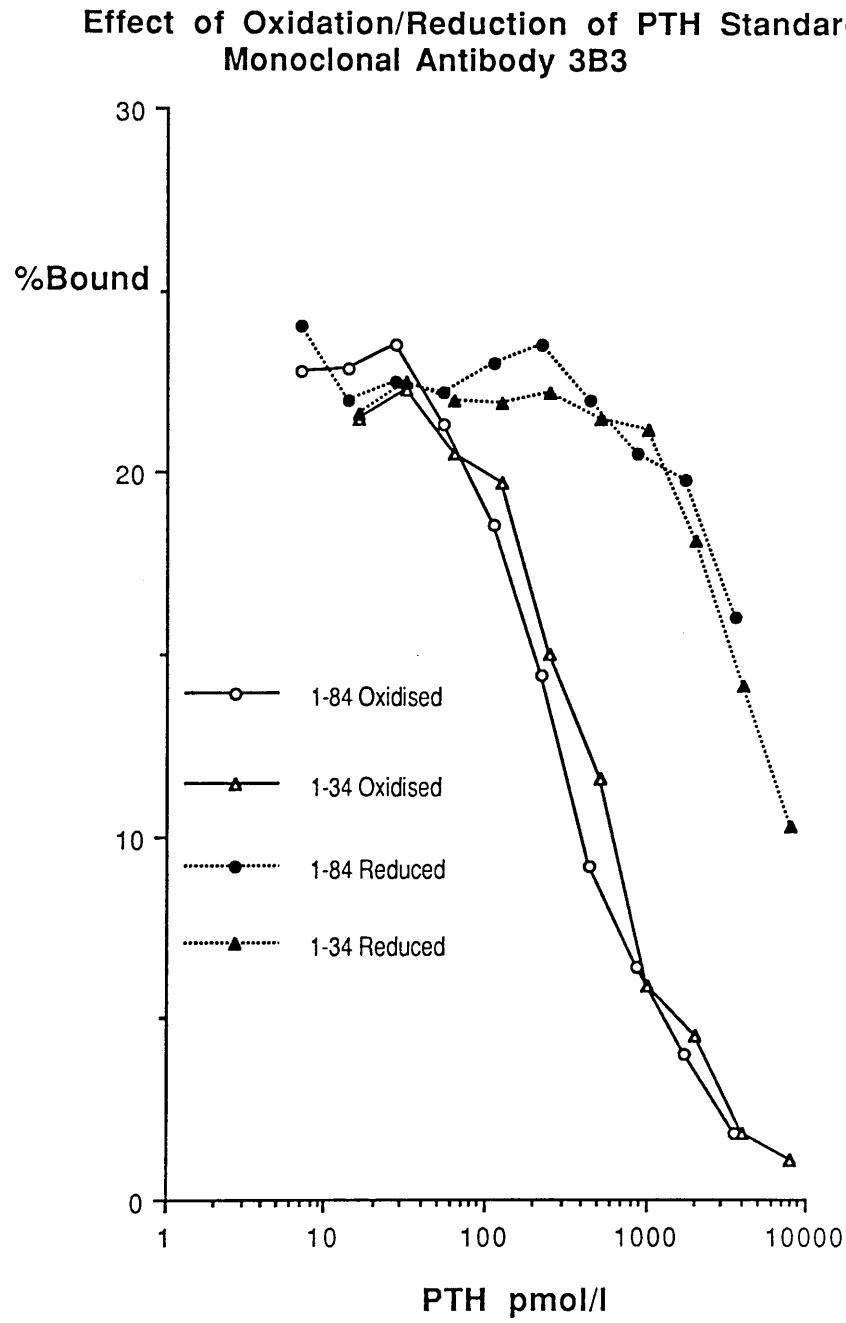


Figure 4.20

Displacement studies on Mab 3B3 with oxidised and reduced PTH peptides. Studies were carried out at a 1,4000 dilution (in barbitone buffer pH 8.6, 0.5% BSA) of 3B3 culture fluid with iodinated PTH (1-34) as radiolabel.

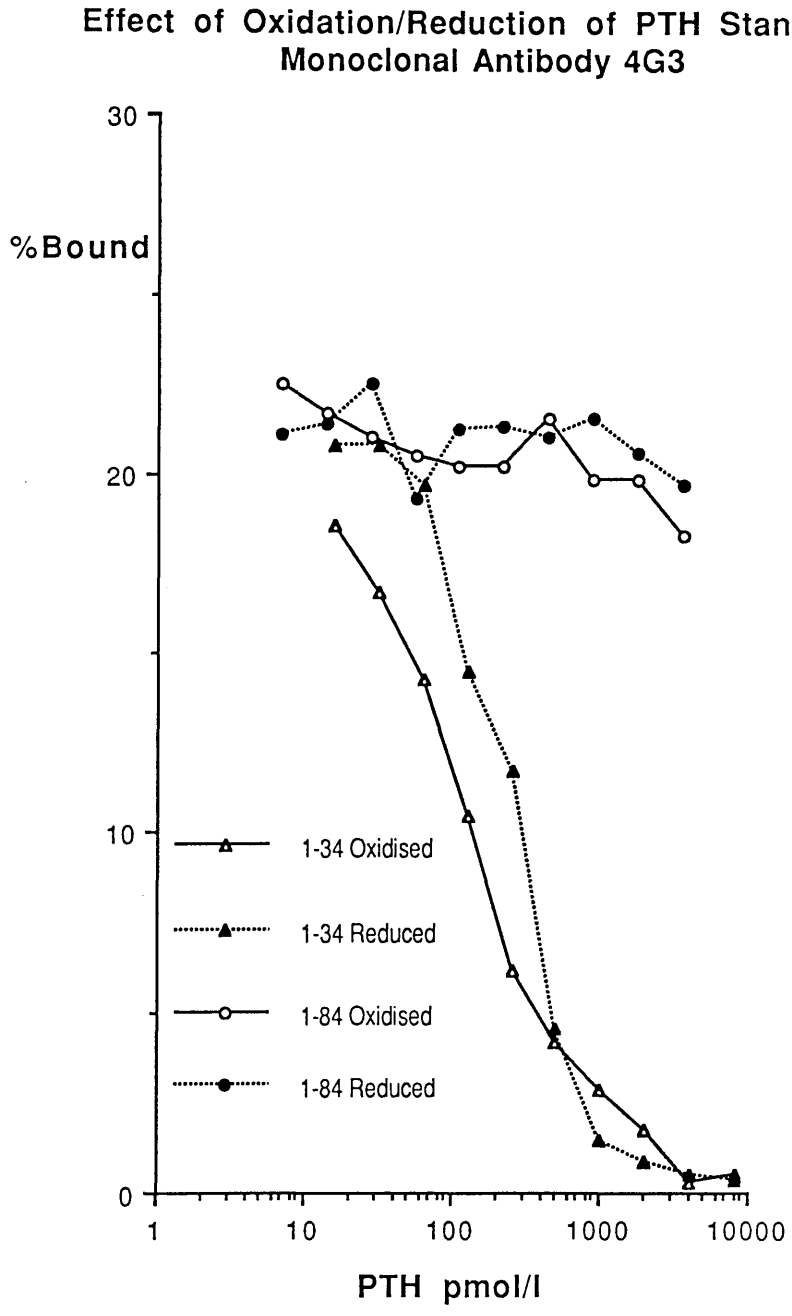


Figure 4.21

Displacement studies on Mab 4G3 with oxidised and reduced PTH peptides. Studies were carried out at a 1,4000 dilution (in barbitone buffer pH 8.6, 0.5% BSA) of 4G3 culture fluid with iodinated PTH (1-34) as radiolabel.

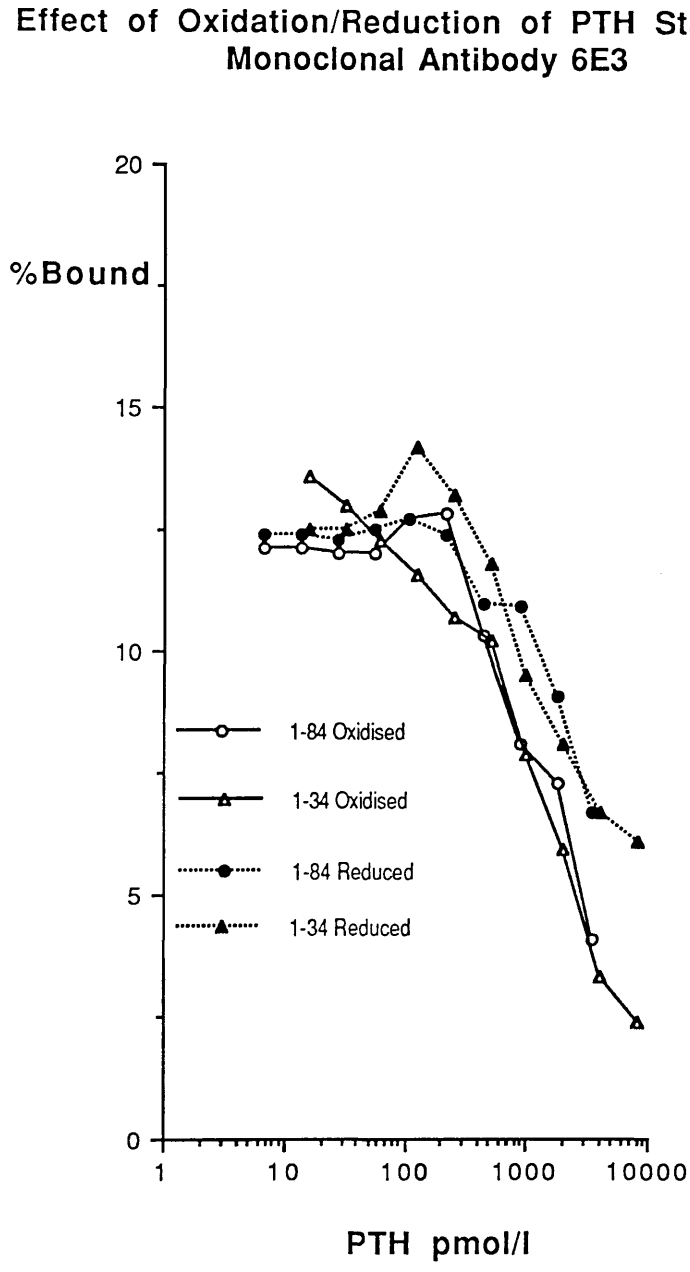


Figure 4.22

Displacement studies on Mab 6E3 with oxidised and reduced PTH peptides. Studies were carried out at a 1,000 dilution (in barbitone buffer pH 8.6, 0.5% BSA) of 6E3 culture fluid with iodinated PTH (1-34) as radiolabel.

However, the assay development data indicated that provided endogenous PTH (1-84) could be oxidised then the measurement of circulating concentrations could be achieved. Therefore the development of an assay system based on these reagents required investigation of the oxidation of circulating PTH (1-84).

3.6 Oxidation of Endogenous PTH (1-84)

Clinical Groups

Serum samples from normal subjects (5), patients with primary hyperparathyroidism (5) and patients with hypercalcaemia of malignancy (5) were treated with hydrogen peroxide (0.1 M) for 2 h at room temperature prior to measurement in the PTH (1-84) IRMA.

Figure 4.23 shows a comparison of the responses obtained in the PTH (1-84) IRMA for the oxidised aliquots of the above samples compared with that obtained for an untreated aliquot for each sample. The responses obtained for the untreated aliquots (shown shaded) were low, similar to previous experiments. Oxidation (shown as shaded + open) increased the responses in all samples. The predicted reference range (Section 3.3) is also indicated on Figure 4.23. The oxidised aliquots from normal subjects had responses falling within the predicted range; in the hyperparathyroid patients the responses were elevated; and in the HCM group the responses were below the reference range. Thus the oxidation of samples prior to assay had increased the response in all samples, but more importantly, dramatically improved the discrimination between the clinical groups. This was consistent with the postulate that the PTH (1-84) IRMA recognised oxidised PTH (1-84) preferentially and hydrogen peroxide treatment converts endogenous PTH (1-84) to the oxidised form.

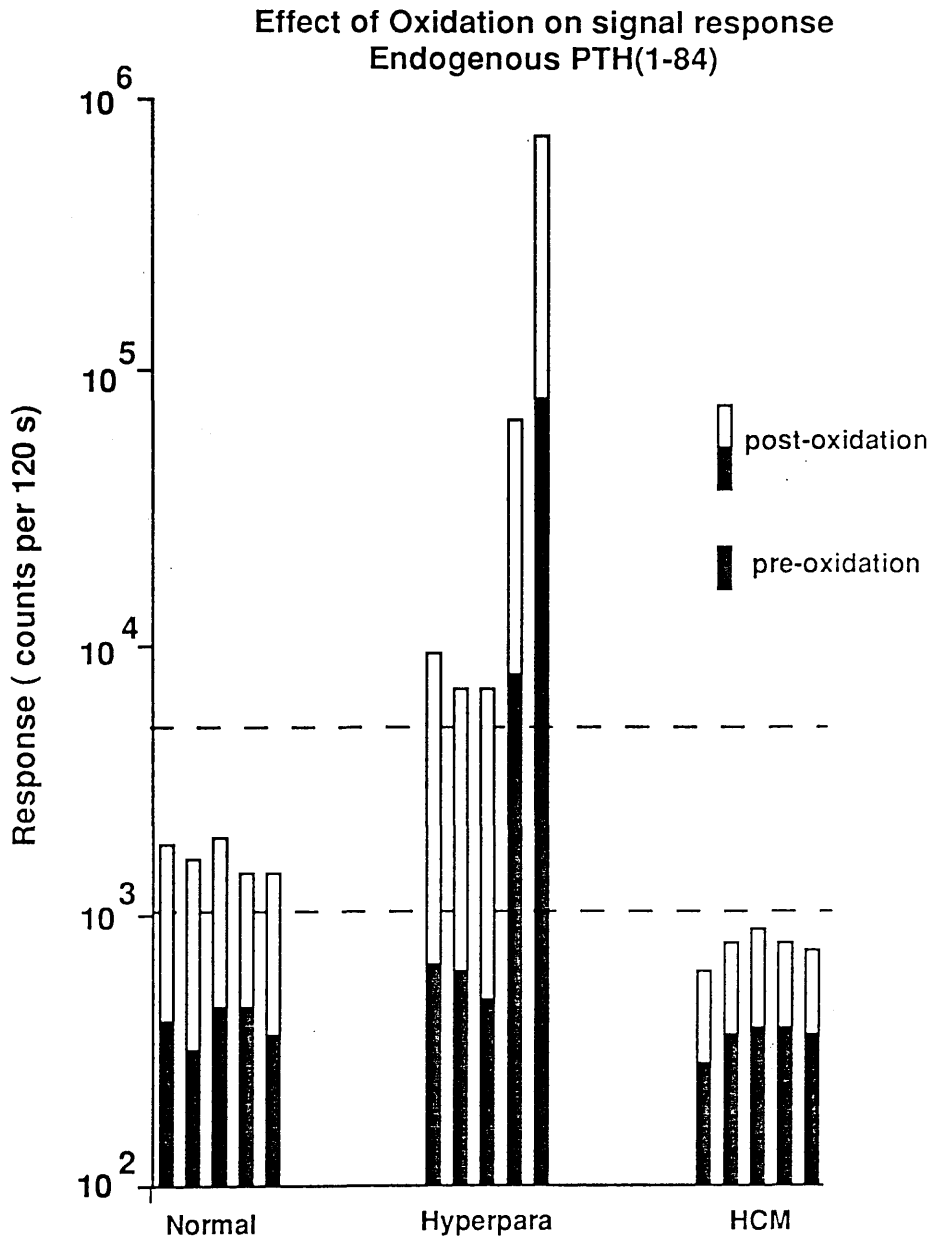


Figure 4.23

Effect of oxidation of endogenous PTH(1-84). Serum samples from normal subjects and patients with either primary hyperparathyroidism (hyperpara) or hypercalcaemia of malignancy (HCM) were treated with hydrogen peroxide (0.1 mol/l) prior to measurement. Responses are shown for the untreated (pre-oxidation) and the hydrogen peroxide treated (post oxidation) aliquots for each sample. The dotted lines indicate the responses corresponding to the lower and upper ends of the expected reference range for normal subjects.

Optimisation of Hydrogen Peroxide Concentration in PTH (1-84) IRMA

As the protocol for the PTH (1-84) IRMA included an overnight incubation it was hoped that the addition of hydrogen peroxide to the solution of radiolabelled 3B3 would be effective rather than have a separate pre-treatment step for each sample.

Patient Samples

Aliquots of samples from patients with various disorders of calcium metabolism were assayed in the PTH (1-84) IRMA with hydrogen peroxide (final assay concentration 0-0.25 mol/l) to the solution of radiolabelled 3B3.

Figure 4.24 shows the results of the hydrogen peroxide optimisation with patient samples. All responses were increased over the control (no hydrogen peroxide added) on the addition of hydrogen peroxide. The response in all samples had a plateau around 0.1 mol/l hydrogen peroxide. It appeared that on the higher concentration (0.25 mol/l) the responses may decrease.

Synthetic PTH (1-84) Standards

The above experiment was repeated with synthetic PTH (1-84) standard diluted in horse serum (Figure 4.25). The responses with synthetic PTH (1-84) also had a plateau around 0.1 mol/l hydrogen peroxide added. There was no significant difference from control (no hydrogen peroxide added) up to 0.125 mol/l - the synthetic standard having oxidised on storage. Responses decreased at 0.25 mol/l hydrogen peroxide.

The experiments on the optimisation of hydrogen peroxide concentration in the PTH (1-84) IRMA confirmed the use of 0.1 mol/l hydrogen peroxide in the oxidation procedure as described by Tashijian et al, 1964. The addition of hydrogen peroxide to the solution of radiolabelled 3B3 obviates the requirement for treatment of samples

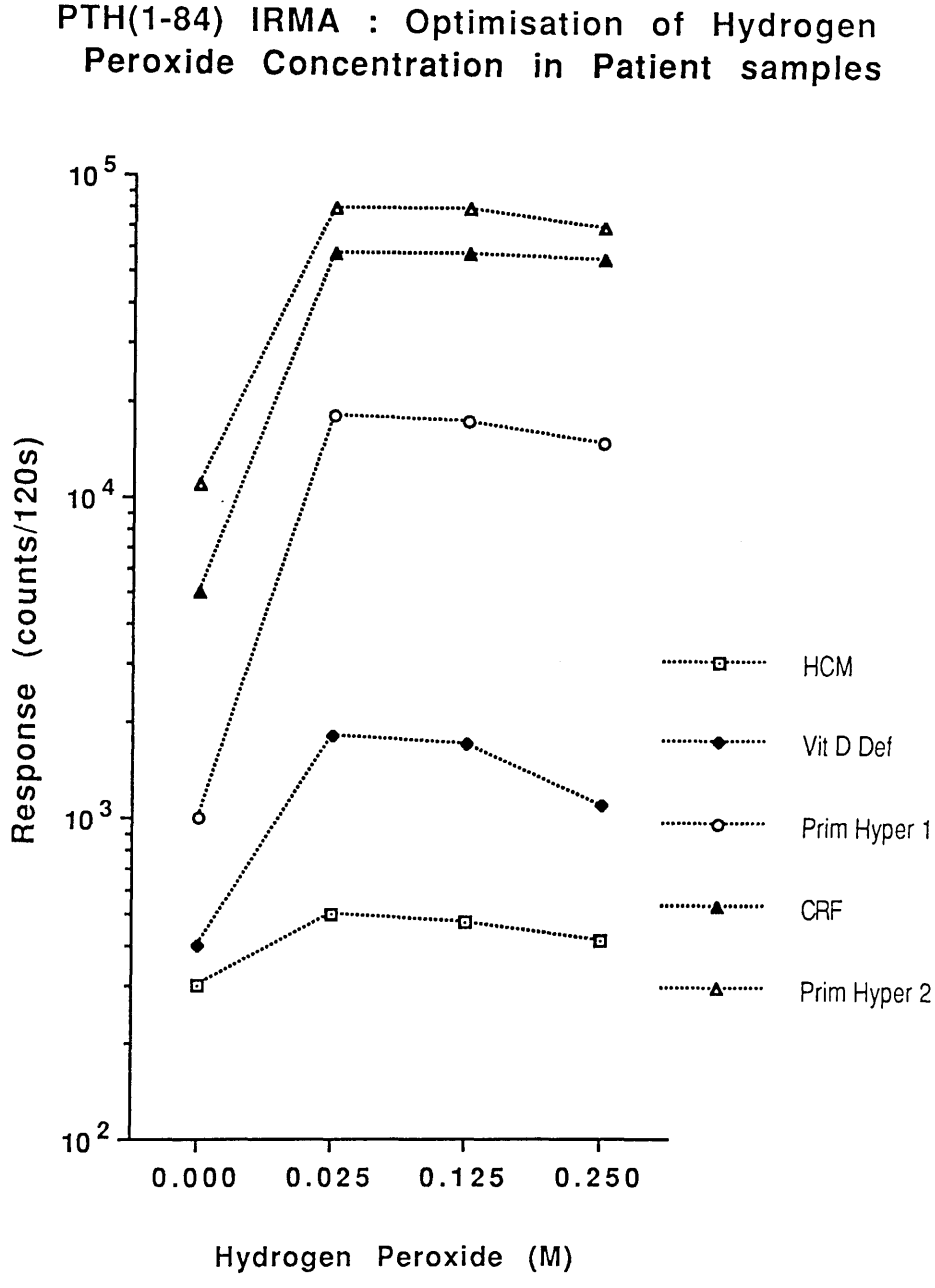


Figure 4.24

Optimisation of the hydrogen peroxide concentration for the measurement of endogenous PTH (1-84). Samples from patients with hypercalcaemia of malignancy (HCM), vitamin D deficiency (Vit D Def); chronic renal failure (CRF) and primary hyperparathyroidism (Prim hyper 1, 2) were assayed at a range of hydrogen peroxide concentrations (0 - 0.25 mol/l) which was added directly to the assay in the solution of radiolabelled 3B3.

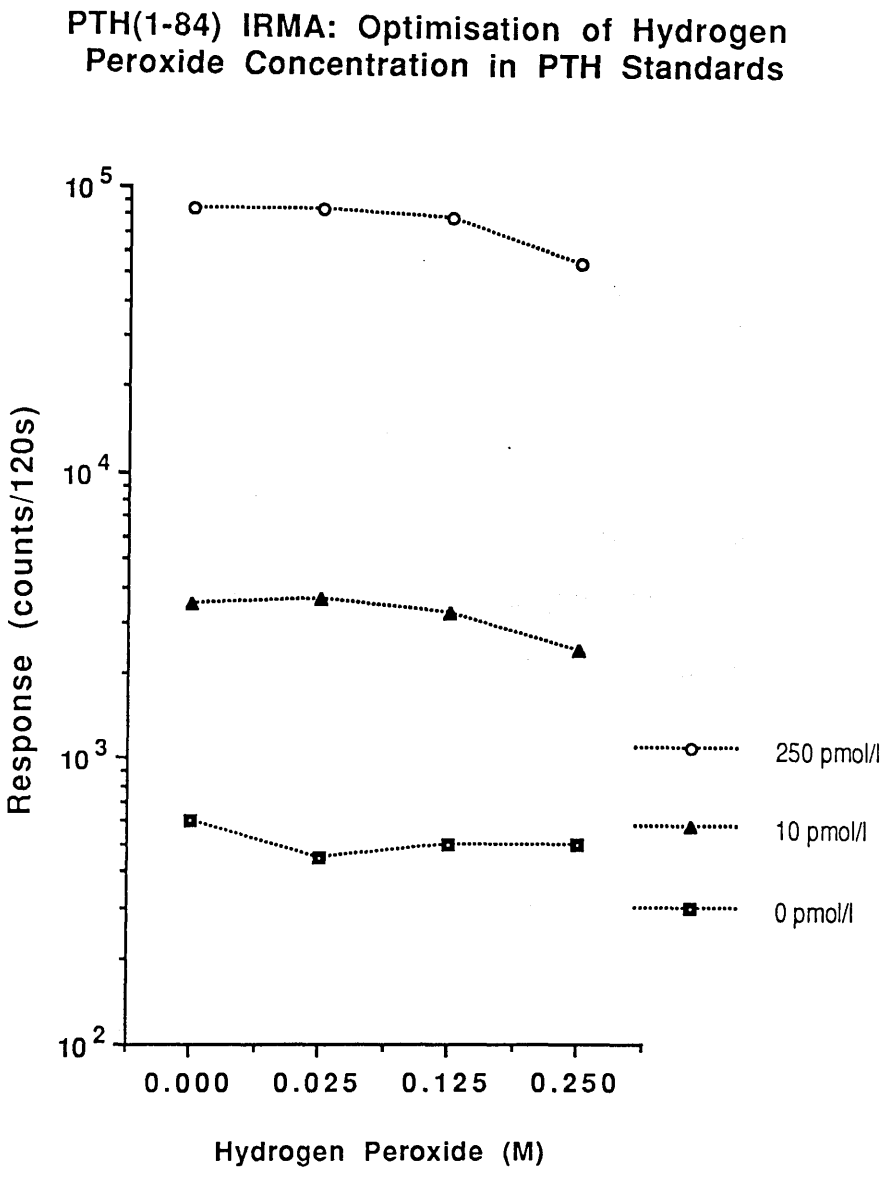


Figure 4.25

Optimisation of the hydrogen peroxide concentration for the measurement of synthetic PTH (1-84). Solutions of PTH (1-84) standard (0, 10 & 250 pmol/l) were assayed at a range of hydrogen peroxide concentrations (0 - 0.25 mol/l) which was added directly to the assay in the solution of radiolabelled 3B3.

prior to assay.

3.7 PTH (1-84) IRMA - Preparation of Standards and Quality Control Pools

Selection of Matrix for Standards

Serum from a patient with hypoparathyroidism had been employed as the matrix for the PTH (1-84) standards in the optimisation studies. As this was in limited supply, possible alternative sources of suitable matrix material were investigated prior to final validation of the assay and application to clinical samples.

Figure 4.26 shows a comparison of the response curves for synthetic PTH (1-84) standard in assay buffer, serum from the hypoparathyroid patient, equine serum, bovine serum and porcine plasma. The response curves diverge from the assay buffer curve at low PTH (1-84) concentrations. This is consistent with the previous findings of higher blanks in the assay buffer at pH 7.0 (Section 3.2). There was no significant difference between the response curves in human, equine or bovine serum or porcine plasma. As equine serum was readily available in large batches, this was chosen as the matrix for PTH (1-84) standards.

Preparation of Standards

Synthetic PTH (1-84) was the most readily available source of material for primary standardisation. In view of the possible instability of PTH (1-84) in serum it was decided to store initial dilutions of the synthetic PTH (1-84) in 0.05mol/l barbitone buffer pH 8.6 containing 0.5% BSA. These were then diluted into the equine serum matrix prior to assay according to the protocol in Table 4.3.

Preparation of Quality Control Pools

Initially quality control pools were prepared by the addition of known amounts of synthetic PTH (1-84) to equine serum. However these proved unstable over the first

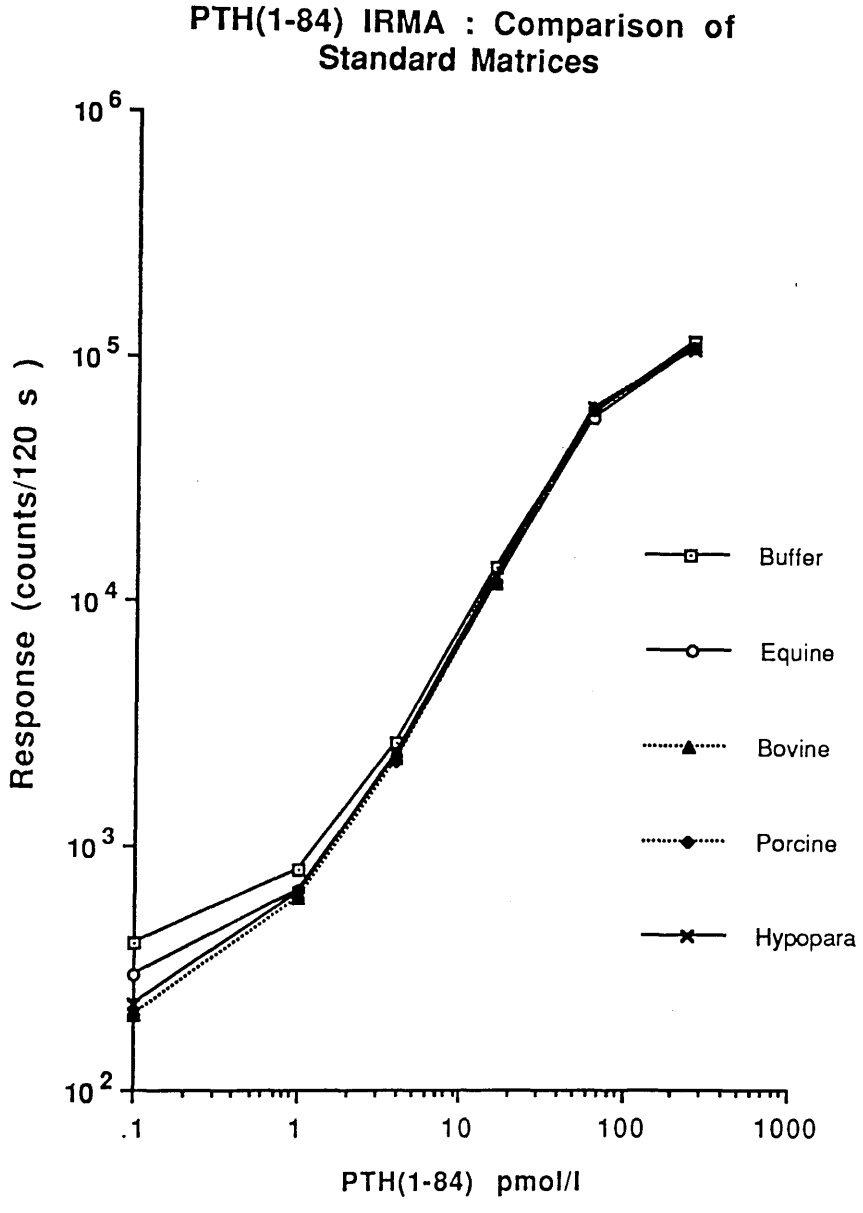


Figure 4.26

Selection of matrix for standards. Response curves for dilutions of PTH (1-84) standard in assay buffer (buffer), equine serum, bovine serum, porcine serum and serum from a patient with hypoparathyroidism (hypopara).

Table 4.3

Preparation of PTH (1-84) standards

(A) Concentrated Standard

1 vial 20 µg PTH (1-84) (Peninsula) made up in 2 ml 0.05 mol/l barbitone buffer pH 8.6, 0.5% BSA.

Aliquots stored at -70°C = 1 µg/100 µl.

(B) Stock Standard

100 µl of concentrated standard (A) made up to 10 ml barbitone buffer pH 8.6, 0.5% BSA.

Aliquots stored at -70°C = 10 ng/100 µl.

(C) Working Standard (Made up fresh for each assay)

100 µl stock standard (B) plus 1.9 ml horse serum = 500 pmol/l.

(D) Standard Curve (Diluted fresh for each assay)

(i)	1 ml working standard (C) + 1 ml equine serum	= 250 pmol/l
(ii)	200 µl (i) + 600 µl equine serum	= 62.5 pmol/l
(iii)	200 µl (ii) + 600 µl equine serum	= 15.6 pmol/l
(iv)	200 µl (iii) + 600 µl equine serum	= 3.9 pmol/l
(v)	200 µl (iv) + 600 µl equine serum	= 0.98 pmol/l

month of usage (Figure 4.27). The measured value in these pools dropped by 30% on average over the 30 day period. Subsequently the quality control pools were prepared by diluting serum from a patient with primary hyperparathyroidism in equine serum. These pools showed no sign of deterioration over a three month period (Table 4.4).

4 VALIDATION OF THE PTH (1-84) IRMA

4.1 PTH (1-84) IRMA Finalised Protocol

The protocol for the PTH (1-84) IRMA derived from the antibody characterisation/compatibility studies and the subsequent optimisation experiments is shown in Table 4.5.

4.2 Potency of Oxidised/Reduced PTH (1-84)

Given the sensitivity of the assay system to the oxidative state of PTH (1-84) the assessment of the potency of oxidised PTH (1-84) and reduced PTH (1-84) was an important validation criterion.

Preparations of oxidised and reduced PTH (1-84) were produced from the same stock aliquot of synthetic PTH (1-84) by the method of Tashijian et al, (1964) (Section 3.5). The preparations were then diluted for assay according to the protocol for assay standards (Section 3.7). Figure 4.28 shown the response curves obtained for the oxidised and reduced standard preparations compared to the routine standard curve. The potency of both oxidised and reduced PTH (1-84) is essentially identical across the reference range of the standard curve (0-250 pmol/l).

4.3 Specificity for PTH (1-84); interference from PTH Fragments

Circulating PTH fragments may be present in excess in serum and competitively inhibit reagent binding. The effect of adding excess PTH fragments to the standard curve was investigated.

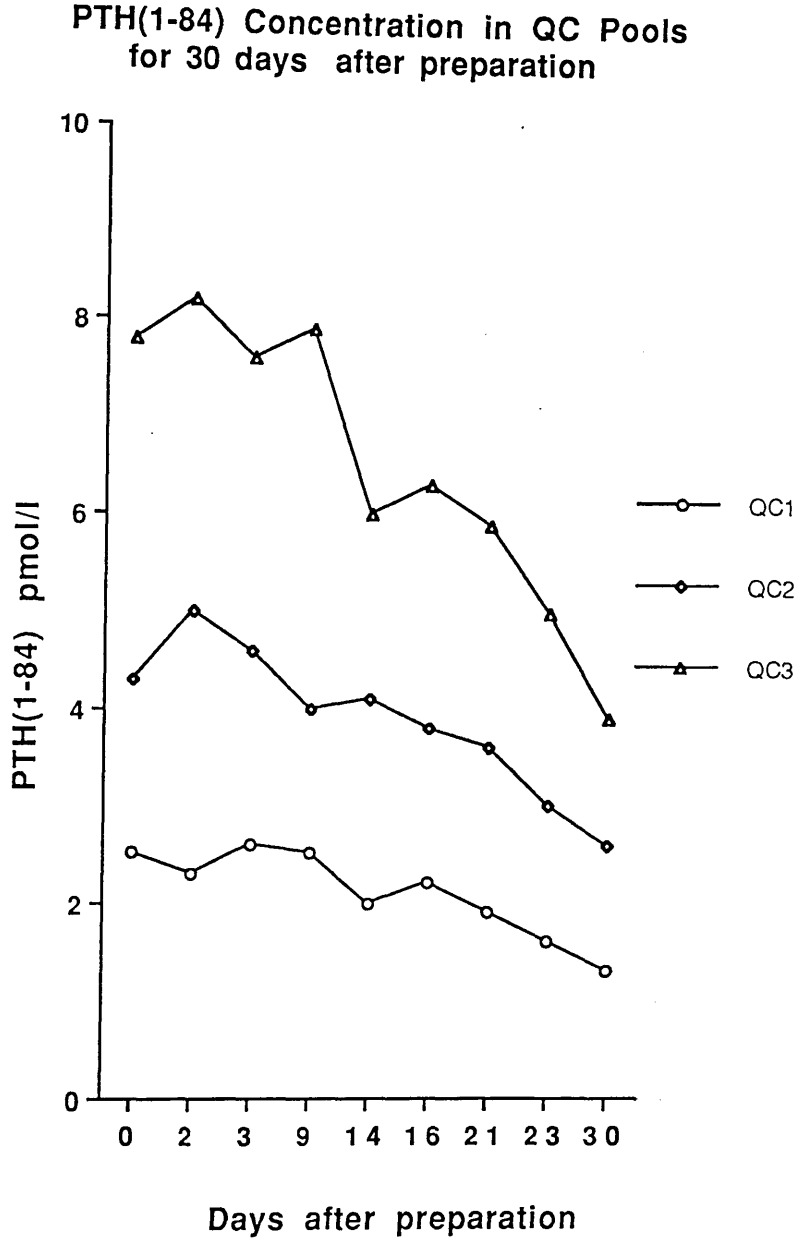


Figure 4.27

PTH (1-84) concentrations measured in quality control pools, prepared by the addition of synthetic PTH (1-84) peptide to equine serum, for 30 days post preparation.

Table 4.4

Assessment of PTH (1-84) assay performance using quality control pools prepared from endogenous PTH

	Month 1	Month 2	Month 3
QC1 Mean:	2.8	2.9	2.6
SD:	0.33	0.40	0.37
CV:	11.7	13.7	12.6
n:	10	10	10
QC2 Mean:	10.6	11.7	11.6
SD:	0.94	1.0	1.1
CV:	8.8	8.5	9.4
n:	10	10	10
QC3 Mean:	17.6	17.8	17.7
SD:	1.6	1.9	1.3
CV:	9.1	10.6	7.3
n:	10	10	10
Mean CV:	9.9%	10.9%	9.8%

Table 4.5

Optimised PTH (1-84) assay protocol

Assay Diluent	0.05 mol/l phosphate buffer pH 7.0 containing NaCl (0.1 mol/l), EDTA (0.05 mol/l), BSA (0.5% w/v) and rat serum (1% v/v).
1st incubation	200 µl sample or standard 100 µl radiolabelled 3B3 (100,000 cpm) diluted in assay diluent containing 0.4 mol/l H_2O_2. overnight at room temperature.
2nd incubation	100 µl ESQ1-Sepharose CL-4B (1 mg/tube). 2 h at room temperature,with shaking.
Wash	2 ml 0.9% saline/Tween 20 (0.2% v/v). Centrifuge and aspirate (x4).
Count	120 secs.

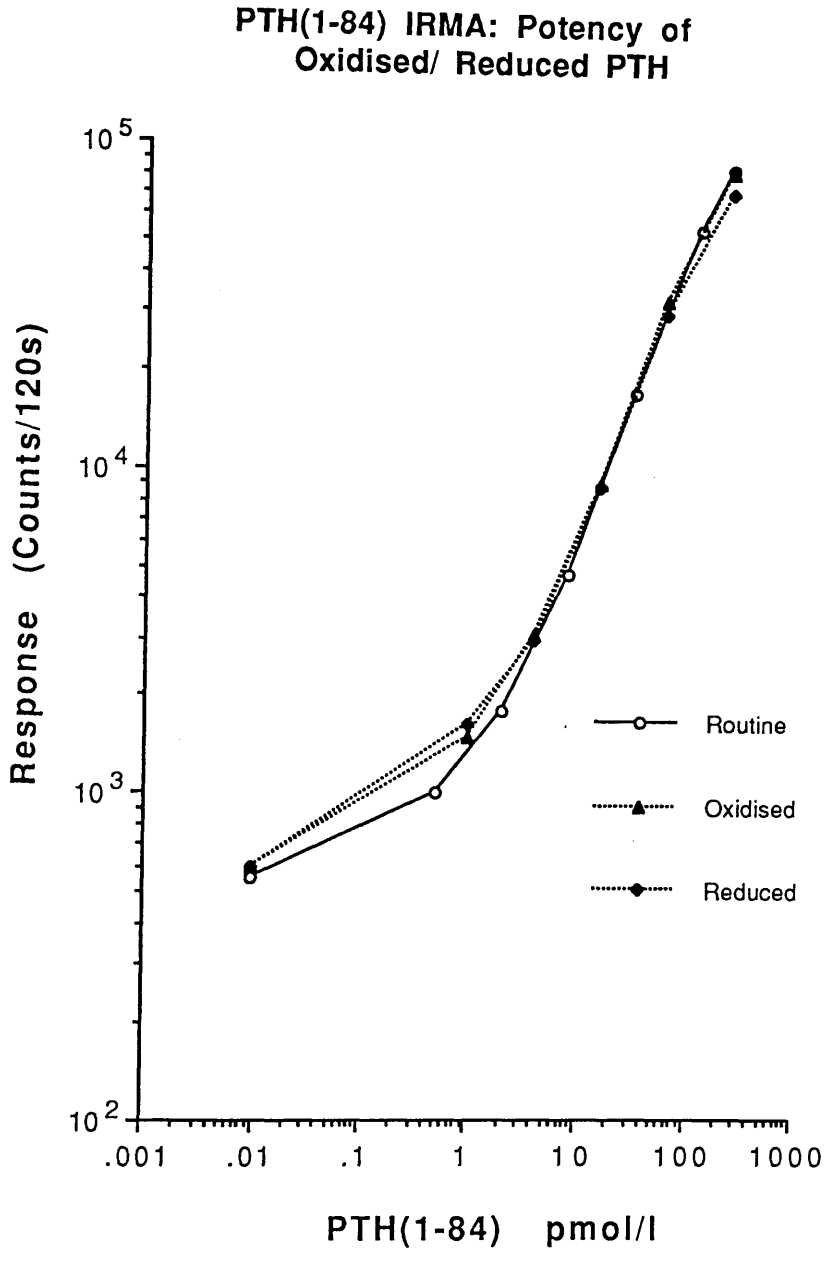


Figure 4.28

Potency of oxidised and reduced PTH (1-84). The response curves for oxidised and reduced synthetic PTH (1-84) prepared by the method of Tashijan 1964 (Section 3.5) are shown compared to the PTH (1-84) standard curve in routine use.

The response curve was unaffected by up to a 200 molar excess of C-terminal fragments PTH (53-84) (Figure 4.29). The N-terminal fragment PTH (1-34) caused a saturation of binding at high PTH (1-84) concentration at a fifteenfold molar excess (Figure 4.29).

The tolerance of a 200 molar excess of C-terminal fragments should prevent interference even in samples from patients with established renal failure where gross elevations of C-terminal fragments can occur. The level of interference by PTH (1-34) is unlikely to be a major problem in clinical samples as recent evidence indicates that circulating levels of PTH (1-34) are low (Bringhurst et al, 1988).

4.4 Reference Interval and Clinical Groups

Serum samples (separated within 30 minutes of venepuncture) were collected for assay in the PTH (1-84) IRMA from the following groups:

Normal subjects: A group of 57 apparently healthy laboratory staff with mean age 30.3 years (range 20-53 years). All samples contained detectable PTH (1-84) concentrations (Figure 4.30). The mean PTH (1-84) concentrations was 2.21 pmol/l (absolute range 0.8-5.0 pmol/l). The reference interval was estimated as 1.0-4.4 pmol/l (range containing 95% of results).

Primary hyperparathyroidism: A group of 27 patients with appropriate biochemistry and surgically proven hyperparathyroidism gave values in the range 5.8-100 pmol/l (mean 21.0 pmol/l).

Hypoparathyroidism: Six patients with established hypoparathyroidism post radical neck surgery gave undetectable (<0.5 pmol/l) PTH (1-84) concentrations.

Hypercalcaemia associated with malignancy (HCM): Fourteen of 18 samples from patients with HCM gave undetectable (<0.5 pmol/l) PTH (1-84) concentrations. Of the four samples with detectable PTH (1-84), two (0.8, 0.9 pmol/l) were from

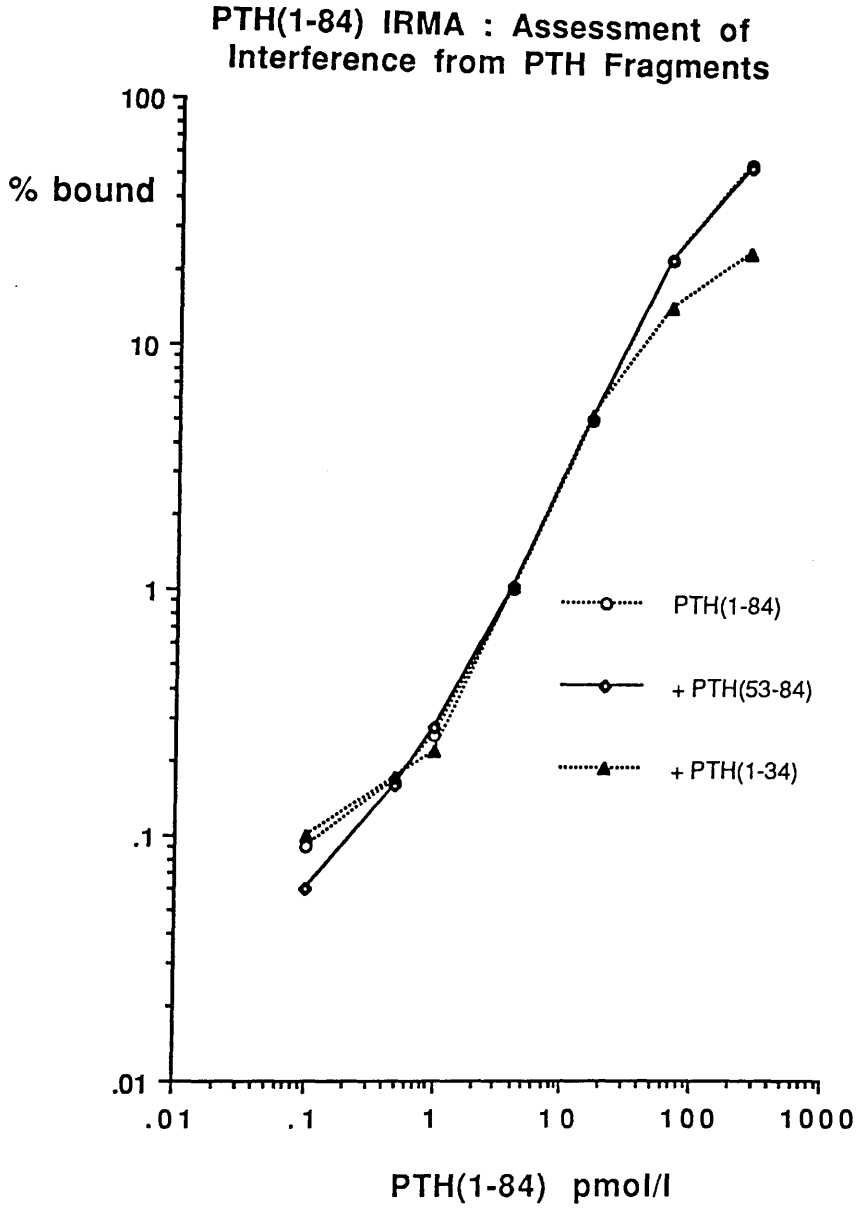


Figure 4.29

Measurement of PTH (1-84) in the presence of PTH fragments. The response curves for PTH (1-84) is shown compared to the curves obtained for PTH (1-84) with an added 200 molar excess of PTH (53-84) and for PTH (1-84) with an added 15 molar excess of PTH (1-34).

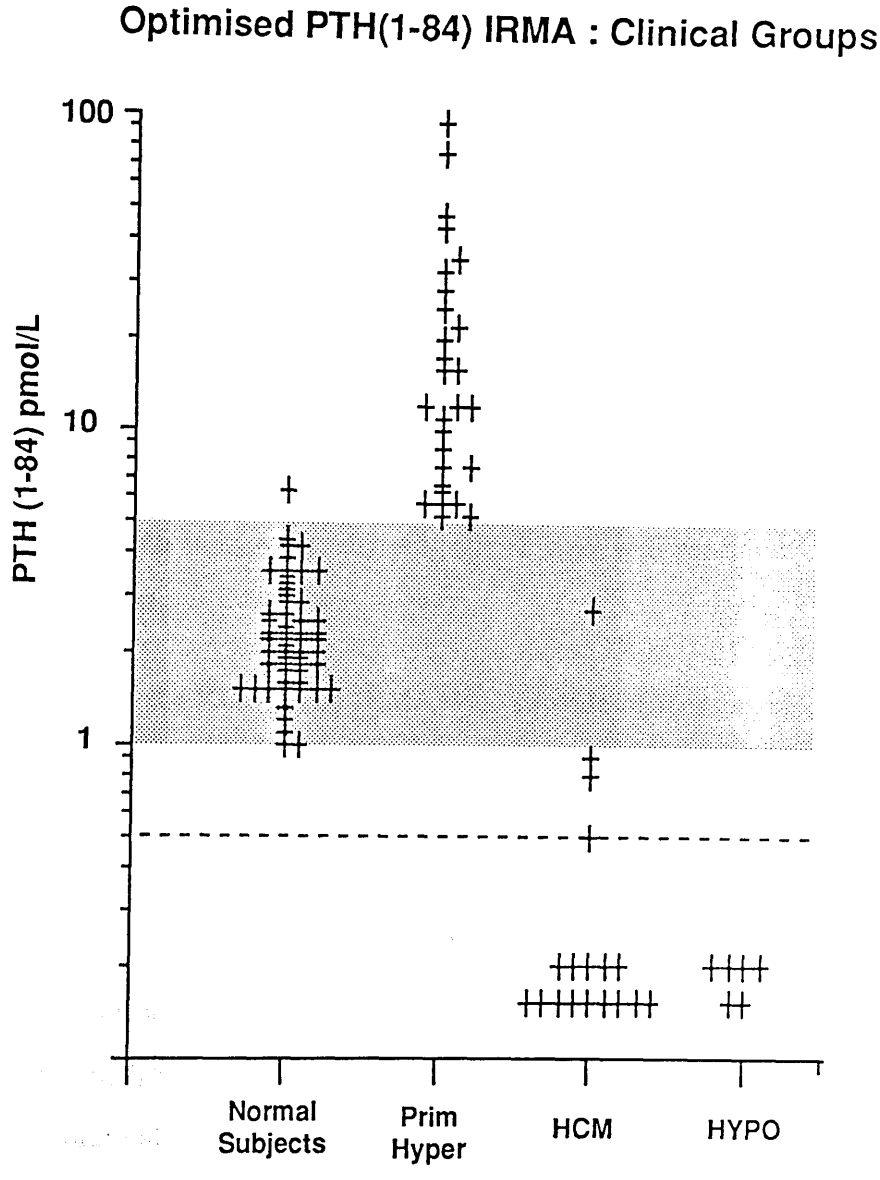


Figure 4.30

PTH (1-84) concentrations measured using the optimised IRMA in normal subjects, patients with primary hyperparathyroidism (prim hyper), hypercalcaemia of malignancy (HCM) or hypoparathyroidism (hypo). The shaded area represents the reference interval estimated from the normal subjects. Also represented as the dashed line is the minimum detection limit of the assay (cf Figure 4.15).

patients with some degree of renal failure. A third (2.7 pmol/l) was from a patient who was found to have an elevated urinary cAMP concentration. The fourth sample gave a result at the detection limit of the assay (0.5 pmol/l).

The PTH (1-84) concentrations in these groups are compared in Figure 4.30. The results from patients with hyperparathyroidism (5.8 - 100 pmol/l) were all above the reference interval (1.0 - 4.4 pmol/l) estimated from the normal subjects. Six of the patients with hyperparathyroidism however, had results in the range 5.2 - 7.0 pmol/l, close to the upper limit of the reference interval indicating there may be some overlap between PTH (1-84) concentrations in normal subjects and patients with hyperparathyroidism. The results in hyperparathyroid patients were well separated from those in patients with HCM.

These results confirmed that the PTH (1-84) IRMA was capable of measuring PTH (1-84) in normal subjects and gave good discrimination between hyperparathyroid patients, normal subjects and patients with HCM.

Oral Calcium Load

The samples from the study described in Section 3.3 were re-analysed in the optimised PTH (1-84) IRMA. The PTH (1-84) concentrations in the twenty normal volunteers pre and post oral calcium load (1 g) are shown in Figure 4.31. All subjects pre calcium load had detectable PTH (1-84) concentrations; mean 1.8 pmol/l (range 1.0 - 3.6 pmol/l). There was a reduction in PTH (1-84) concentrations in all subjects 2 h post oral calcium, mean 0.92 pmol/l which was significant on a paired difference t-test ($p < 0.0001$). The results confirm the ability of the PTH (1-84) IRMA to measure detectable PTH (1-84) concentrations in normal subjects and to detect changes in PTH (1-84) concentrations within the reference interval.

4.5 Recovery of PTH (1-84) added to Human Serum

Intra-assay recovery: Synthetic PTH (1-84) (10 pmol/l) was added to ten serum

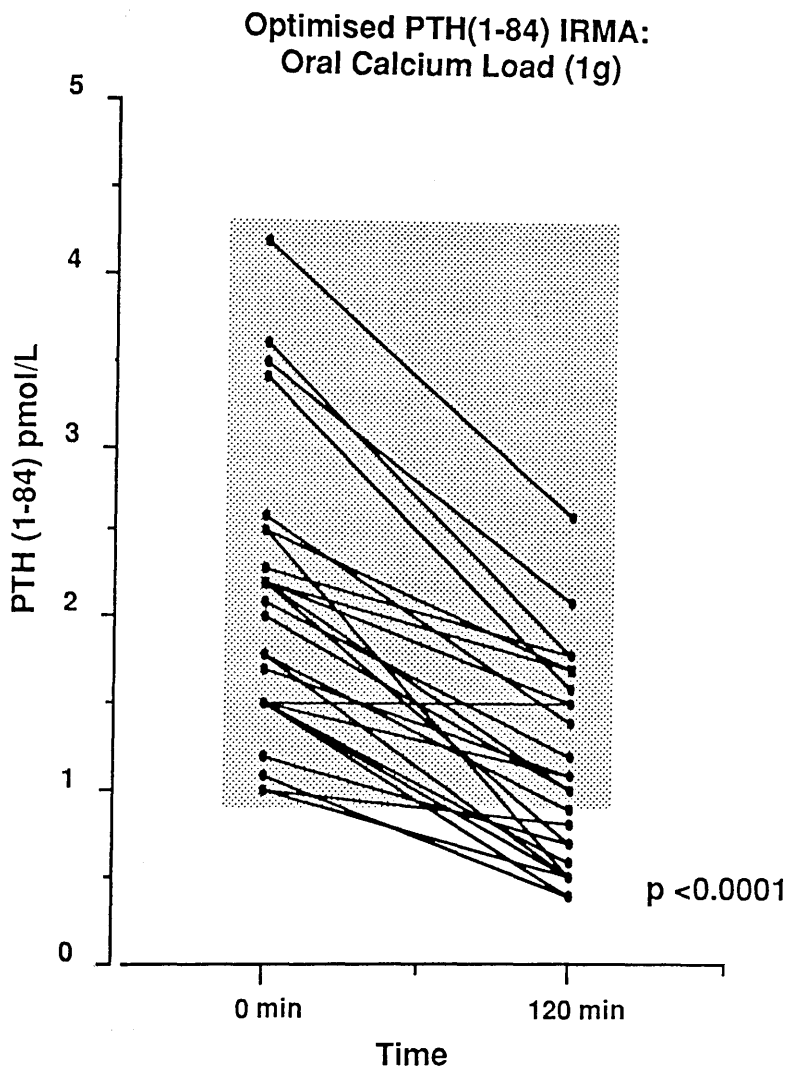


Figure 4.31

PTH (1-84) concentrations measured using the optimised IRMA in normal subjects pre (0 min) and post (120 min) an oral calcium load (1 g). (cf Figure 4.16).

samples (4 normal subjects, 3 hyperparathyroid patients and 3 patients with chronic renal failure). The results are shown in Table 4.6(i).

Inter-assay recovery: Pooled normal human serum was spiked with 5, 10 and 20 pmol/l synthetic PTH (1-84) and assayed in the PTH (1-84) IRMA (x5). The results are shown in Table 4.6(ii).

Recovery of human PTH (1-84) (IRP 79/500): The experiment in (ii) above was repeated using the IRP 79/500 human PTH as both the added recovery material and for the assay standard curve. The results are shown in Table 4.6(iii).

The recovery experiments indicated that the optimised PTH (1-84) IRMA gave good recovery of both synthetic and human PTH (1-84) from human serum. There is a slight over-recovery (107 - 110%) at 10 pmol/l added which appears to increase at 20 pmol/l added (116 - 120%). This level of recovery was not expected to compromise the clinical utility of the assay. The results however indicate that the equine serum adopted for the routine production of standards is perhaps not the ideal matrix for the standard curve.

4.6 Dilution of Patient Samples

Serum samples from a patient with primary hyperparathyroidism (PTH (1-84) 80 pmol/l) and three patients with elevated PTH (1-84) concentrations (50, 80, 100 pmol/l) and varying serum creatinine concentrations (690, 830 and 1300 µmol/l respectively) due to chronic renal failure were analysed at various dilutions. The responses were plotted against the theoretical PTH (1-84) value and compared to the synthetic PTH (1-84) standard response curve (Figure 4.32).

All samples diluted essentially parallel to the synthetic PTH (1-84) response curve with no evidence of interference from PTH fragments.

Table 4.6

Recovery of PTH (1-84) added to human serum

(i) Intra-assay recovery: synthetic PTH (1-84)

	Basal	**+ 10 pmol/l PTH (1-84)**
Mean PTH (1-84)	6.9 pmol/l	17.6 pmol/l
Range	1.5-15.0 pmol/l (n = 10)	12.0-25.0 pmol/l (n = 10)
Mean recovery 107% (98 - 110%)		

(ii) Inter-assay recovery: synthetic PTH (1-84)

	PTH (1-84) (SD) pmol/l	**Recovery % (range)**
Basal	2.04 (0.23)	-
+5	7.44 (0.57)	108 (88-124)
+10	12.72 (1.13)	107 (94-118)
+20	25.24 (2.43)	116 (112-124)

(iii) Recovery of human PTH (1-84) (IRP 79/500)

	PTH (1-84) (SD) pmol/l	**Recovery % (range)**
Basal	4.5 (0.46)	-
+5	10.0 (0.91)	110 (91-124)
+10	15.9 (1.49)	114 (86-127)
+20	28.4 (3.00)	120 (115-128)

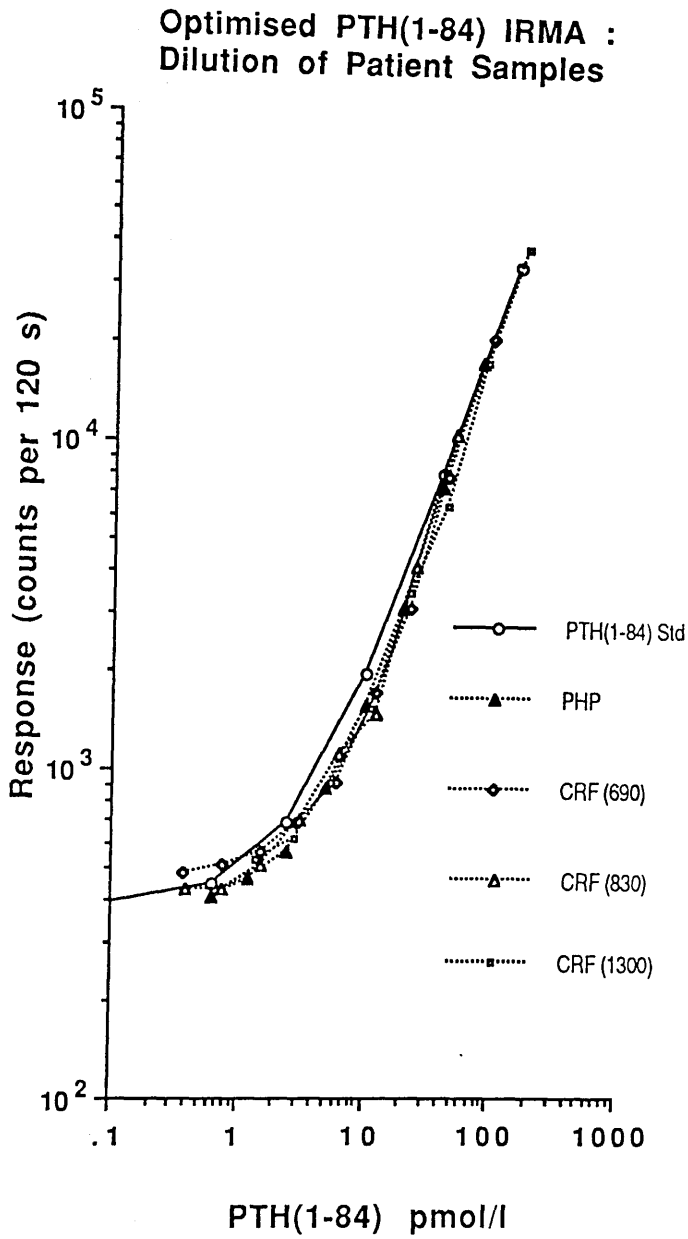


Figure 4.32

Measurement of endogenous PTH (1-84) in pathological samples. Response curve for synthetic PTH (1-84) standard compared with those for dilution of samples from patients with primary hyperparathyroidism (PHP) or with chronic renal failure with a serum creatinine of 600 µmol/l (CRF 600), 830 µmol/l (CRF 830) and 1300 µmol/l (CRF 1300).

5 STABILITY OF PTH (1-84) IN BLOOD SAMPLES

As a final assay validation prior to initiating studies on circulating PTH (1-84) concentrations the stability of PTH (1-84) in blood samples and the comparability of the results obtained with the PTH (1-84) assay developed in this study with those of published assays was investigated.

For the measurement of immunoreactive PTH it had been recommended that samples were obtained in the morning (0700-0900 h) after an overnight fast followed by rapid separation and storage at -20°C (Stitch 1980, Di Bella and Hawker 1982). With the development of assays for intact PTH (1-84) the stability of PTH (1-84) in blood samples has been re-examined both during assay validation (Brown et al 1987) and more extensively during method evaluation (Newman and Ashby 1988).

These studies reported a gradual loss (18-30%) of both synthetic and endogenous PTH (1-84) in whole blood or serum incubated overnight at room temperature and have recommended rapid separation and freezing of samples. Endogenous PTH (1-84) was found to be stable on storage at -20°C. The present study therefore aimed merely to confirm these data with the in-house assay and is therefore of limited scope rather than an extensive investigation of PTH (1-84) stability.

Blood from a normal subject and a patient with primary hyperparathyroidism was taken into plain, lithium heparin and lithium heparin with aprotinin (4000 KIU; 0.2ml Trasylol) containing tubes. Aliquots were separated within 30 minutes of venesection and incubated at either room temperature or 4°C for up to 24h.

The PTH (1-84) concentrations measured in the samples from the normal subject and the patient with primary hyperparathyroidism are shown in Figures 4.33 and 4.34 respectively. There was a drop in PTH (1-84) concentrations over the 24 h period in the samples from both the normal subject (mean 23.9%) and the patient with hyperparathyroidism (mean 39.2%). There was no significant difference observed in

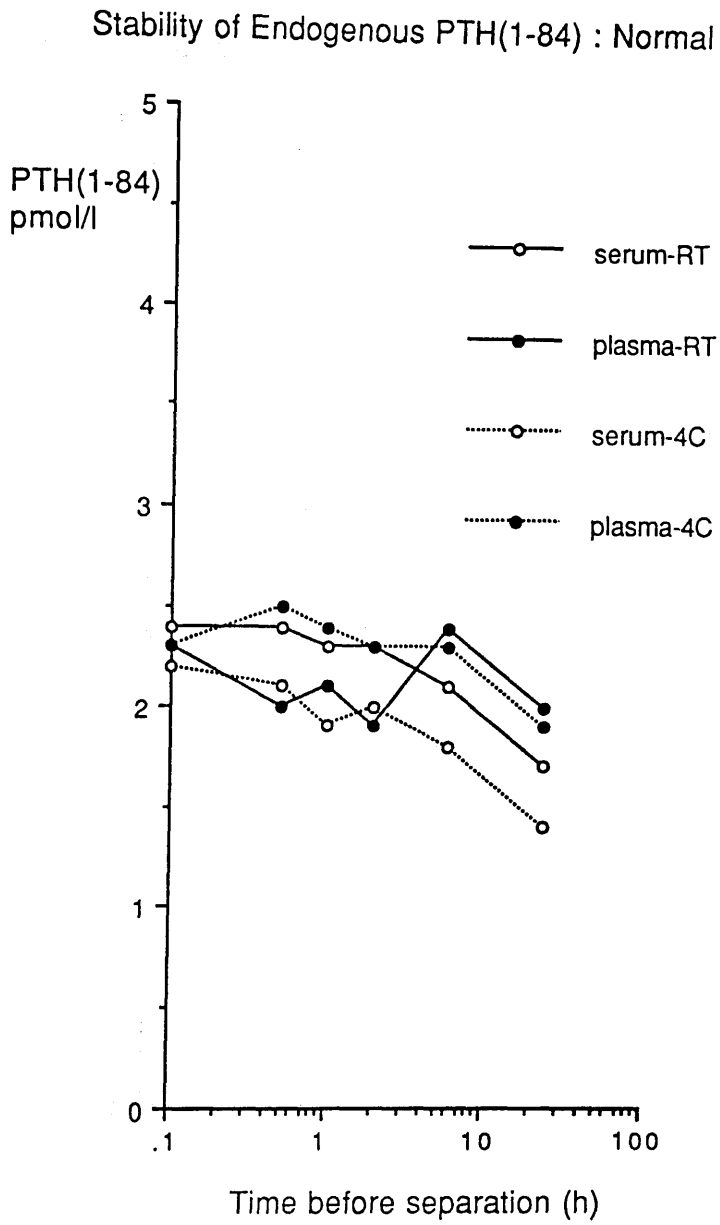


Figure 4.33

The stability of endogenous PTH (1-84) in samples from a normal subject. Aliquots of serum or plasma were incubated at either room temperature (RT) or 4°C (4C) then stored at -50°C prior to assay (within 4 days).

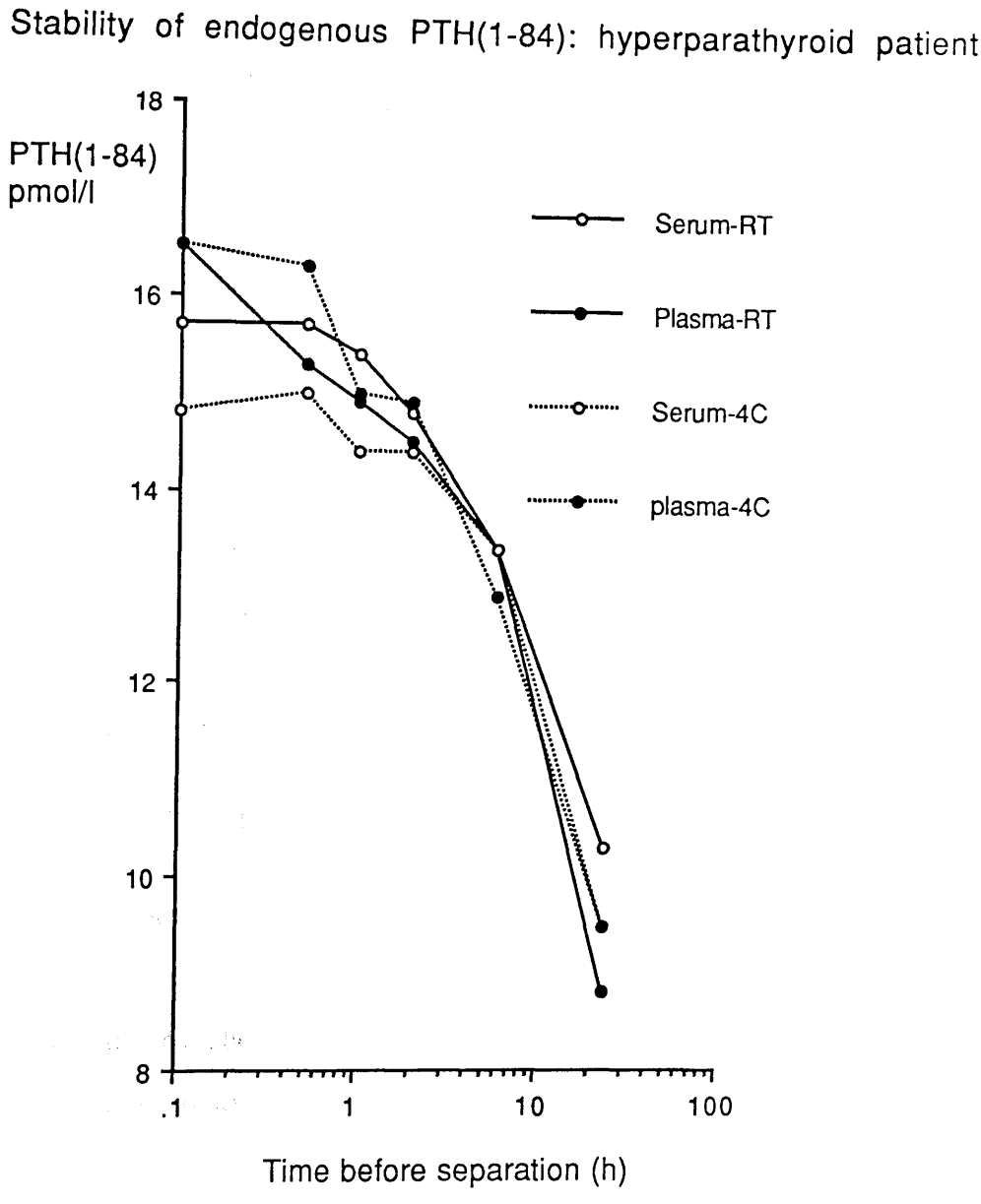


Figure 4.34

The stability of endogenous PTH (1-84) in samples from a patient with primary hyperparathyroidism. Aliquots of serum or plasma were incubated at either room temperature (RT) or 4°C (4C) then stored at -50°C prior to assay (within 4 days).

the PTH (1-84) concentrations between serum and plasma samples or with the temperature of incubation. The addition of aprotinin had no effect on the decrease in PTH (1-84) concentrations over the incubation period (data not shown).

The results confirm a gradual loss of endogenous PTH (1-84) concentrations on incubation at 4°C and room temperature. The stability of endogenous PTH (1-84) (from a patient with hyperparathyroidism) stored over a period of several months at -20°C was demonstrated during the preparation of quality control pools. While this study involves samples from a limited number of subjects the results are are in agreement with published data (Brown et al 1987, Newman and Ashby 1988). Thus whereas short delays in separations and freezing would not normally have major affects on the clinical value of PTH (1-84) concentrations there is the possibility that misclassification of patients with mild primary hyperparathyroidism may occur. Therefore for clinical and physiological studies samples were separated within 30 minutes of venepuncture and stored frozen at -20°C.

6 CORRELATION WITH PUBLISHED PTH (1-84) METHODS

At the time of initiating the studies on the circulating concentrations of PTH (1-84) two methods for the measurement of intact PTH (1-84) were available commercially. The assay validation studies (above) had shown the performance of the in-house assay developed in this study to be comparable to the published data from these assays in terms of sensitivity, precision and discrimination between clinical groups. It was of further interest to assess the correlation between the in-house PTH (1-84) assay and each of these methods on the same clinical samples. Thus the study was aimed to investigate briefly the degree of correlation between these methods and was not designed to be an extensive comparison of methods.

The PTH (1-84) concentrations measured in a range of clinical samples using the in-house assay were compared to those measured using the Allegro^{R} method (Nichols Institute) an immunoradiometric assay employing two affinity purified polyclonal

antisera (Nussbaum et al 1987) and the Magic Lite^R system (Ciba-Corning) an immunochemiluminometric assay employing an affinity purified polyclonal N-terminal antibody and a C-terminal Mab (Brown et al 1987).

The comparison of PTH (1-84) concentrations measured by the in-house method and the Allegro^R method is shown in Figure 4.35. There is good correlation between the methods (r^2=0.95) the results using the Allegro method were on average 10% higher. The correlation between the the in-house and Magic Lite^R methods was also good (r^2=0.975) with the Magic Lite method results on average 20% lower than those from the in-house method (Figure 4.36).

Although on a limited number of samples the results indicate a good correlation between the methods across a range of clinical samples (including primary hyperparathyroidism and renal failure). The differences in the actual PTH (1-84) concentrations observed in the patient samples were also apparent in the standards for these methods and reflected differences in primary calibration between the methods.

7 CONCLUSIONS

The sensitivity of the N-terminal Mab to 3B3 to the oxidation state of PTH initially caused difficulty in the assay development. It is however a characteristic previously described for polyclonal antisera to PTH (Tashijian et al, 1964) and is probably not unusual in antisera raised to N-terminal PTH peptides. The fact that this effect is not commonly encountered is probably because assay systems employing polyclonal antisera - i.e. raised to multiple epitopes on PTH - would not be expected to show the same sensitivity to the oxidation state of PTH as a system using a Mab raised to a single epitope. It is not possible from the data generated from the present study to assess the effect of oxidation on the production of antibodies to PTH. It is likely that it plays a role both in the production of an immune response and also in the ability of antibody-screening assays to detect the response. The oxidation of samples prior to assay to allow quantitation of total oxidised peptide has been described in an assay

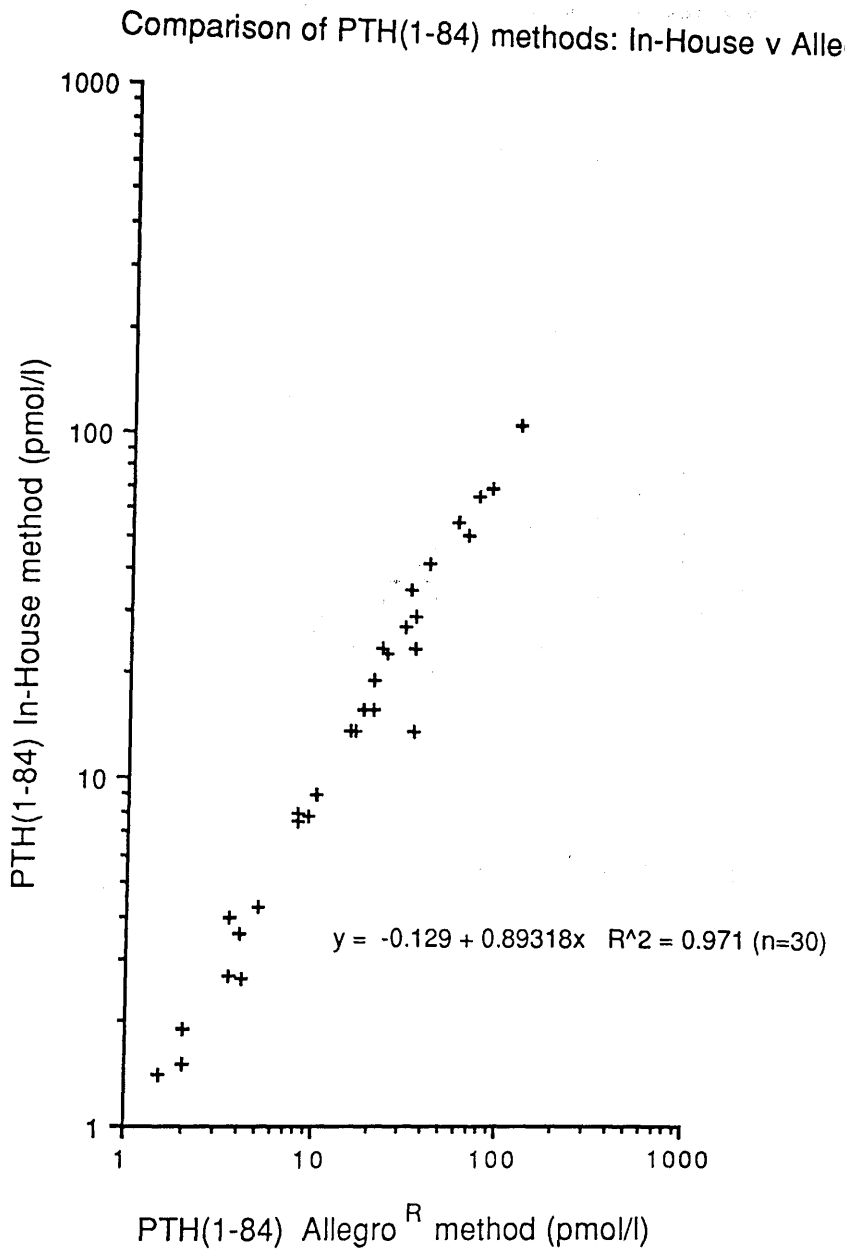


Figure 4.35

The correlation between the PTH (1-84) concentrations measured using the in-house assay with those obtained using the Allegro^{R} method in a range of clinical samples.

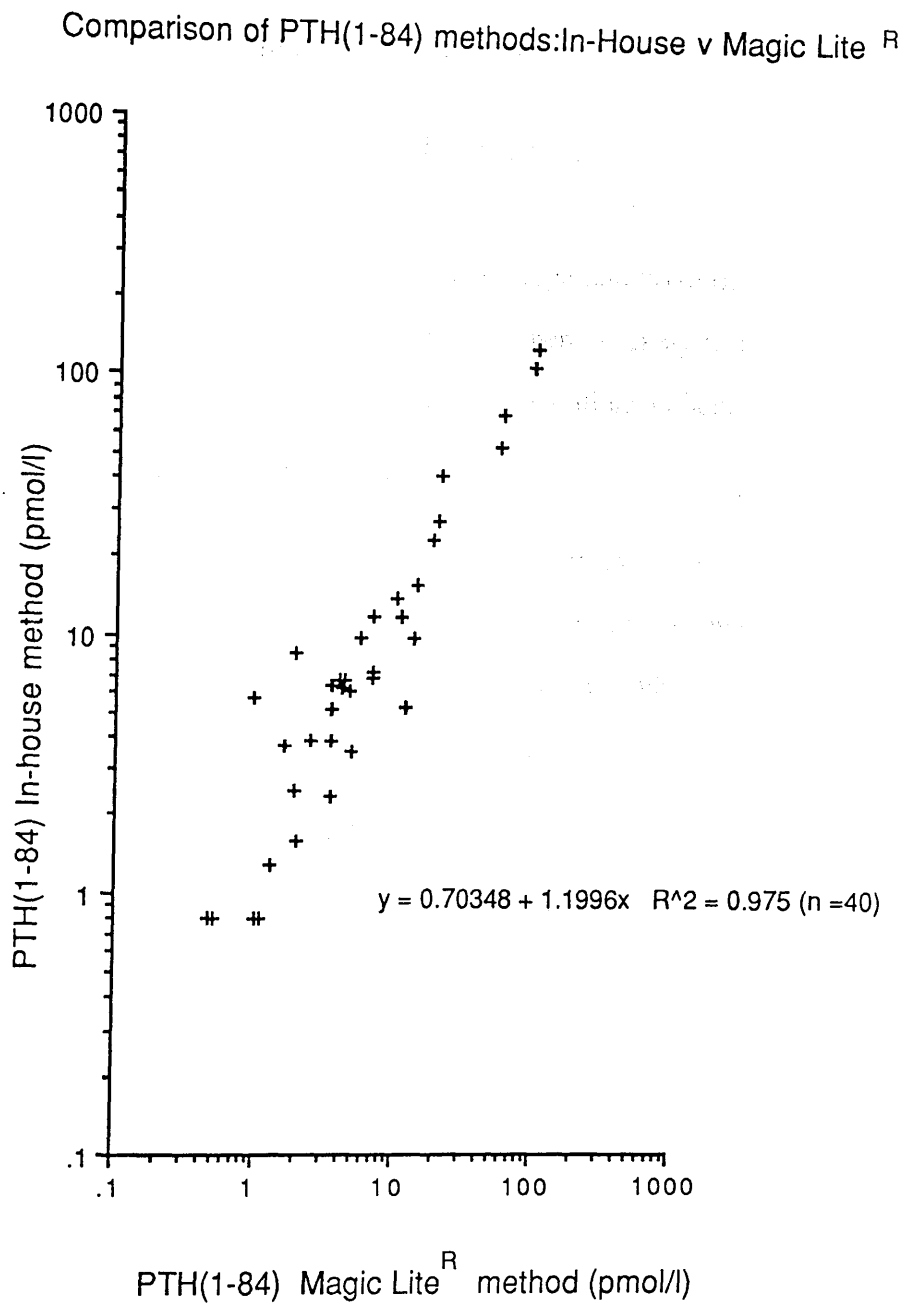


Figure 4.36

The correlation between the PTH (1-84) concentrations measured using the in-house assay with those obtained using the Magic Lite^{R} method in a range of clinical samples.

for the measurement of methionine enkephalin (Clement-Jones et al, 1980). The validation studies have shown the assay system developed to give quantitative recovery of both oxidised and reduced PTH (1-84).

The assay has also been shown to be sufficiently sensitive to measure circulating concentrations of PTH (1-84) in normal subjects. The results of the assay allow good discrimination between patients with primary hyperparathyroidism and those with hypercalcaemia associated with malignancy. There is likely to be, however, some degree of overlap between the PTH (1-84) concentrations in both these groups with those found in normal subjects.

The assay is specific for PTH (1-84) in that it is unaffected by an excess of N or C-terminal fragments of PTH. In particular the assay has sufficient capacity to cope with the high levels of C-terminal fragments found in chronic renal failure.

Thus the performance of the PTH (1-84) IRMA developed compares favourably with other published assays (Brown et al, 1986, Nussbaum et al, 1987). In addition, the PTH (1-84) concentrations measured using the in-house assay show good correlation with those obtained using these methods.

CHAPTER 5

STUDIES ON THE CIRCULATING CONCENTRATIONS OF PTH (1-84)

CHAPTER 5 : STUDIES ON THE CIRCULATING CONCENTRATIONS OF PTH (1-84)

1 INTRODUCTION

The development of two-site immunometric assays for PTH (1-84) has been an important advance in the laboratory assessment of patients with disorders of calcium metabolism and particularly with regard to the differential diagnosis of hypercalcaemia. The specificity for PTH (1-84) and the improved sensitivity and precision has allowed greater discrimination between clinical groups than could be achieved with previous immunoassay methodology (Nussbaum et al, 1987, Brown et al, 1987, Logue et al, 1990a).

Similarly studies on the circulating concentrations of PTH in normal and pathological states have been limited by PTH immunoassay methodology. N-terminal assays, while measuring biologically active PTH, in general lacked the sensitivity to measure circulating PTH concentrations in normal subjects (Segre et al, 1975, Armitage 1986). C-terminal immunoassays, measuring biologically inactive PTH fragments of long half life, were not useful in following short term fluctuations in biologically active PTH and were markedly influenced by renal function (Di Bella et al, 1978b, Armitage 1986).

Thus the ability to measure PTH (1-84), free of interference from PTH fragments, in normal subjects and to follow changes within the reference interval with confidence offers the potential to study the physiology and control of PTH (1-84) secretion more clearly than previously possible.

2 STUDIES ON THE CIRCADIAN SECRETION OF PTH (1-84)

2.1 Introduction

The majority of biochemical, physiological and behavioural phenomena of the human

organism show profound diurnal variation ie regular changes from day to night (Turek 1985, Van Cauter and Aschoff 1989). In nature these diurnal rhythms are normally synchronised with 24 h environmental rhythms such as the light-dark cycle. However, even under constant environmental conditions, most diurnal rhythms persist. This finding has led to the demonstration that these diurnal fluctuations are endogenous rhythms and are driven by an internal biological clock (Pittendrigh 1981). These rhythms are referred to as 'circadian' rhythms, from the Latin circa diem, meaning about a day, because their period under constant conditions is close to but rarely exactly 24 h. In mammals, the paired suprachiasmatic nuclei (SCN) of the hypothalamus have been demonstrated to control the timing of a large variety, if not all, circadian rhythms. So far no other circadian 'clock' has been anatomically identified in mammals (Turek 1985).

The secretion of many hormones has been shown to be strongly modulated by circadian rhythmicity eg melatonin, cortisol, testosterone and the anterior pituitary hormones adrenocorticotrophic hormone (ACTH), growth hormone, thyroid stimulating hormone (TSH) and prolactin (Desir et al, 1981, Van Cauter and Honinckx 1985, Weeke and Gunderson 1978, Linkowski et al, 1987).

A series of studies were carried out to investigate the characteristics and control of the circadian rhythm of PTH (1-84) secretion. Prior to reporting the results a description of the general design of the studies and the methods employed is given in Sections 2.2 and 2.3.

2.2 General Study Design

Subjects were studied over a 24 h period. Venous blood samples were obtained through indwelling cannulae at 30 minute intervals. Timed urine collections were taken every 4 h during the day, one sample prior to retiring to bed and one sample on awakening in the morning.

Within an experiment all subjects ate identical diet on the day of sampling, with meals being consumed at fixed times. The meals content for the normal subjects was intended to represent approximately their usual diet. All patients were hospitalised during the study and ate hospital diet at identical times.

Throughout the periods of study subjects and patients were ambulent but avoided exercise. Within an experiment the subjects and patients lay down to sleep at the same time. Ethical committee approval was obtained for the study, and all patients gave informed consent before inclusion in the study.

2.3 Methods

Assays

Intact PTH (1-84) was measured using the in-house IRMA (Logue et al, 1990a). Plasma cAMP was measured using a commercial kit (Amersham International). Urinary cAMP was measured using an in-house RIA method (O'Reilly et al, 1986). Nephrogenous cAMP was calculated by the method of Broadus et al, (1977). Serum total calcium, phosphate, albumin and creatinine were measured on a Hitachi 704 using standard methodologies, and calcium was adjusted for albumin concentration (Gardner et al, 1981).

Prolactin, HGH, TSH and LH were measured using immunometric assays:-

Prolactin : IRMA (NETRIA, St Bartholomews, London) with a detection limit of 30 mU/l and a working range of 60-3500 mU/l.
HGH: in-house IRMA with a detection limit of 0.1 mU/l and a working range of 0.5-200 mU/l.

TSH: in-house IRMA with a detection limit 0.2 mU/l and working range of 0.5-150 mU/l.

LH: DELFIA (Pharmacia) with a detection limit of 0.12mU/l and working range of 0.4 to 250 mU/l.

All samples from an individual subject were analysed in the same assay batch.

Statistical analysis of data

Statistical analysis was performed using a paired difference t-test to analyse significant changes within 24 h profiles. The Mann-Whitney U-test was used to analyse differences between groups.

Cosinor analysis: Statistical analysis of circadian rhythm parameters was carried out using the Cosinor technique (Nelson et al, 1979). In this procedure a cosine curve with a period of 24 h is fitted to the data for each individual using the method of least squares. An F statistic was then used to test the zero amplitude hypothesis for a circadian rhythm. The following parameters were calculated: the mesor (rhythm adjusted mean), the amplitude (half the total extent of the predicted change) and the acrophase (crest time of the best fit cosine function, in relation to local midnight). The mean cosinor data were then plotted on a polar coordinate graph. The mean amplitude and acrophase of the rhythm were displayed by means of the length and angle of the vector. The envelope representing the bivariate statistical confidence region was also derived.

Cross-Correlation Analysis: the PTH (1-84) and prolactin data for each individual over the 24 h period were smoothed using a 3 sample moving average technique to minimise perturbations due to either pulsatile secretion or assay noise. Cross - correlation coefficients, the correlation between the PTH (1-84) data of an individual versus successive lags of their prolactin data (1lag = sample interval = 30min) were estimated using the Statsgraphics Package (Statistical Graphics Corporation Rockville MD 20852).

2.4 The circadian rhythm of intact PTH (1-84) and in nephrogenous cAMP in normal subjects and patients with primary hyperparathyroidism

Introduction

Previous studies on the secretion of PTH over a 24 h period in normal subjects using assays mainly specific for the biologically inactive C-terminal region of PTH have reported nocturnal rises in immunoreactive PTH concentrations (Jubiz et al, 1972, Sinha et al, 1975) which may also be sleep related (Kripke et al, 1978).

The biological action of PTH is mediated by cyclic adenosine monophosphate (cAMP). The measurement of the nephrogenous component of urinary cAMP (NcAMP) has been shown to be a good index of PTH bioactivity in vivo (Broadus et al, 1977). Studies on cAMP metabolism have reported both the presence (Murad and Pak 1972, Sagel et al, 1973) and the absence (Shaw et al, 1977) of circadian variation in total urinary cAMP excretion. There have been no reports of circadian variation in NcAMP.

Patients with primary hyperparathyroidism have inappropriately detectable or elevated serum concentrations of PTH (1-84) in the presence of hypercalcaemia (Nussbaum et al, 1987, Blind et al, 1988, Chu and Chu 1988) and elevated NcAMP in the majority of cases (Broadus et al, 1977, 1978). Some of the available evidence indicates that in primary hyperparathyroidism the adenomatous parathyroid tissue functions autonomously whilst other evidence suggests that the gland is not totally autonomous and an alteration in the set-point for PTH secretion exists (Parfitt 1969a, Brown 1983, Gardin et al, 1988).

Thus we might expect that the circadian rhythm of PTH (1-84) and NcAMP observed in normal subjects could be lost in primary hyperparathyroidism. Studies measuring N and C terminal PTH have shown either the existence (Sinha et al, 1975, Lo

Cascio et al, 1982) or absence (Riggs et al, 1971, Jubiz et al, 1972) of a circadian rhythm of PTH in primary hyperparathyroidism.

In this study the circadian secretion of PTH (1-84), NcAMP, serum calcium and phosphate in normal adult male and premenopausal female volunteers is investigated and compared to the results obtained in patients with primary hyperparathyroidism.

Subjects

Adult males:

Six healthy male volunteers (aged 29-40; mean 33 years) were studied over the same 24 h period. The study period was from 1400 to 1330 h the following day, with food being consumed at 1800, 2300, 0800 and 1300 h. The male subjects lay down at 0100, were asleep by 0200 and were awakened at 0700 h.

Premenopausal females:

Three premenopausal female healthy volunteers (aged 27-45 yrs) were studied for a 24 h period from 1900-1900 h (due to the timing of the study period the female subjects ate their evening meal 2-3 h later than the male subjects). The female subjects lay down at midnight were asleep by 0100 and were awakened at 0800 h.

Hyperparathyroid patients:

Six patients (three male, three female) with primary hyperparathyroidism (surgically proven with histological diagnosis age 47-58 years). All patients were hospitalised during the study, they ate hospital diet at similar times to the normal subjects. The patients lay down at 1100 were asleep by 1200 and were awakened at 0700 h.

Results

The mean 24 h profiles of PTH (1-84) and NcAMP in the six male subjects are shown in Figure 5.1. PTH (1-84) rose from 0130 h in a broad peak through the night. The concentrations from 0200 to 0600 h were significantly ($P<0.05$) elevated compared to baseline (1400-1800 h). The PTH (1-84) concentrations in all subjects

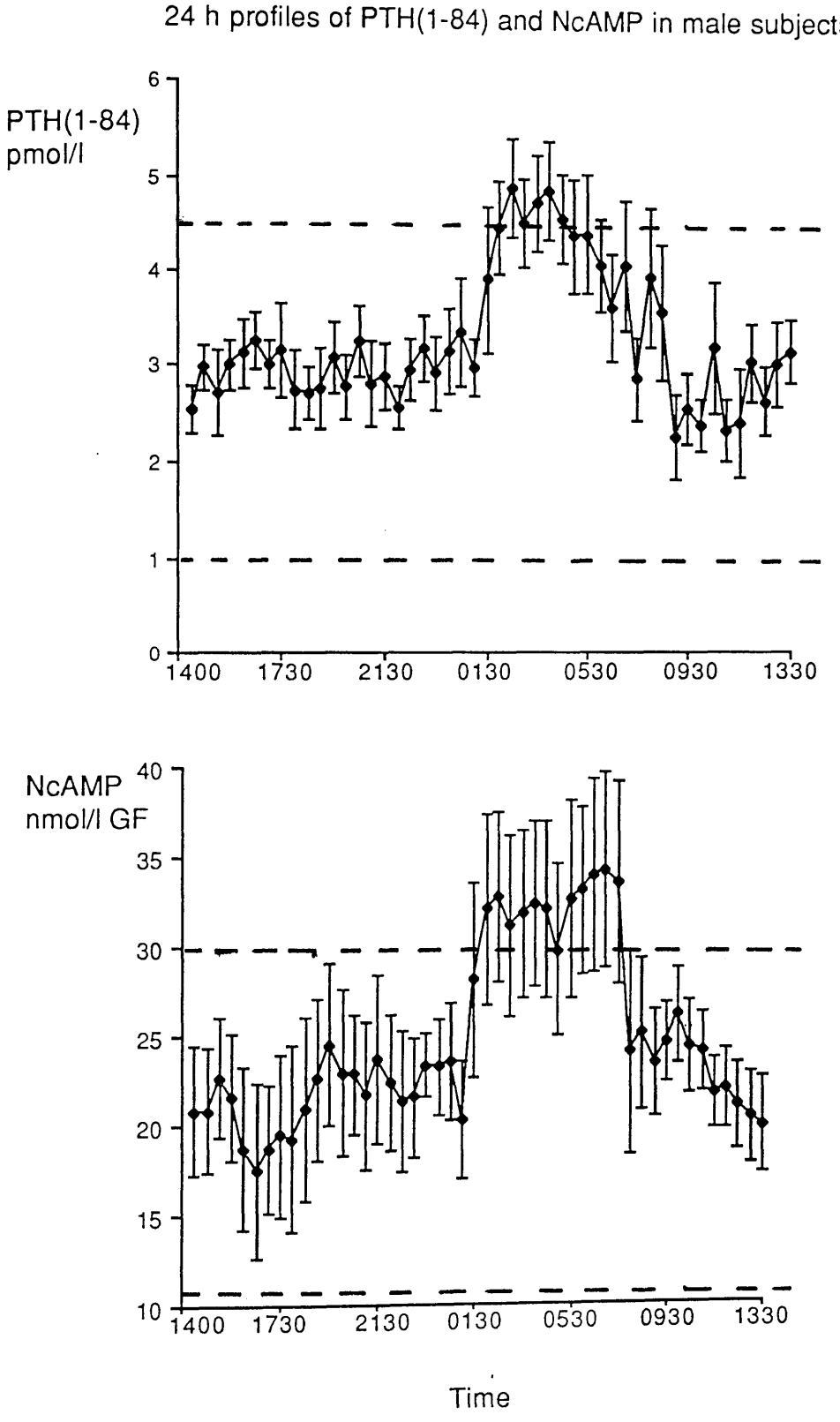


Figure 5.1

The mean (± SEM) concentrations of PTH(1-84) and NcAMP in six normal male subjects sampled at 30 minute intervals over a 24 h period. The dotted lines indicate the reference intervals for normal male subjects

returned to baseline values by 1000 h although the rate of fall varied markedly between individuals. The mean intra-individual CV between 0630 and 1000 h was 30% (range 12.9-42.5) compared to 17% (12.6-23.6) in the basal period.

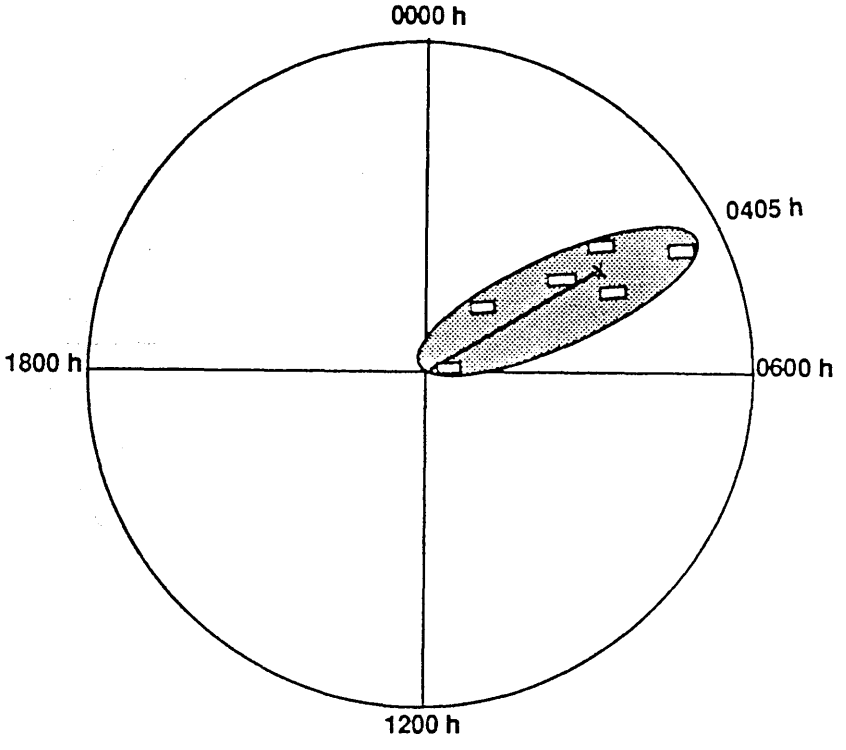
The mean cosinor analysis for PTH (1-84) in the male subjects is shown in Figure 5.2. The polar co-ordinate plot indicates the tight phase and frequency synchronisation in the PTH (1-84) of the six subjects over the 24 h period. The results confirm a significant circadian rhythm in PTH (1-84) of mean amplitude 0.72 from a mesor concentration of 3.3 pmol/l with the acrophase occurring at 0405 h.

The mean NcAMP concentrations in the male subjects rose throughout the night in parallel to the PTH (1-84). The NcAMP concentrations at eight of the eleven time points during the period 1200 to 0700 h were significantly ($P<0.05$) elevated compared to the baseline individual means. The mean cosinor data for NcAMP in the six male subjects is shown in Figure 5.3. Although there is phase synchrony in the NcAMP concentrations of the six subjects occurring at 0524 h, the inter-individual variability is such that the mean amplitude of 5.9 from the mesor concentration of 25 nmol/l glomerular filtrate is not statistically significant at the 5% level.

The 24 h profiles of PTH (1-84) and NcAMP concentrations in the female subjects are shown compared to the mean profile for male subjects in Figure 5.4. The PTH (1-84) concentrations are higher throughout the 24 h period than the corresponding profile in male subjects (mean 24 h PTH (1-84) in female subjects was 5.0 vs 3.3 pmol/l in the males $P<0.001$). Similarly the NcAMP concentrations are higher in the female subjects than in the males (24 h mean NcAMP 28.6 vs 24.8 nmol/l GF $p<0.01$). It is apparent that the difference in PTH and NcAMP concentrations between the males and females is most evident in the late afternoon and early evening.

Cosinor analysis of the data from the female subjects confirms a significant circadian rhythm in PTH (1-84) of mean amplitude 0.69 pmol/l from a mesor concentration of

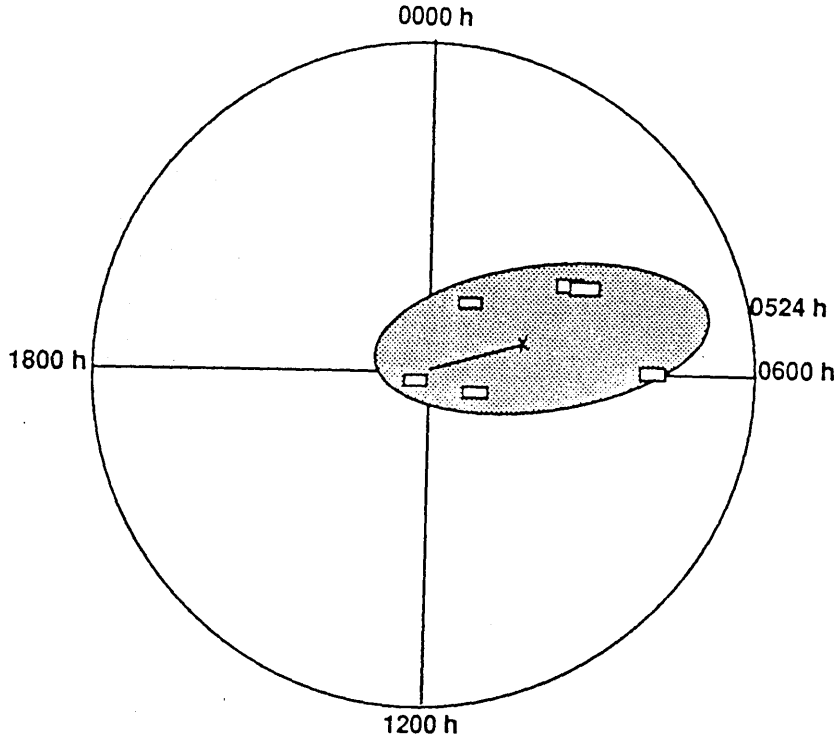
Cosinor analysis of the PTH (1-84) concentrations in normal male subjects over a 24 h period



Acrophase : 0405 h
Mesor : 3.3 pmol/l
Amplitude : 0.72 pmol/l ($p < 0.05$)

Figure 5.2: Mean cosinor analysis of intact parathyroid hormone concentrations for the six normal subjects during the 24 h period. Polar co-ordinate plot.
The mean amplitude of the circadian rhythm is plotted, as distance from the origin, against the angle from 0000 h corresponding to the time of mean acrophase (⊣). The shaded area represents the statistical confidence (95%) limits of the estimated mean parameters. The amplitude and acrophase for the individual subjects are also indicated (▭).

Cosinor analysis of the NcAMP concentrations in normal male subjects over a 24 h period



Acrophase : 0524 h
Mesor : 25.0 nmol/l GF
Amplitude : 5.9 nmol/l GF

Figure 5.3: Mean cosinor analysis of nephrogenous cyclic adenosine monophosphate concentrations for the six normal subjects over the 24 h period. Polar co-ordinate plot. The shaded area represents the statistical confidence limits. (GF = glomerular filtrate)

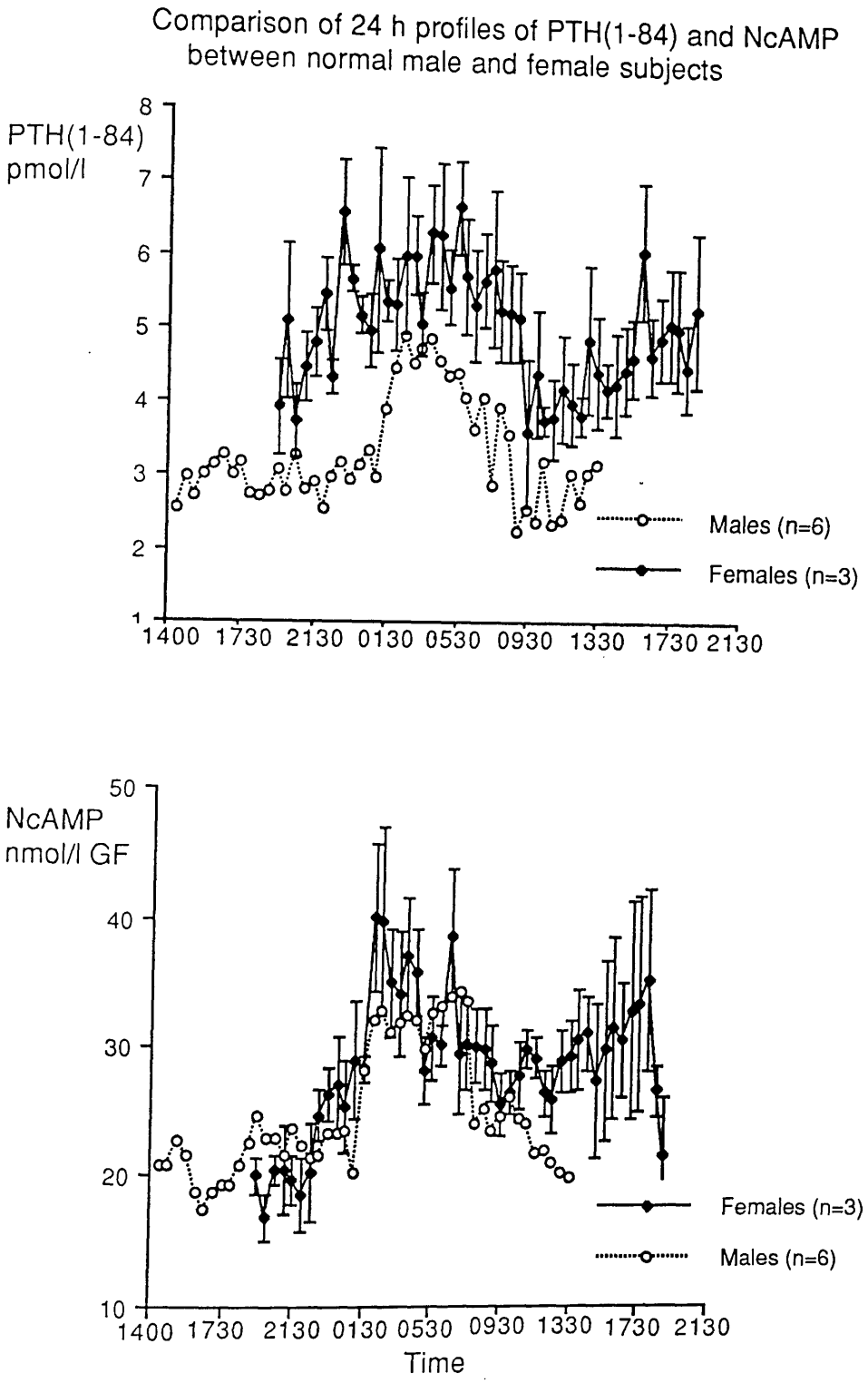


Figure 5.4

The mean (± SEM) concentrations of PTH (1-84) and NcAMP in three normal premenopausal females sampled at 30 minutes over a 24 h period are shown compared to the mean profiles obtained for the normal male subjects. Female subjects were studied from 1900-1830 h, the male subjects from 1400-1300 h.

5.0 pmol/l with the acrophase occurring at 0302 h ($p<0.05$). The mean amplitude of 3.55 from a mesor NcAMP concentration of 28.6 nmol/l GF occurring at an acrophase of 0736 h was not statistically significant at the 5% level.

PTH (1-84) and NcAMP values showed a wide scatter in the patients with hyperparathyroidism therefore for clarity of presentation the results were expressed as percentage of the individual's 24 h mean. The 24 h profiles for PTH (1-84), NcAMP (both expressed as percentage of the individual 24 h mean), adjusted serum calcium and serum phosphate for normal subjects and patients with primary hyperparathyroidism are shown in Figures 5.5 and 5.6 respectively. The measured PTH (1-84) and NcAMP concentrations throughout the 24 h period are summarised and compared to those found normal male subjects in Table 5.1. The results of the cosinor analysis of the data for the four measurements in both of these groups are shown in Table 5.2.

The circadian rhythm observed for PTH (1-84) in the normal subjects with a significant rise in PTH (1-84) overnight, the acrophase occurring at 0405 h, was lost completely in the patients with primary hyperparathyroidism with no synchronised rise in PTH (1-84) observed throughout the 24 h period. In normal subjects the greatest variability in serum PTH (1-84) concentrations occurs between 0630-1000 h. In patients with primary hyperparathyroidism PTH (1-84) concentrations occasionally fell within the reference range and in one patient these results coincided with normal values for adjusted calcium.

Similarly the significant rise in NcAMP observed in in the normal subjects was abolished in patients with primary hyperparathyroidism. In two patients values for NcAMP within the reference range were obtained intermittently throughout the 24 h period.

The three male patients with primary hyperparathyroidism had the highest concentrations of PTH (1-84) and NcAMP (Table 5.1). As these patients also had the

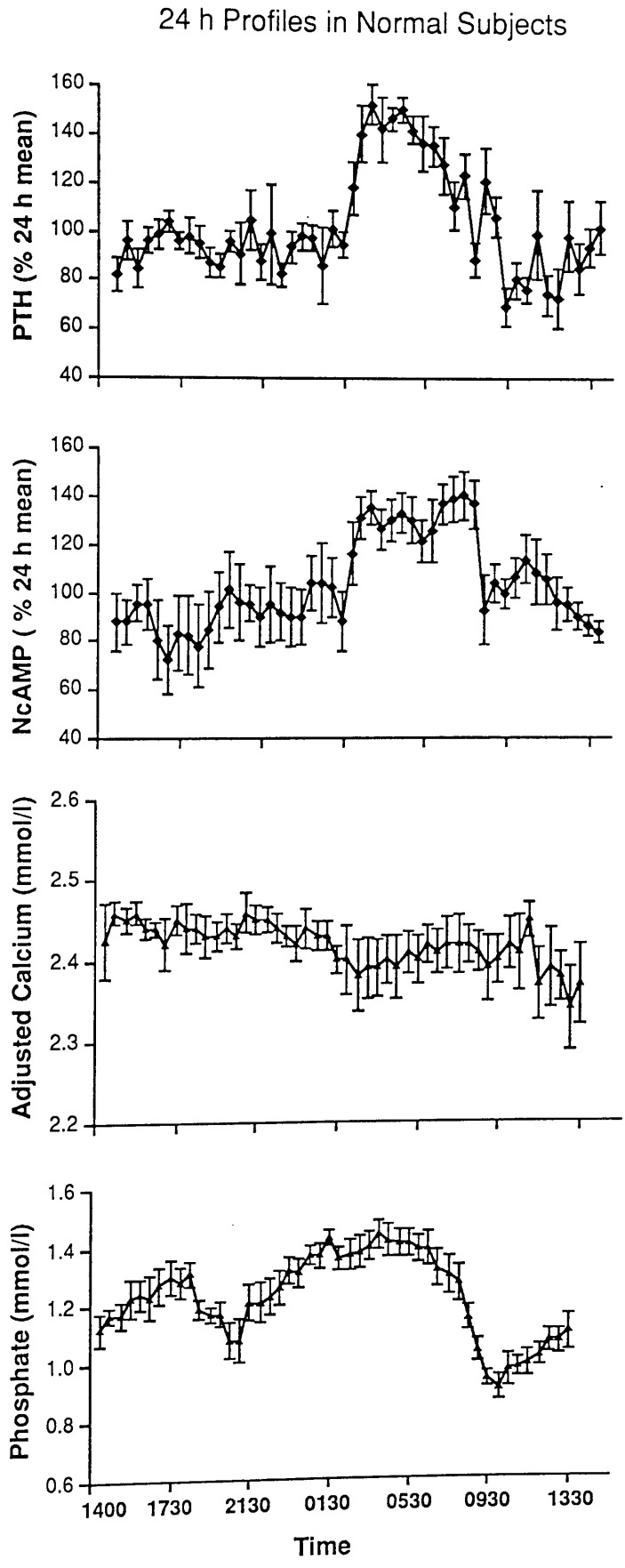


Figure 5.5

The mean (± SEM) 24 h profiles (expressed as % of the individuals 24 h mean) of PTH (1-84), NcAMP, adjusted calcium and phosphate in the six normal subjects.

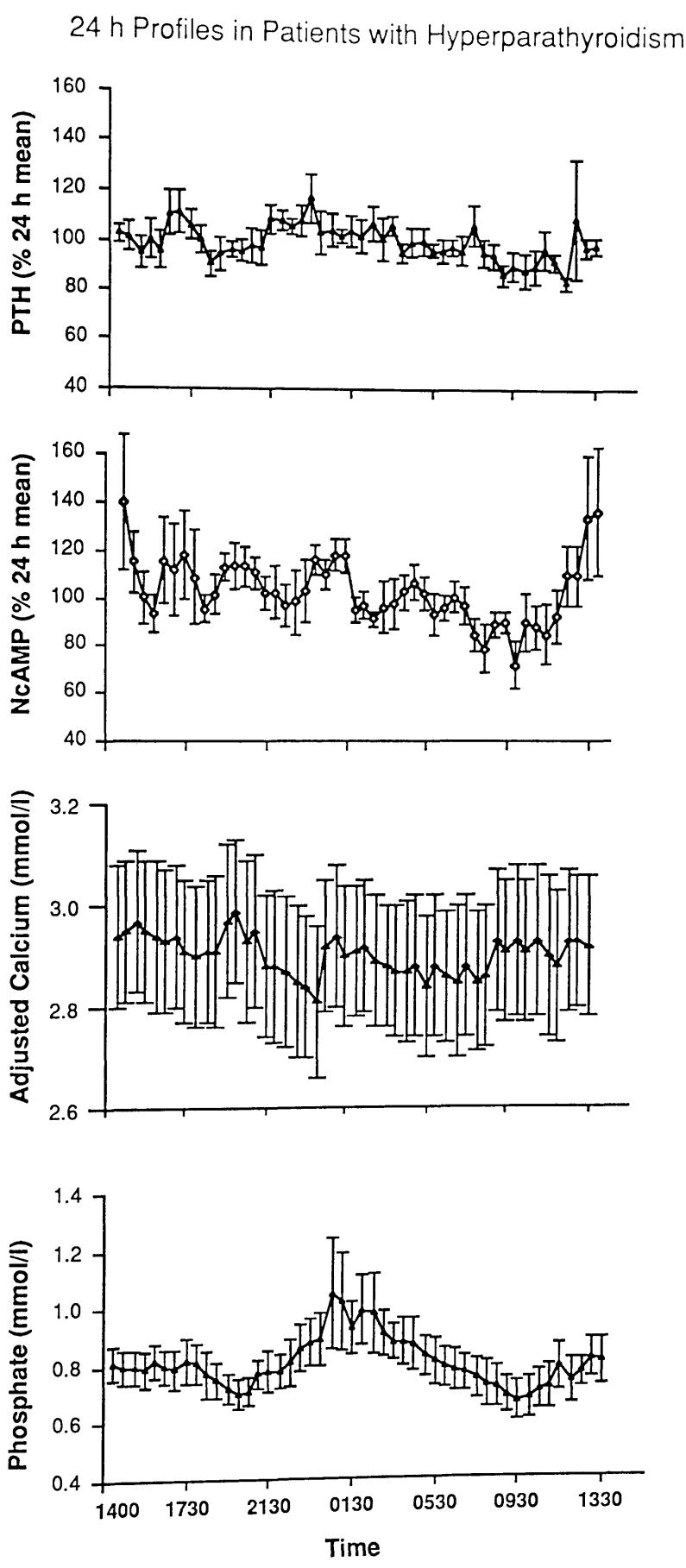


Figure 5.6

The mean (± SEM) 24 h profiles (expressed as % of the individuals 24 h mean) of PTH (1-84), NcAMP, adjusted calcium and phosphate in the six patients with primary hyperparathyroidism.

Table 5.1

PTH (1-84) and N-cAMP concentrations in normal subjects over the 24 h period

		PTH (1-84) (pmol/l)			N-cAMP (nmol/l GF)		
Normal Subjects:	Sex	24 h Mean	Range	CV	24 h Mean	Range	CV
1	M	2.3	1.1-4.5	36.7	23.0	4.9-26.9	37.5
2	M	3.9	1.6-6.5	25.1	19.4	6.7-32.3	32.0
3	M	4.6	2.1-7.6	32.2	33.4	14.2-56.7	38.8
4	M	3.2	1.4-5.6	35.2	27.0	10.6-40.6	31.4
5	M	2.6	0.9-4.2	30.6	13.9	4.5-22.7	33.4
6	M	3.2	2.0-4.2	14.8	31.5	20.6-39.7	13.3
Hyperparathyroid Subjects:							
TC	M	13.9	11.2-17.2	11.0	46.6	20.7-72.9	25.3
PT	F	7.3	4.7-11.9	20.4	37.7	13.8-74.4	43.8
EC	M	20.8	12.6-25.8	10.9	104.0	81.9-126.0	10.3
SB	F	5.5	4.0-12.0	22.7	54.7	30.3-75.6	23.6
TD	M	24.6	16.0-33.0	14.4	73.9	42.6-101.4	17.6
EMcC	F	13.1	9.0-20.0	19.1	38.8	18.2-99.0	44.6

Table 5.2

Cosinor analysis of 24 h profiles

	Acrophase	Mesor	Amplitude
Normal Subjects			
PTH(1-84)	0405	3.3	0.72 ($p < 0.05$)
N-cAMP	0524	25.0	5.9 ns
Adjusted calcium	2003	2.4	0.02 ns
PO_4	0155	1.2	0.14 ($p < 0.001$)
Hyperparathyroid Patients			
PTH(1-84)	2339	14.2	0.80 ns
N-cAMP	1804	58.0	5.6 ns
Adjusted calcium	1613	2.9	0.03 ns
PO_4	0116	0.8	0.07 ns

highest concentrations of adjusted calcium this probably reflects the severity of the condition rather than a fundamental difference in PTH (1-84) secretion between men and women.

Adjusted calcium concentrations varied little during the 24 h period in both groups. In the normal subjects a transient statistically significant ($p<0.05$) decrease (compared to the individual means between 1400-1800 h) in mean adjusted calcium from 2.44 to 2.40 mmol/l was observed at 0130 h. There was also a small but significant drop in adjusted calcium from 2.42 to 2.38 mmol/l ($p<0.05$) observed at 1230 h (relative to mean levels between 0600 and 0800 h). In the hyperparathyroid patients a decrease from 2.94 to 2.84 mmol/l ($p<0.05$) at midnight (relative to the individual mean between 1400-1800 h) was observed. Predictably the mean adjusted calcium concentration in the patient group was higher than that in the normal subjects throughout the 24 h period ($p<0.001$).

Serum phosphate was significantly lower in the primary hyperparathyroid patient group throughout the 24 h ($p<0.001$). A marked synchronised rhythm in phosphate consisting of a transient decrease following the evening meal a significant nocturnal rise and then an early morning fall was observed in both groups. The percentage change in phosphate was greater in normal subjects than in the patients with primary hyperparathyroidism.

Discussion

In normal male subjects both serum PTH (1-84) and NcAMP concentrations rise significantly during the night, with mean values above the day-time reference ranges for these analytes. The cosinor analysis confirms a significant circadian rhythm in PTH (1-84), with an acrophase at 0405 h. This is consistent with the night-time rise in immunoreactive PTH previously reported using C-terminal specific assays (Jubiz et al, 1972, Sinha et al, 1975) and also with the hypothesis that the rise may be sleep related (Kripke et al, 1978). The circadian variations in PTH (1-84) are

accompanied by parallel variations in NcAMP levels. This strongly supports the conclusion that the PTH (1-84) released through the night is physiologically active.

The circadian rhythm of PTH (1-84) seen in male subjects is also present in premenopausal females. The pattern of secretion however shows some notable differences between the male and female subjects. The PTH (1-84) concentrations are set higher throughout the 24 h period in the females and in particular during the early evening. This is reflected in the cosinor analysis which confirms a higher mesor concentration in the females but similar amplitude of rhythm to the male subjects. In contrast to our data no sex based difference in PTH (1-84) 24 hour profiles was noted in a previous study (Calvo et al, 1988). A more recent study has however shown that a low calcium and high phosphate diet causes a persistent elevation in PTH (1-84) secretion throughout a 24 h period (Calvo et al, 1990). The increase in the early evening PTH (1-84) concentrations relative to the nocturnal peak seen in our female subjects is more consistent with studies in both males and females carried out in USA which showed biphasic profiles (Markowitz et al, 1988, Calvo et al, 1988). It has been postulated from these and our own studies that there are dietary influences on the secretion of PTH (1-84) particularly in the early evening.

The protocol adopted for these studies involved the subjects following a standard regime of eating times and approximate meal content within each experiment. The choice of food was intended however to reflect the usual diet of the subjects. Thus it is probable that the differences between the pattern of secretion of PTH (1-84) in female and male subjects seen in this study reflect differences in diet and food intake between the groups. The data on the female group is however based on only three subjects and further studies which take account of dietary influences are required to define the parameters of the circadian secretion of PTH (1-84) in female subjects.

Although the NcAMP concentrations rose significantly in both male and female subjects, the cosinor analysis failed to detect a significant phase and frequency

synchronised rhythm. This is probably related to the small number of subjects and the inherent variability in this parameter which is calculated from four separate measurements (cAMP and creatinine both in plasma and urine). It is of interest however that the NcAMP concentrations remained elevated (0600-0700 h) after the main peak of PTH (1-84).

The circadian rhythm of PTH (1-84) is not present in patients with primary hyperparathyroidism. This loss of PTH (1-84) rhythm is reflected in the absence of the circadian rhythm for NcAMP in primary hyperparathyroidism.

These observations raise important questions for the control of PTH (1-84) secretion in normal and abnormal states. Previous studies have demonstrated a fall in ionised calcium during the night in both normal subjects and patients with hyperparathyroidism (Lo Cascio et al, 1982). In this study we have also shown a transient but significant lowering of adjusted calcium in both groups between midnight and 0200 h. The aetiology of such a transient fall in calcium is unclear.

A reduction in ionised calcium might be expected to trigger a compensatory surge in PTH (1-84) secretion and therefore provide a mechanism for the nocturnal rise in PTH (1-84) in normal subjects. However this explanation appears to be an oversimplification for it is known that continuous infusion of calcium into normal subjects at a dose just sufficient to obliterate the nocturnal fall in serum calcium does not eliminate the nocturnal rise in PTH. Predictably infusion of calcium at higher doses leads to decreased nocturnal PTH secretion (Jubiz et al, 1972, Sinha et al, 1975). Clearly therefore factors other than changes in ionised calcium concentration are important in the circadian rhythm of PTH (1-84) in normal subjects.

Patients with primary hyperparathyroidism have been shown to respond to the lowering of serum calcium with increased PTH secretion (Murray et al, 1972, Lockefeer et al, 1974). It has been further suggested that the basis of primary hyperparathyroidism is an alteration in the calcium-PTH set point rather than

autonomous secretion of PTH by the parathyroid gland (Brown 1983, Parfitt 1969a, Gardin et al, 1988). In this study we have shown the absence of a synchronised nocturnal increase in PTH (1-84) in the hyperparathyroid patients despite the detection of a transient lowering in adjusted calcium consistent with previous data on ionised calcium (Lo Cascio et al, 1982). Although the precise incremental fall in serum calcium required to produce a response in hyperparathyroid patients has not been defined this is further evidence against the fall in calcium being the main trigger to circadian changes in PTH (1-84).

If changes in calcium concentration do not fully explain the circadian rhythm of PTH (1-84) then the influence of phosphate must also be considered. This study demonstrates a pronounced circadian rhythm of phosphate in normal subjects. A small decrease in phosphate which may be due to the movement of phosphate intracellularly to support carbohydrate metabolism occurs following the evening meal. This is followed by a much larger increase in phosphate prior to the nocturnal surge of PTH (1-84). This increase could be regarded as the stimulus for PTH (1-84) release, which in turn would cause phosphaturia and the observed fall in serum phosphate. However it has been reported that the phosphate circadian rhythm can be abolished by prolonged fasting but that the nocturnal rise in PTH persists in these individuals (Jubiz et al, 1972). Furthermore, suppression of PTH secretion by infusion of an appropriate dose of calcium results in an exaggerated phosphate circadian rhythm (Jubiz et al, 1972, Sinha et al, 1975). Thus it would appear that phosphate has little or no effect on the secretion of PTH during the night but that the PTH status of the individual may well influence the extent of the phosphate circadian rhythm. Further support for this latter conclusion is obtained from the phosphate results in the hyperparathyroid patients in this study for the phosphate rhythm is attenuated in the presence of consistently elevated PTH (1-84) concentrations.

An important incidental observation from this study is that the discrimination between normal subjects and patients with primary hyperparathyroidism is time dependent.

The elevated night-time PTH (1-84) concentrations had returned to basal values in all subjects by 1000 h. However the the fall to baseline concentrations varied between individuals, which gave rise to increased intra-individual variation between 0630 and 1000 h. These results have implications for the assessment of the PTH (1-84) concentrations in an individual. Early morning samples, such as those typically collected from hospital in-patients, may give spuriously elevated concentrations. Thus using PTH (1-84) as the discriminant the optimal time of sampling is 1000-1600 h when the variability within and between subjects is at its minimum. Similarly the optimal time for sampling using NcAMP as discriminant is between 1100 and 1500 h and not first thing in the morning as recommended by previous authors (Broadus et al, 1977, Holmegaard 1982).

Combining the available data leads to the conclusion that changes in calcium and phosphate either alone or in combination can not fully explain the circadian rhythm of PTH (1-84) secretion in normal subjects. It remains to be established whether the rhythm is modified by the age or sex of the subject, but is tempting to speculate that neuroendocrine factors from the hypothalamus or other higher centres may exert influence on PTH (1-84) secretion as well as on the the hormones of the anterior pituitary. Further work is required to establish whether the loss of circadian rhythm of PTH (1-84) in primary hyperparathyroidism is an early event in the natural history of the disease or whether it is a secondary consequence of increases in the plasma ionised calcium beyond critical threshhold concentrations.

These findings may be of physiological significance for it has been shown in animal models that continuous infusions of PTH are not effective in promoting bone growth; increased bone formation requires a regimen resulting in transient rises in PTH by daily injections (Podbesek et al, 1983, Malluche et al, 1982). Thus the night time rise in PTH (1-84) may be essential to normal bone physiology. Further studies on the control of the circadian secretion of PTH (1-84) are required, particularly in female subjects, to evaluate the possible role of neuroendocrine factors in the

pathogenesis of bone disorders such as osteoporosis.

2.5 The circadian secretion of PTH (1-84): Temporal correlation with the secretion of prolactin, HGH, TSH and LH.

Introduction

Studies on PTH (1-84) secretion have demonstrated the circadian rhythm in intact PTH (1-84) and its association with parallel changes in nephrogenous cyclic adenosine monophosphate (NcAMP), a measure of the biological activity of PTH, in normal subjects (Calvo et al, 1988, Markowitz et al, 1988, Logue et al, 1989). It has further been shown that the synchronised circadian rhythm in both PTH (1-84) and NcAMP is absent in patients with primary hyperparathyroidism (Logue et al, 1990b). The mechanism responsible for the PTH (1-84) rhythm remains unclear and it has been concluded from these and previous studies (Kripke et al, 1978) that the PTH (1-84) rhythm cannot be accounted for by changes in serum calcium or phosphate concentrations and therefore that the central nervous system may play a role either directly or indirectly in modulating normal PTH (1-84) secretion.

The secretion of many hormones has been shown to be strongly modulated by circadian rhythmicity eg melatonin, cortisol, testosterone and the anterior pituitary hormones adrenocorticotrophic hormone (ACTH), growth hormone (HGH), thyroid stimulating hormone (TSH) and prolactin (Desir et al, 1981, Van Cauter and Honinckx 1985, Weeke and Gunderson 1978). It was interest therefore to investigate whether there was any commonality between the secretion of PTH (1-84) and other circadian hormones over a 24 h period. Prolactin because of its noted association with calcium metabolism was of particular interest.

In the light of evidence that exogenous PTH can stimulate prolactin in normal subjects (Issac et al, 1978) and that prolactin has been reported to stimulate PTH release *in vitro* (Maggliola et al, 1981) a possible inter-relationship between these

two hormones has been postulated. Previous studies employing basal serum samples have failed to confirm a relationship between PTH and prolactin (Raymond et al, 1982, Fiore et al, 1984).

In view of the evidence of a relationship between PTH (1-84) and prolactin and the well defined circadian rhythm of prolactin secretion it was decided to investigate if there was any commonality of secretion between these hormones over a 24 h period in normal subjects and hyperparathyroid patients. The secretion of other anterior pituitary hormones known to be elevated during the night HGH, TSH and LH was also investigated.

Subjects and Methods

The samples from the normal subjects and patients with hyperparathyroidism obtained in the study on the circadian secretion of PTH (1-84) and NcAMP (Section 2.4) were assayed for prolactin. The samples from the normal male subjects were also assayed for HGH, TSH and LH.

The assay methods are as described in Section 2.3. The data from individuals was analysed using cross correlation analysis as described in section 2.3.

Results

The 24 h profiles for serum PTH (1-84) and prolactin in the six individual male subjects are shown in Figures 5.7 and 5.8. On visual examination of the profiles there is a strikingly similar pattern in PTH (1-84) and prolactin concentrations throughout the 24 h period. Two main periods of increased secretion in the six male subjects occur at 1600-1900 h and 0200-0600 h for PTH (1-84) and 2000-2200 h and 0400-0800 h for prolactin. Although there is variation in the relative height and duration of both peaks between subjects they are closely associated for both PTH (1-84) and prolactin within each individual with increases in PTH (1-84) occurring before those in prolactin in every case.

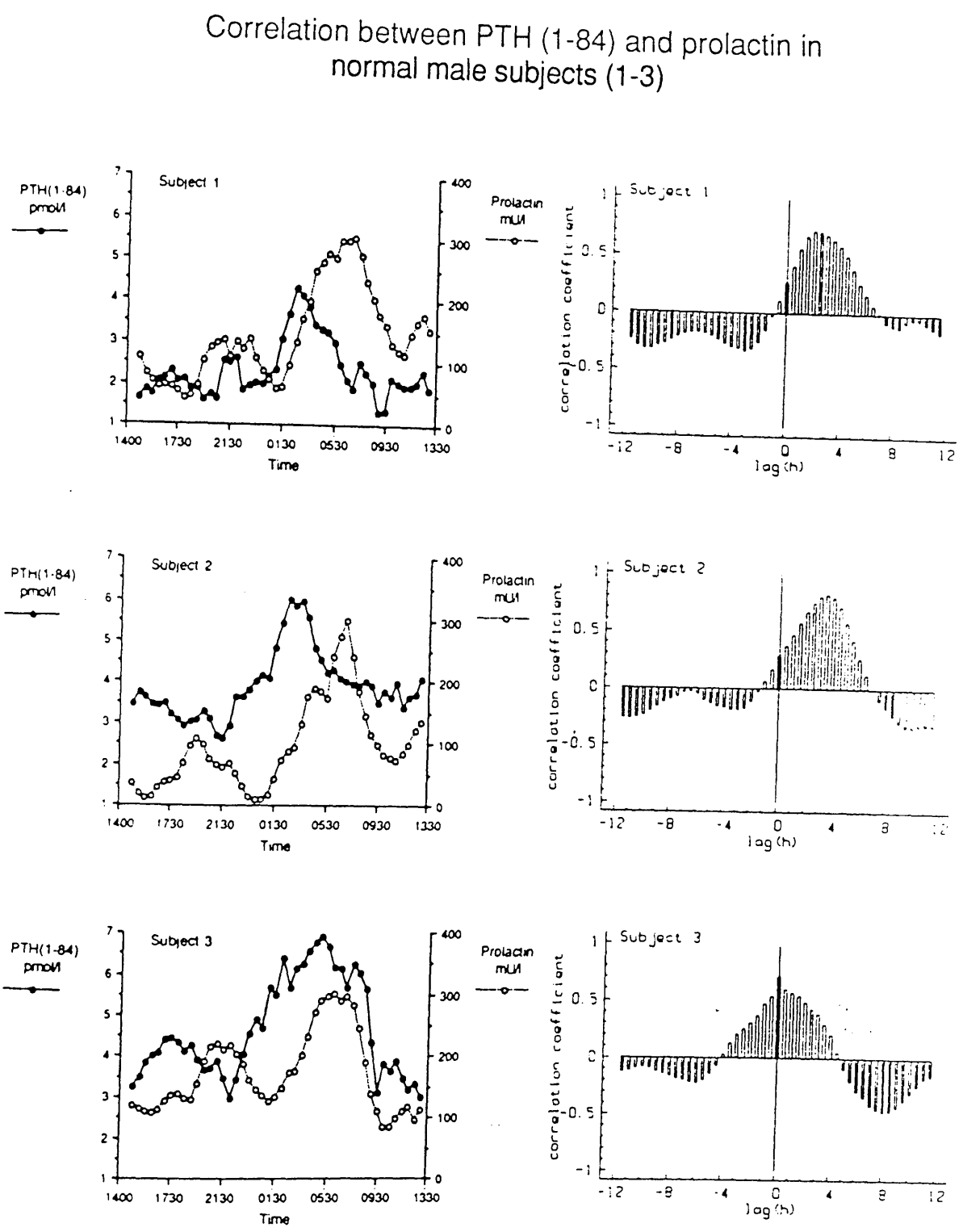


Figure 5.7

24 h profiles for PTH (1-84) and prolactin (smoothed using a three sample moving average) in normal male subjects. Associated with each profile is a plot of the cross-correlation coefficients for the PTH (1-84) data vs successive lags of the prolactin data (1 lag = sampling interval = 30 min).

Correlation between PTH (1-84) and prolactin in normal male subjects (4-6)

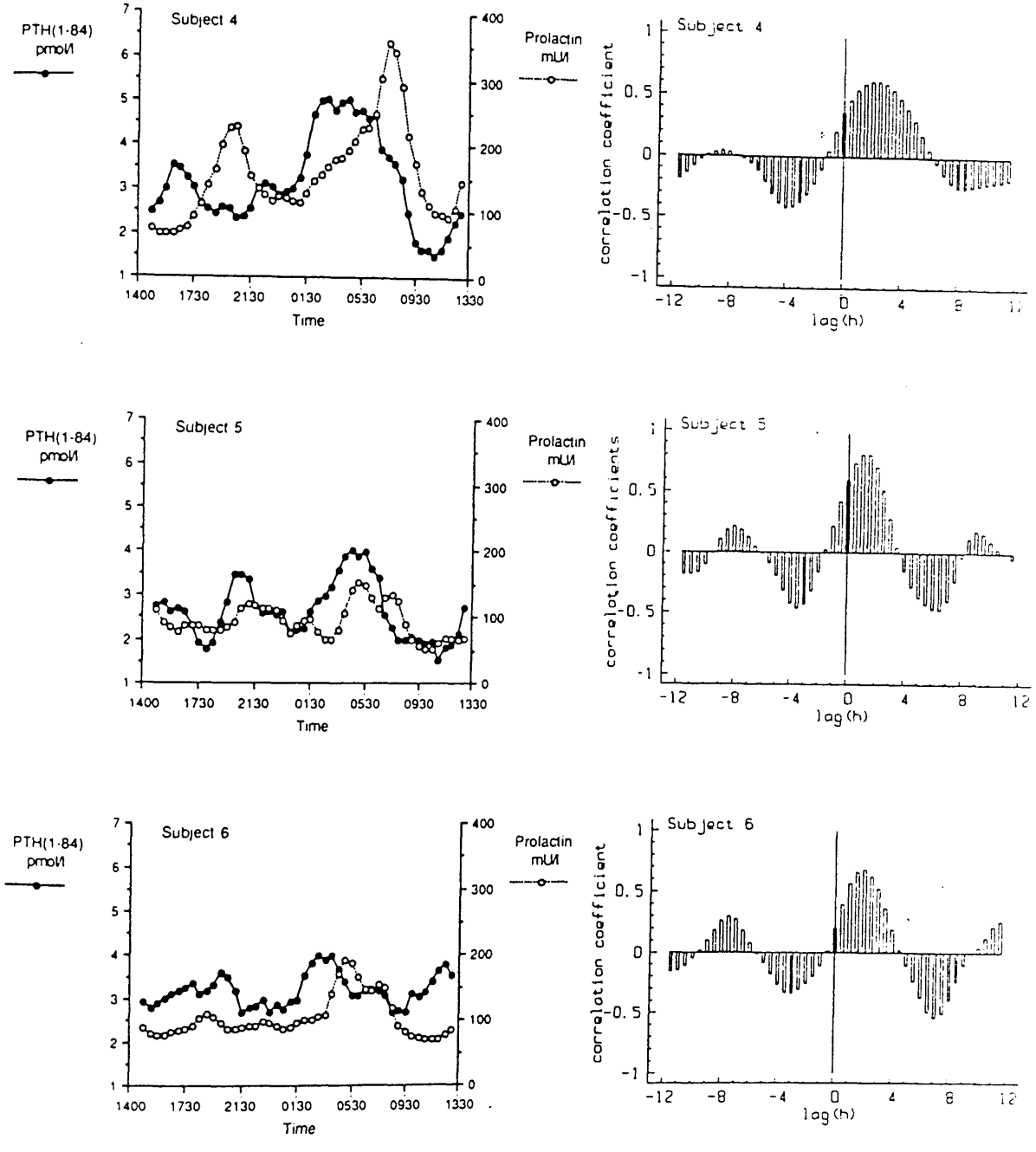


Figure 5.8

24 h profiles for PTH (1-84) and prolactin (smoothed using a three sample moving average) in normal male subjects. Associated with each profile is a plot of the cross-correlation coefficients for the PTH (1-84) data vs successive lags of the prolactin data (1 lag = sampling interval = 30 min).

Table 5.3

Cross-correlation analysis of 24 h profiles of PTH (1-84), prolactin and adjusted calcium in normal men.

	PTH v Prolactin		PTH v Adjusted calcium	
	Maximum r	Lag time (h)	Maximum r	Lag time (h)
Subject				
1	0.73	-2.0	-0.43	4.0
2	0.83	-3.5	-0.58	0.0
3	0.66	-0.5	-0.66	9.5
4	0.62	-2.0	-0.36	2.5
5	0.81	-1.0	-0.46	0.5
6	0.68	-2.0	-0.49	10.5

The data from each individual were smoothed using a method of three-sample running means. A series of correlation coefficients were calculated between the PTH(1-84) data of an individual versus successive lags [1 lag = 30 min] of their prolactin and adjusted calcium data. The maximal correlation obtained and the corresponding lag time are shown for individual subjects.

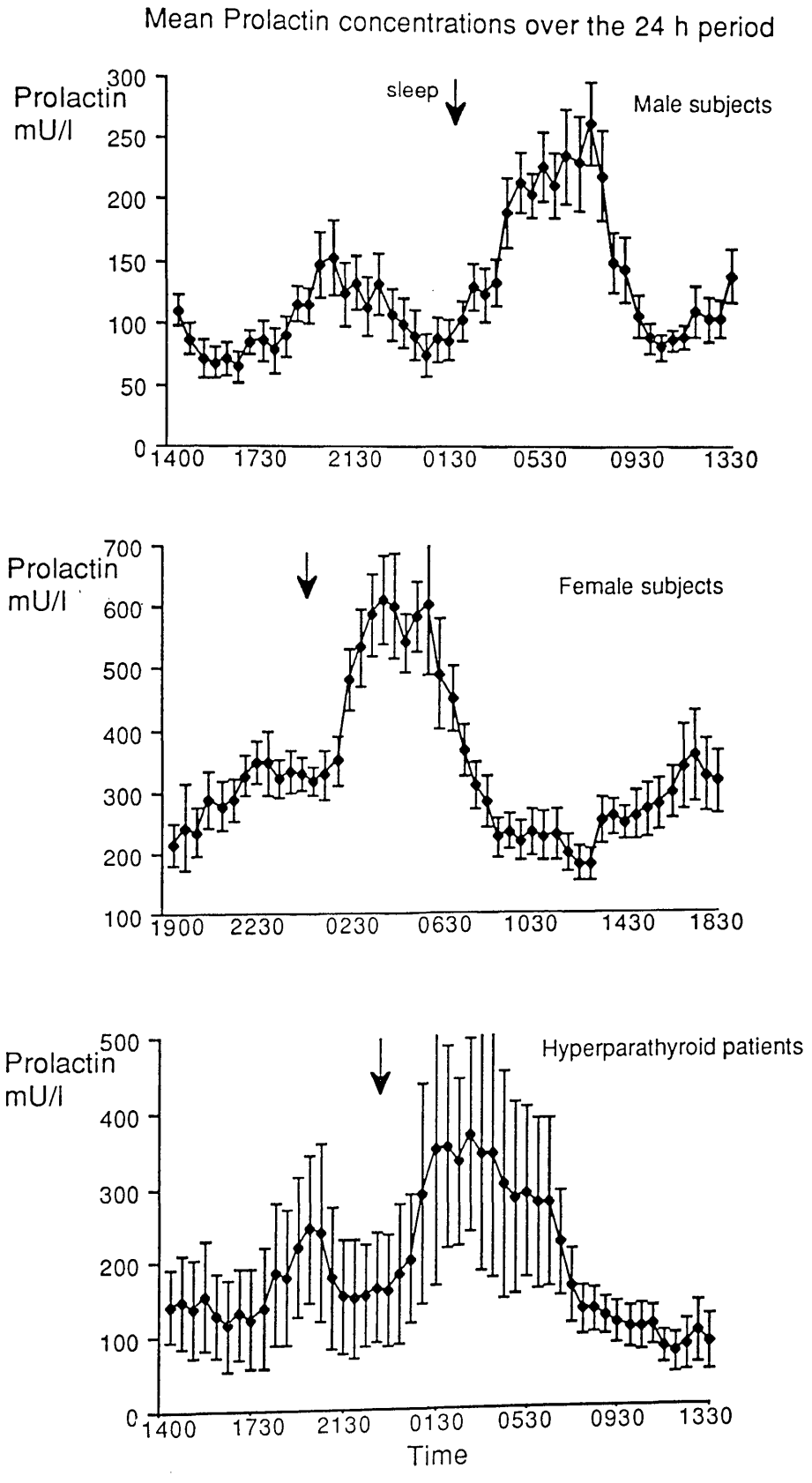


Figure 5.9

A comparison of the mean (± SEM) concentrations of prolactin over the 24 h period between normal male and female subjects and patients with primary hyperparathyroidism. Time of laying down to sleep is indicated by the arrow.

Associated with the 24 h profiles in Figures 5.7, 5.8 is a plot of the estimated cross-correlations for the PTH (1-84) versus successive lags of the prolactin data in each of the male subjects. This analysis confirms a strong temporal relationship between PTH (1-84) and prolactin secretion with maximal correlation coefficients of 0.73, 0.83, 0.66, 0.62, 0.81, and 0.68 in the six subjects, respectively. The maximum cross-correlation coefficients and the corresponding lag times obtained for the PTH (1-84) versus the prolactin data in each of the male subjects are shown in Table 5.3. Also shown for comparison are the same parameters for PTH (1-84) versus serum adjusted calcium.

The mean concentrations of prolactin in the male subjects, premenopausal females and hyperparathyroid patients over the 24 h period are shown in Figure 5.9. The prolactin profiles are qualitatively similar in males and females although the prolactin concentrations are consistently higher and the sleep related prolactin peak is of greater amplitude in women (24 h mean in female subjects = 339 vs 127 mU/l in males $p<0.01$). Cross correlation analysis confirms a similar temporal relationship between PTH (1-84) and prolactin in the female subjects,with a mean maximal $r=0.67$ (range 0.61-0.78) occurring at a mean lag time of -1.7 h (range -0.5 to -4.0), to that observed in the male subjects.

The mean prolactin 24 h profile in hyperparathyroid patients is also qualitatively similar to that of the normal male subjects both showing the early evening rise and the nocturnal peak shortly after the onset of sleep. The mean correlation coefficient between PTH (1-84) and prolactin concentrations in the hyperparathyroid patients however was 0.19 (range -0.61 to 0.43) with a mean lag time of 0.9 h (range -2.0 to 7.0).

The mean 24 h profiles for HGH, TSH and LH in the six normal male subjects are shown in Figures 5.10. The maximal correlation coefficients and the corresponding lag times for the PTH (1-84) data versus successive lags of HGH, TSH and LH data

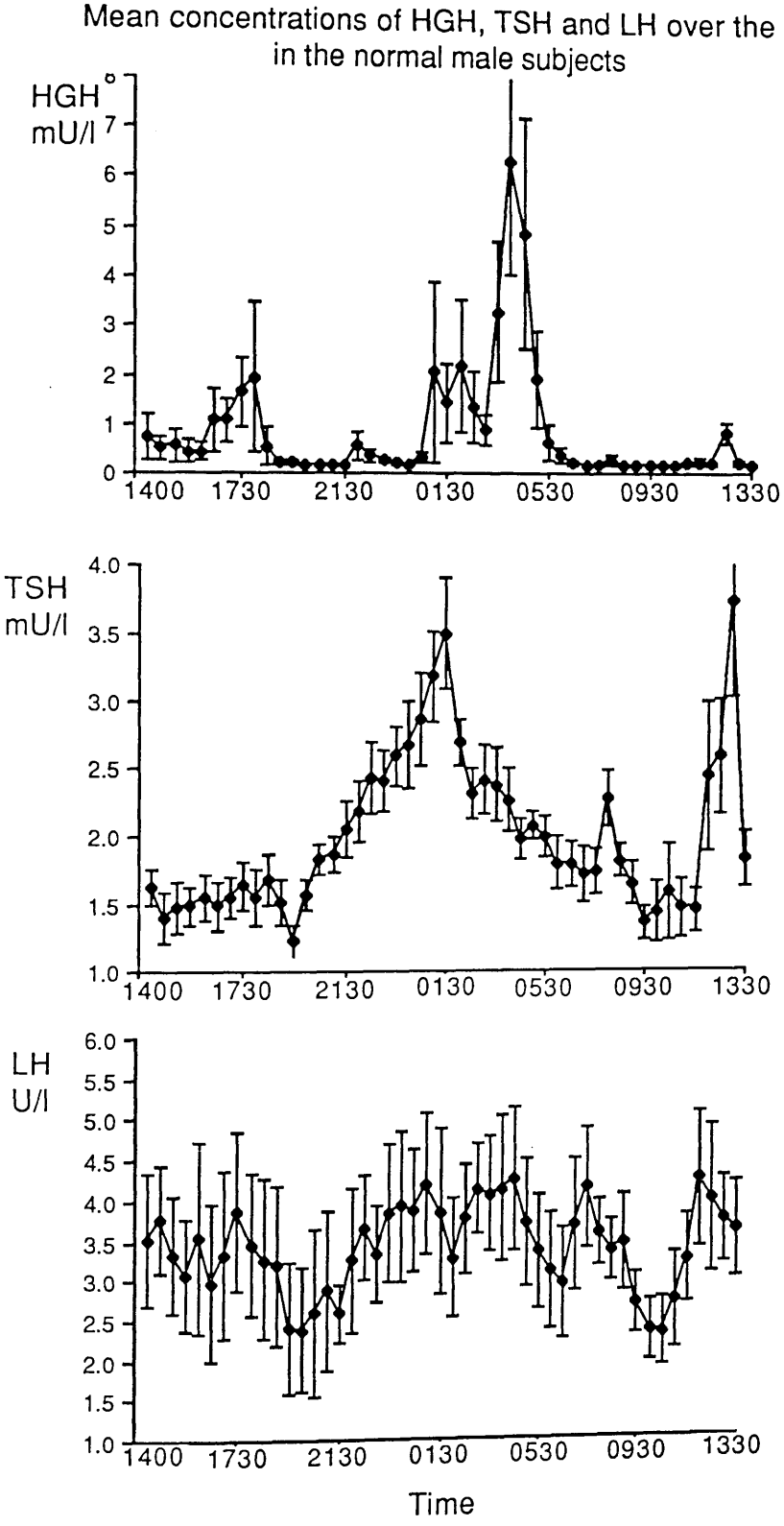


Figure 5.10

The mean (± SEM) concentrations of HGH, TSH and LH in the six normal male subjects over the 24 h period.

Table 5.4

Cross-correlation analysis of PTH (1-84) concentrations versus adjusted calcium, prolactin, HGH, TSH and LH concentrations over a 24 h period in six normal males

Subject	Adj Ca		Prolactin		HGH		TSH		LH	
	r	lag	r	lag	r	lag	r	lag	r	lag
1	-0.43	4.0	0.73	-2.0	0.77	1.0	0.61	-1.5	0.56	0.0
2	-0.58	0.0	0.83	-3.5	0.51	1.0	0.37	-1.5	-0.81	7.0
3	-0.66	9.5	0.66	-0.5	0.44	-2.5	0.74	-3.0	0.56	0.5
4	-0.36	2.5	0.62	-2.0	0.62	0.0	0.72	-2.0	-0.54	-10.0
5	-0.46	0.5	0.81	-1.0	0.54	-1.0	0.55	-4.5	-0.52	6.0
6	-0.49	10.5	0.68	-2.0	0.61	-1.0	0.33	-3.0	0.40	0.0
Mean r range	-0.49 (-0.36 - -0.66)		0.72 (0.62 - 0.83)		0.58 (0.44 - 0.77)		0.55 (0.33 - 0.74)		-0.06 (-0.81 - 0.56)	
Mean lag range	4.5 (0.0 - 10.5)		-1.8 (-3.5 - -0.5)		-0.42 (-2.5 - 1.0)		-2.6 (-4.5 - -1.5)		0.6 (-10.0 - 7.0)	

The data from each individual were smoothed using a method of three-sample running means. A series of correlation coefficients were calculated between the PTH (1-84) data of an individual versus successive lags [1 lag = 30 min] of their prolactin and adjusted calcium data. The maximal correlation obtained and the corresponding lag time are shown for individual subjects.

in the individual subjects are shown in Table 5.4. Also included for comparative purposes is the data for adjusted calcium and prolactin. PTH (1-84) secretion over the 24 h period shows the strongest temporal correlation with prolactin secretion (mean r = 0.72; mean lag time = -1.8 h (-3.5 to -0.5)). There is also a good correlation between PTH (1-84) secretion and that of HGH (mean r=0.58; mean lag time -0.42 h (-2.5 to 1.0)) and TSH (mean r = 0.55: mean lag time -2.6 h (-4.5 to -1.5)). By comparison the correlation with adjusted calcium is weaker (mean r = -0.49) within widely varying lagtimes (0.0 to 10.5 h). PTH (1-84) shows no correlation with LH secretion (mean r = -0.06; lag times -10.0 to 7.0 h).

Discussion

The analysis of the individual PTH (1-84) profiles, smoothed using the three sample moving average, shows the early evening rise in PTH (1-84) more clearly than does the mean data (Section 2.4, Figure 5.1). This indicates that the early evening rise in PTH (1-84) is less synchronised between subjects than is the nocturnal rise.

The individual profiles from the six normal men reveal two periods of increased PTH (1-84) secretion (1600-1900 h - 0200-0600 h). The broad rise in intact PTH (1-84) through the night (0200 - 0600 h) is in agreement with earlier work detailing the nocturnal rise in immunoreactive PTH (Jubiz et al, 1972, Kripke 1978). The timing of the earlier peak is consistent with more recent studies on immunoreactive PTH in which biphasic PTH profiles were observed, although in contrast to our data, the early evening peak was more pronounced being similar in terms of height and duration to the rise through the night. This may reflect either the influence of dietary calcium and phosphate on the early evening rise or the differences in PTH assay methodology (Calvo et al, 1988, Markowitz 1988, Calvo et al, 1990).

The prolactin profiles in the male subjects showing two main peaks of increased secretion at 2000-2200 h and 0400-0800 h in each subject are in agreement with published data. (Desir et al, 1982). The patterns of prolactin secretion between

female and male subjects are qualitatively similar. The amplitude of the night time rise is higher in the female subjects which is also consistent with earlier studies which found nocturnal peak levels in women up to two fold higher than in men (Sassin, 1972). The mean 24 h profile for prolactin in the hyperparathyroid patients also shows good qualitative agreement with that of the normal subjects. However the loss of circadian rhythm of PTH (1-84) in hyperparathyroid patients (Section 2.4 above, Logue et al, 1990b) means that there is no consistent cross correlation between PTH (1-84) and prolactin secretion in these patients.

The most significant finding from the present study is that over a 24 h period there is a strong temporal correlation (range 0.62-0.83) between PTH (1-84) and prolactin secretion in individual normal subjects with changes in PTH (1-84) occurring approximately 2 h before those in prolactin (lag range 0.5-3.5 h). By comparison the negative correlation between PTH (1-84) and serum adjusted calcium is weaker (range 0.36-0.66) with variable lag times (lag range 0-10 h) - an observation that both challenges the view that PTH (1-84) and plasma ionised calcium are always inversely linked and also suggests that other factors may be implicated in the control of tonic PTH (1-84) secretion.

The controlling mechanism for the circadian rhythm in PTH (1-84) remains unclear. In accordance with previous studies (Markowitz et al, 1988, Mallette 1989) we observed a small fall in adjusted calcium occurring at 1230 h prior to the early evening rise in PTH (1-84) seen in the individual subjects. Similarly we found a small transient drop in adjusted calcium at 0130 h prior to the nocturnal rise (Section 2.4 above, Logue et al, 1990b). While it is tempting to speculate a fall in calcium acting as trigger to rises in PTH (1-84) this appears to be an over simplification. The nocturnal rise in PTH has been shown to persist during continuous infusion of calcium (Jubiz et al, 1972) and it has been noted that the nocturnal rise in PTH (1-84) occurs at a time when ionised calcium is rising (Markowitz et al, 1988). It is noteworthy that separate studies find falling ionised or adjusted calcium around

midday which suggests either dietary influence or an endogenous circadian rhythm of calcium. In addition the fall in adjusted calcium around midday is 3 to 4 h prior to the noted early evening peak in PTH whereas studies using EDTA infusion indicate a much more rapid response in PTH (1-84) to a lowering of serum calcium (Hackeng et al, 1986).

Prolactin has been shown to play a definite role in calcium metabolism only in aquatic habitat vertebrates . However prolactin can stimulate avian renal 25-OH-Vitamin D_3-1a-hydroxylase (Spanos et al, 1976 a) and increase 1,25-$(OH)_2$-Vitamin D_3 levels in the hen (Spanos 1976b). In addition ovine prolactin has been reported to stimulate PTH release from bovine parathyroid cells *in vitro* (Magliola et al, 1981) and exogenous PTH has been shown to stimulate prolactin release in normal men (Issac et al, 1978). It has been postulated from these acute experiments at non-physiological concentrations that prolactin may play a role in the regulation of calcium metabolism through a direct effect on calcitropic hormone concentrations (Fiore et al, 1984). However studies measuring PTH and prolactin concentrations on basal samples from normal and hyperprolactinaemic subjects have produced conflicting results that have failed to confirm a direct inter-relationship between these two hormones (Raymond et al, 1982, Fiore et al, 1984).

The results of the present study demonstrate that the secretion of PTH (1-84) and prolactin are, however, highly correlated over a 24 h period within individual normal subjects. Thus while previous evidence from the acute experiments might suggest a direct positive feedback loop between PTH (1-84) and prolactin the data from this study indicates that while PTH (1-84) may be affecting the secretion of prolactin the reverse is unlikely. Our data are more suggestive of a common controlling factor and indirect coupling of the secretion of these hormones possibly via the neural network of the suprachiasmic nuclei which are responsible for the timing of circadian rhythms (Turek 1985). Inconsistencies with previous studies probably reflect differences in hormonal effects at pharmacological and pathological

concentrations.

It appears therefore that the circadian changes in PTH (1-84) in normal subjects are entrained to an endogenous circadian clock which is temporally coupled to prolactin secretion. The nocturnal rise in prolactin is known to be sleep-related (Sassin et al, 1973) and there is also evidence that the nocturnal rise in PTH (1-84) is sleep related (Kripke et al, 1978) raising the possibility of neuroendocrine control mechanisms common to both PTH (1-84) and prolactin.

In addition to the strong temporal relationship found between PTH (1-84) and prolactin the 24 h profile of PTH (1-84) in the six normal male subjects also shows a better correlation and more consistent lag times with TSH and HGH secretion than with variations in adjusted calcium. The 24 h profile for TSH has been shown to result from the interaction of sleep and circadian rhythmicity. The evening rise in TSH occurring well before the onset of sleep and reflects circadian timing (Parker et al, 1976). During sleep an inhibitory influence is exerted on TSH secretion (Parker et al, 1987) such that during sleep deprivation the evening rise of TSH is prolonged and enhanced. Similarly while HGH secretion is strongly associated with sleep such that adult males typically show a single HGH pulse coincident with sleep there evidence for a weak circadian rhythm modulating the occurrence of and height of HGH secretory pulses (Van Cauter and Refetoff 1985).

The lack of correlation between the PTH (1-84) and LH secretion reflects the more pulsatile characteristics of the LH profile.The secretion of LH is known to be strongly pulsatile in nature, and while there is circadian variation in the pulse frequency in children at puberty resulting in higher LH levels during the night, in males reaching adulthood the circadian variation is dampened and becomes undetectable (Turek and Van Cauter 1988). In the majority of male adults the secretion remains pulsatile and may vary considerably between individuals (Veldhuis 1987).

Based on observations in animal models that continuous infusions of PTH are not effective in promoting bone growth and that increased bone formation requires a regimen resulting in transient rises in PTH by daily injections (Podbesek et al, 1983, Malluche et al, 1982) it has been postulated that the night time rise in PTH (1-84) may be an important factor in normal bone physiology (Calvo et al, 1988, Logue et al, 1990b). Further studies on the control of the circadian secretion of PTH (1-84) and its relationship to prolactin secretion are required, particularly in female subjects, to evaluate the possible role of neuroendocrine factors in the pathogenesis of bone disorders such as osteoporosis. It is also of importance to establish whether manipulation of the circadian PTH (1-84) profile may have a role in the treatment of such disorders.

2.6 Control of the circadian rhythm of PTH (1-84) secretion - evidence from a sleep shift experiment.

Introduction

The profile of PTH (1-84) secretion in normal man is characterised by a late afternoon/early evening rise and a nocturnal surge through the night (Section 2.4 above, Logue et al, 1989). The mechanism responsible for the PTH (1-84) rhythm remains unclear and it has been concluded from these and previous studies (Kripke et al, 1978, Markowitz et al, 1988) that the PTH (1-84) rhythm cannot be accounted for by changes in serum calcium or phosphate concentrations and therefore that the central nervous system may play a role either directly or indirectly in modulating normal PTH (1-84) secretion.

Prolactin secretion follows a similar pattern over a 24 h period in normal male subjects with an early evening rise and a major night time elevation occurring shortly after the onset of sleep (Sassin 1973, Desir et al, 1982). Cross correlation analysis of the 24 h profiles confirmed a strong temporal correlation between PTH (1-84) and

prolactin secretion in normal male and female subjects with the changes in prolactin secretion occurring approximately 2 h after those in PTH (1-84) (Section 2.5 above, Logue et al, 1990c). It has been postulated therefore that there may be neuroendocrine control common to PTH (1-84) and prolactin secretion .

The overall circadian characteristics (excluding food intake or dietary effects) of a 24 h hormonal profile generally result from the effects of circadian rhythmicity (intrinsic effects of time of day irrespective of the sleep or waking state), sleep (intrinsic effects of sleep irrespective of the time of day) and pulsatile secretion occurring at frequencies of minutes or hours. To delineate the relative roles of circadian and sleep effects, strategies based on the fact that circadian rhythmicity needs several days to adapt to abrupt shifts of the sleep-wake cycle have been employed. Thus by shifting the sleep times by 7-12 h, masking effects of sleep on circadian inputs are removed and the effects of sleep at an abnormal circadian time can be revealed.

Studies have shown prolactin secretion to be strongly associated with sleep in that daytime naps are associated with rises in prolactin (Parker et al, 1973) and acute reversal or delay of the the sleep-wake cycle is accompanied by prolactin elevation during daytime sleep (Sassin et al, 1973). There is also evidence that the nocturnal surge in PTH is sleep related (Kripke et al, 1978). A sleep-shift experiment in which the time of sleep was delayed by 7 h was carried out on six normal male subjects to asses the relative roles of circadian and sleep effects on the 24 h profile of PTH (1-84) secretion.

Subjects and methods

Six normal male subjects aged 30-41 yrs were studied on two separate occasions. In study A (circadian) (samples from circadian study in Section 2.4 above) the subjects slept from 0100-0800 h and in study B (sleep shift) the subjects were awake through the night and slept from 0800-1400 h.

Results

The 24 h profiles for PTH (1-84), prolactin and NcAMP during the circadian and sleep shift studies are shown in Figures 5.11. For clarity of presentation the mean profiles have been smoothed using the three sample moving average technique and error bars have been omitted.

The timing of the nocturnal rise in PTH (1-84) appears unaltered despite the shift in the sleep-wake cycle although it appears that the start of the PTH (1-84) peak is attenuated (2300-0300 h). The NcAMP rises in parallel to the PTH (1-84) in both studies but the rise during the sleep shift experiment is reduced, mean NcAMP from 0130 to 0700 h during the sleep shift study was 23.8 nmol/l GF compared to 31.9 ($p<0.01$) in the circadian study. The nocturnal prolactin peak seen in the circadian study is absent in the sleep shift study. However a rise in prolactin occurs coincident with the time of delayed sleep in the sleep shift study.
There were no significant differences observed in the 24 h profiles for adjusted calcium or phosphate between the studies.

Discussion

Early studies using this type of experimental design demonstrated that the secretion of some hormones is primarily modulated by circadian rhythmicity, eg cortisol and melatonin, while others such as prolactin and growth hormone are strictly sleep dependent. The complete distinction between circadian dependent or sleep-related rhythms was the basis of a two-oscillator model for the generation of circadian rhythms (Moore-Ede et al, 1982). The model postulated two separate, but interacting pacemakers, one (referred to as X) driving deep circadian rhythmicity and the other (referred to as Y) driving the rest activity-cycle. According to this model the sleep-related rhythms were assumed to be controlled by the Y oscillator and the circadian rhythms would be driven by the master circadian pacemaker X.

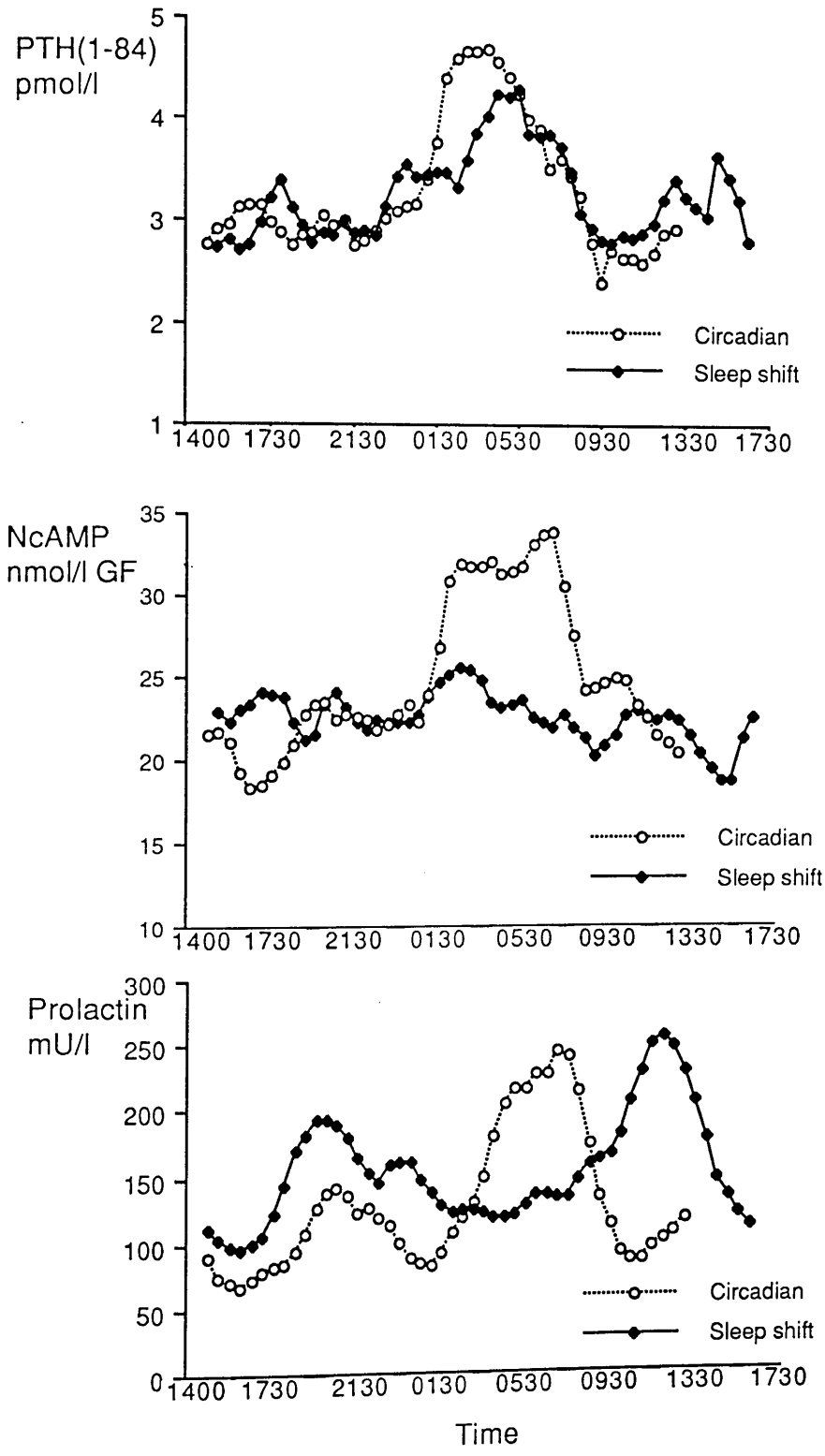


Figure 5.11

A comparison of the 24 h profiles (smoothed using a three sample moving average) for PTH (1-84), NcAMP and prolactin between the circadian and sleep shift studies in the normal male subjects.

The shift in the timing of the prolactin peak seen in this acute sleep shift experiment is consistent with early studies on prolactin secretion which had shown that daytime naps are associated with increases in prolactin (Parker et al, 1973) and that acute reversal or delay of the sleep wake cycle is accompanied by an increase in prolactin during daytime sleep (Sassin et al, 1973). These studies lead to the conclusion that the prolactin secretion was primarily controlled by sleep without inherent circadian rhythmicity. However more recent studies on repeated sleep-shifts as part of a jet-lag study had shown a splitting of the prolactin peak revealing an endogenous circadian component to prolactin secretion in addition to the sleep-related component. (Desir et al, 1982). It is now accepted that sleep effects and circadian effects interact to produce the overall temporal pattern of the majority of hormones and that different portions of the wave shape of the hormonal pattern adapt at different rates to manipulations of the sleep wake-cycle. The data on prolactin is consistent with a circadian control strongly influenced by sleep but with some underlying circadian modulation (Van Cauter and Refetoff 1985).

Nocturnal PTH concentrations have been shown previously to be temporally correlated with cycles of stage 3 and 4 sleep (Kripke et al, 1978). In this acute sleep-shift experiment the timing of the main nocturnal PTH (1-84) rise did not alter significantly from the normal circadian pattern. However it appears that the early part of the peak (2300-0300 h) is suppressed. Thus the PTH (1-84) data would be consistent with a primarily circadian rhythm which may be modified by sleep. Another example of this type of control is that of TSH which is primarily circadian but suppressed by sleep such that in sleep deprivation experiments the evening rise in TSH is enhanced and prolonged (Parker et al, 1987).

Therefore while the secretion of PTH (1-84) and prolactin follow well defined circadian patterns in normal subjects such that there is a strong temporal correlation between these hormones over a 24 h period the mechanism by which they are

entrained to the biological clock appears to be fundamentally different.

However, the evidence linking prolactin to PTH and calcium metabolism remains (Spanos et al, 1976, Magliola et al, 1981, Fiore et al, 1984). The recent finding that prolactin can stimulate parathyroid hormone related peptide in breast milk (Thiede and Rodan 1988) adds weight to the association of prolactin and calcium metabolism. The comparison of the NcAMP profiles between the studies indicates that while the NcAMP peak remains coincident with the PTH (1-84) peak in the sleep shift experiment it is reduced in size. It is interesting to speculate whether the night time rise in NcAMP during the normal sleep wake cycle has a prolactin dependent component, perhaps related to the production of parathyroid hormone related peptide.

Thus while the results of sleep shift experiment argue against a direct neuroendocrine link between PTH (1-84) and prolactin secretion the circadian studies so far demonstrate that these hormones are maintained by the circadian clock in a distinct phase relationship throughout a 24 h period in normal subjects. Given the postulated role of prolactin in calcium metabolism it may be that this high degree of temporal organisation allows concerted metabolic effects between these hormones.

2.7 Control of the circadian rhythm of PTH (1-84) secretion - preliminary evidence of modification of the rhythm by a 96 h fast.

Introduction

The secretion of PTH (1-84) in normal subjects follows a defined circadian rhythm. Over a 24 h period the PTH (1-84) concentrations in normal subjects follow a biphasic pattern of an early evening rise and a nocturnal peak. (Markowitz et al, 1988, Calvo et al, 1988, Logue et al, 1989). However the mechanism responsible for the circadian rhythm remains unresolved.

The most consistent relationship throughout our studies and those of others is that

between PTH and serum phosphate concentrations. The rhythm in serum phosphate is qualitatively similar and coincident with that of PTH (1-84) (Markowitz et al, 1988, Calvo 1988, Logue et al, 1990b). The strongest evidence against a direct link between the circadian secretion of PTH (1-84) and phosphate is that of Jubiz et al, (1972) where it was shown that the circadian rhythm in serum phosphate was abolished by a 96 h fast while the PTH rhythm was unaffected.

It was decided therefore to re-evaluate the association between the secretion of PTH (1-84) and phosphate by investigating the effect of a prolonged fast in normal subjects. The preliminary results from three male volunteers are presented below.

Subjects

Three male subjects (aged 35-41 yrs) who had also participated in the circadian study (Section 2.4) were fasted (water only) for four days. They were sampled every 30 minutes from the third (1500 h) to the fourth (1430 h) day of fasting. Throughout the study the subjects were ambulant but avoided exercise and followed a normal sleep wake cycle.

Results

The mean 24 h concentrations of PTH (1-84), adjusted calcium and serum phosphate found in the fasting study are shown compared to the profiles obtained in the circadian study (Section 2.4) in Figure 5.12.

PTH (1-84) concentrations were higher compared to the fed state (mean of 5.2 vs 3.3 pmol/l $p<0.01$) and were outwith the reference range for normal subjects (1-5 pmol/l) for much of the 24 h. The synchronised nocturnal peak between subjects is not present in the fasting study. PTH (1-84) secretion appears more pulsatile in nature with concentrations being highest in the afternoon.

The mean 24 h adjusted calcium was decreased compared to the circadian study (2.33 versus 2.22 mmol/l. $p<0.01$).

Comparison of the 24 h profiles of PTH(1-84), adjusted calcium and phosphate between the circadian and fasting studies

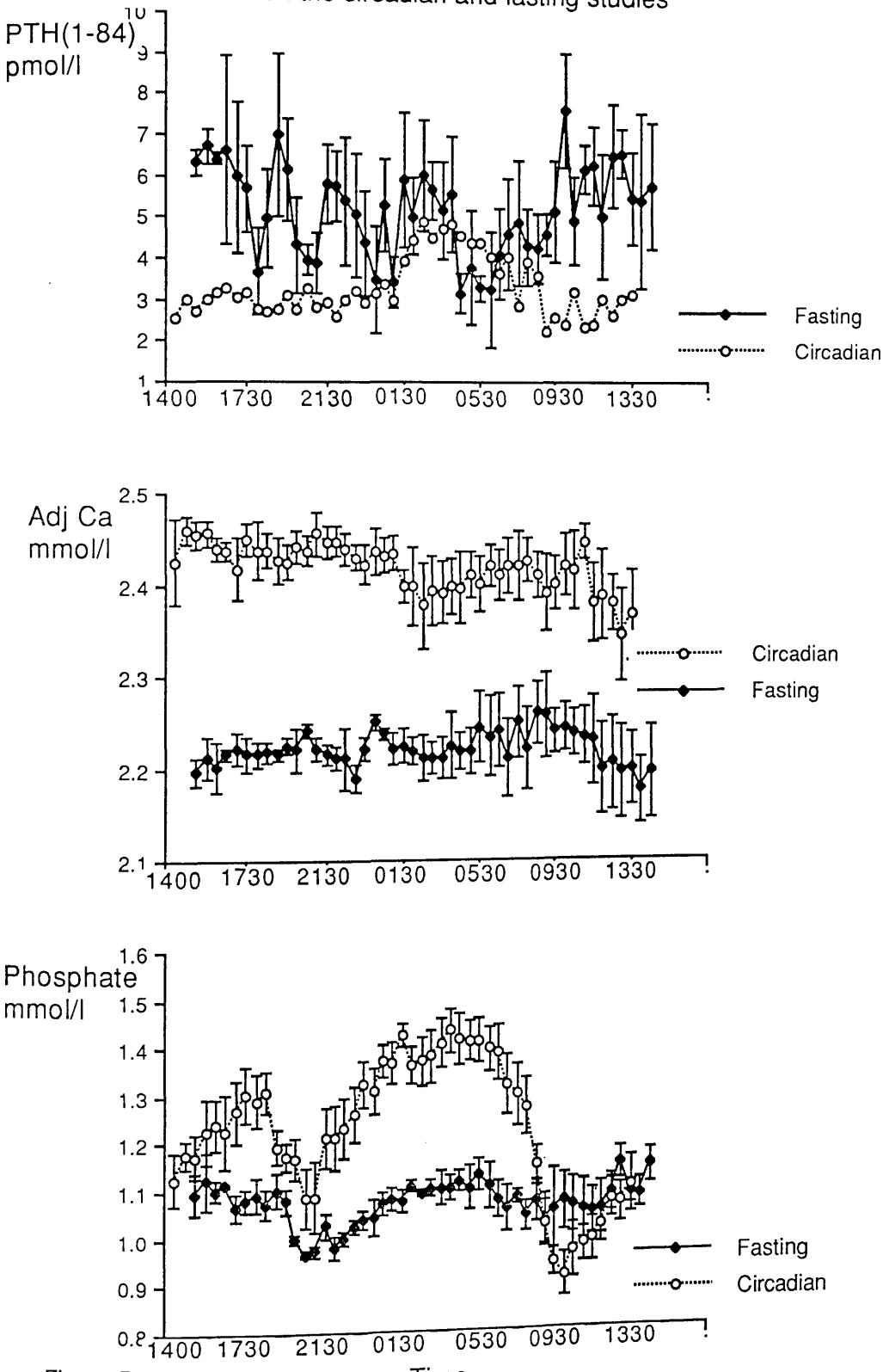


Figure 5.12

The mean (±SEM) PTH (1-84) concentrations in the fasting study shown in comparison to the mean PTH (1-84) profile (smoothed) in the circadian study. Also shown is a comparison of the mean (± SEM) concentrations of adjusted calcium and phosphate between the fasting and circadian studies.

The serum phosphate concentrations were reduced and the rhythm attenuated on fasting; fasting mean 1.07 mmol/l (range 0.97-1.15) compared to 1.23 mmol/l (range 0.91-1.43) in the fed state.

Discussion

The results of this study are based on the first three of six subjects and therefore should be regarded as preliminary.

The decrease in serum adjusted calcium is in agreement with previous data on fasting (Statland et al, 1973, Elia et al, 1984). Dietary manipulation has been shown to have a major impact on the circadian phosphate rhythm (Jubiz et al, 1972). The reduction in serum phosphate concentrations observed on fasting is consistent with the previously reported loss of circadian rhythm in phosphate under these conditions (Jubiz et al, 1972). Comparison of the 24 h profiles in this study however suggests a markedly attenuated rhythm rather than a complete loss. This probably reflects the increased sampling frequency in this study.

However in contrast to the unaltered PTH circadian rhythm reported by Jubiz et al, (1972) the PTH (1-84) profile was markedly different between the fasting and fed states. The PTH (1-84) concentrations were elevated during the day and appeared pulsatile in nature with marked between subject variability such that the synchronised nocturnal peak was not detected. The differences between the studies most probably reflect the combination of improved methodology, allowing the measurement of intact PTH (1-84) rather than immunoreactive PTH, and the increased sampling frequency employed in this study.

The marked attenuation of the serum phosphate rhythm produced on fasting in this study is therefore also accompanied by an elevation in PTH (1-84) concentrations and loss of circadian rhythmicity in PTH (1-84). Thus it is no longer appropriate to exclude an interaction between the circadian changes in serum phosphate and those of

PTH (1-84) on the basis of the dissociation of these rhythms during a prolonged fast. Simplistically it could be argued that if under normal conditions the circadian rhythm in PTH (1-84) were triggered by increases in serum phosphate the attenuated phosphate rhythm observed on fasting would result in a reduction in PTH (1-84) concentrations. The elevated PTH (1-84) concentrations seen in this study are therefore probably secondary to the reduction in serum calcium. While it is not possible from this study to evaluate the role of serum phosphate and calcium in the circadian secretion of PTH (1-84) under normal conditions the data from this study are more consistent with the postulate that the PTH (1-84) status may modulate the phosphate rhythm (Jubiz et al, 1972, Markowitz et al, 1988, Logue 1990b). Recent studies on the effect of dietary manipulation on PTH (1-84) secretion have also shown persistently elevated PTH (1-84) concentrations in response to a high phosphorous, low calcium diet (Calvo et al, 1990). Thus it is clear that both diet and food intake have a marked influence on the circadian profile of PTH (1-84).

While full analysis of the pulsatile nature of the PTH (1-84) must await the completion of this study (with more subjects) there appears to be an increase in the pulsatility of PTH (1-84) secretion on fasting. Growth hormone is known to to increase during fasting (Cahill et al, 1966) and it has recently been shown that there is a significant increase in both the frequency and amplitude of the pulsatility of growth hormone secretion with the appearance of ultradian frequencies (Ho et al, 1988). It has been postulated from these and animal studies that the function of the signal mode, ie continuous versus pulsatile secretion, can mediate differential metabolic actions of growth hormone, and therefore play a role in the switch from the predominantly gluconeogenic to the lipolytic source of metabolic fuel with prolonged fasting. Similarly the pattern of secretion has been linked to differential effects of PTH (1-84) concentrations on type 1, cAMP mediated, and type 2, inositol phosphate mediated, receptors (Hesch et al, 1988). It is possible therefore that the pulsatile secretion of PTH (1-84) represents an adaptation to the fasting state perhaps

facilitating the mobilisation of calcium as it has been calculated that approximately half the quantities of calcium and phosphate excreted in urine during a prolonged fast had been mobilised from bone (Elia et al, 1984).

Despite the increased PTH (1-84) concentrations during the day the night time concentrations are not significantly different between the fasting and circadian studies. Therefore although the PTH (1-84) concentrations did not show a synchronised night time rise from the elevated basal concentrations it remains possible the neuroendocrine or sleep related factors are responsible for PTH (1-84) secretion during the night. It is also possible that fasting *per se* suppressed the expected nocturnal surge in PTH (1-84) as has been shown for TSH (Romijn et al, 1990).

In conclusion the preliminary analysis of the effects of a four day fast in normal subjects has shown that while the serum phosphate rhythm is greatly reduced the circadian rhythm of PTH (1-84) does not remain unaltered as previously reported. This has implications for the investigation of the control of PTH (1-84) secretion as a possible role for serum phosphate has been excluded on the basis of previous studies on fasting (Jubiz et al, 1972).

2.8 Conclusion

There is general agreement that serum calcium concentration is the main regulator of PTH (1-84) secretion. There is little evidence however that this relationship is dominant within the physiological range and it is has been postulated that non-ionic influences such as intrinsic pulsatility and adrenergic activity under the control of the central nervous system are of importance in modulating PTH (1-84) secretion particularly during sleep (Kripke et al, 1978, Markowitz et al, 1988). The improved sensitivity and specificity of the two-site immunometric assay has allowed the definition of the circadian rhythm of PTH (1-84) secretion in normal subjects. The studies of this project have indicated that the circadian secretion of PTH (1-84) could

not fully be explained by changes in serum adjusted calcium. There is a strong correlation between changes in serum phosphate and PTH (1-84) concentrations under normal conditions. The overall results are consistent with the postulate that the PTH status modulates the rhythm in serum phosphate, however, the fasting study has indicated that a possible role for phosphate in the generation of the circadian profile of PTH (1-84) cannot be excluded.

The secretion of PTH (1-84) over a 24 h period is strongly correlated with other hormones which are entrained to the circadian 'clock' and are elevated through the night, most notably prolactin which has a known association with calcium metabolism. These results were suggestive of neuroendocrine control common to PTH (1-84) and prolactin especially as evidence had suggested that the nocturnal PTH (1-84) peak was, like that of prolactin, sleep related. The dissociation of the secretion during sleep shift indicated however that these hormones are entrained to the circadian 'clock' by different mechanisms. PTH (1-84) secretion appears to have an underlying endogenous rhythm which may be modified by sleep whereas prolactin is strongly associated with sleep but has been shown to have an endogenous component. Nevertheless under normal conditions the PTH (1-84) concentrations are maintained in a defined phase relationship to those of prolactin over a 24 h period. The secretion of PTH (1-84) particularly during the night is therefore likely to have a component of neuroendocrine control linking it to the pacemaker of circadian rhythms.

In addition to the effects of endogenous rhythmicity and of sleep, discussed above, the role of diet and food intake should also be considered. A diet low in calcium and high in phosphate has been shown to cause persistently elevated PTH concentrations in normal subjects (Calvo et al, 1990). The differences in PTH (1-84) concentrations between male and female subjects found in this study was most likely diet related. The elevated PTH (1-84) concentrations and increased pulsatility seen on fasting may represent a positive adaptation to fasting to enable mobilisation of calcium

presumably from bone. It is also possible however that the increased PTH (1-84) concentrations reflect an absence of suppression, normally present due to food intake, of the tonic secretion of PTH (1-84). Suppression could result from the calcium content of the diet or indirectly via other hormones influenced by food intake. PTH (1-84) concentrations have been shown to suppress during an insulin tolerance test in normal subjects (Fisher et al, 1990). Whether this is a centrally mediated stress effect or a direct action of insulin remains to be established.

The profile of PTH (1-84) concentrations over a 24 h period in normal subjects is therefore multifactorial in origin. Further studies are required to elucidate the relative contributions of endogenous rhythmicity, sleep, diet and food intake to the generation of the circadian rhythm of PTH (1-84) secretion in normal subjects and in the pathophysiology of calcium metabolism.

3 STUDIES ON THE PULSATILE SECRETION OF PTH (1-84)

3.1 Introduction

The studies on circulating concentrations of PTH (1-84) over a twenty four hour period revealed a defined circadian pattern of secretion in normal subjects (Section 2 above; Logue et al, 1989). Most hormones are secreted as a series of pulses rather than continuously and therefore, in addition to circadian, also show ultradian (i.e. occurring at intervals shorter than 24 h) variations in serum concentrations. While the pulse generators appear anatomically distinct from the neural structures responsible for circadian timing (Pohl et al, 1983) interactions between the circadian rhythmicity and pulsatility occur via the modulation of the amplitude, frequency and phase of the pulses.The circadian pattern of ACTH release has been shown to be obtained by the circadian modulation of pulse amplitude and frequency (Van Cauter and Honinckx 1985). The frequency and amplitude of LH pulses are modulated during the

nocturnal hours by circadian rhythmicity and/or sleep (Soules et al, 1985).

Virtually all endocrine glands exhibit an episodic rather than continuous pattern of hormone secretion (Urban et al, 1988). The resultant pulsatile mode of hormone delivery is believed to convey important physiological information to the target cells with variations in pulse amplitude and frequency being important modulators of target organ response (Gross et al, 1987). The investigation of the pulsatile release by the measurement of circulating concentrations of hormones requires sensitive and precise assay methodology ideally using small sample volumes and having the capability to process large numbers of samples. Previous methodology made the study of PTH difficult although studies suggested pulsatility was present in dogs (Fox et al, 1981) and in man (Robinson et al, 1982). More recently Dixit et al, (1987) demonstrated pulsatile release of bioactive PTH in normal subjects with pulses occurring every 10-15 minutes and an increase in amplitude and frequency following stimulation by EDTA infusion. The development of two-site immunometric assays for PTH (1-84) has allowed more detailed studies of ultradian variations in the circulation and PTH (1-84) concentrations have been shown to vary in a pulsatile fashion with peaks approximately every 10 minutes in normal subjects (Harms et al, 1989).

The main reason for investigating the pulsatile release of a hormone is to understand its role in disease states. It has been shown that pulse amplitude and frequency modulation disturbances can have the same dramatic consequences for the expression of an endocrine disease as a glandular defect for example disturbed GnRH secretion in polycystic ovarian disease and Klinefelters syndrome (Kazer et al, 1987, Spratt et al, 1987). Indeed Harms et al, (1989) reported a decreased PTH (1-84) pulse amplitude and frequency in patients with osteoporosis.

A study of the pulsatile nature of PTH (1-84) secretion under basal conditions and following EDTA infusion in normal subjects and patients with primary

hyperparathyroidism was therefore undertaken.

3.2 Subjects and Methods

Normal Subjects

Four healthy male volunteers (aged 31-42; mean 35 years) were studied on two separate occasions. In the first study the subjects were studied under basal conditions for a period of 60 minutes. Venous blood samples were obtained through an indwelling cannula at 0.5 minute intervals. The subjects were then studied (approximately one year later) before and during an EDTA infusion (24 mg/kg/h). In this study the subjects were sampled for 30 minutes (0.5 minute intervals) under basal conditions and then infused with EDTA for 90 minutes. Samples were withdrawn (0.5 minute intervals) during the last 30 minutes of the EDTA infusion. The studies were carried out between 1200 and 1400 h to minimise the effects of circadian changes in PTH (1-84) concentrations.

Hyperparathyroid patients: Four patients (two male, two female) were studied with primary hyperparathyroidism (subsequently surgically proven with histological diagnosis; aged 47-58 years). The patients were studied before and during EDTA infusion according to the protocol used with the normal subjects.

Intact PTH (1-84) was measured using the in-house IRMA (Logue et al, 1990a). All samples from an individual subject were analysed in the same assay batch.

The PTH (1-84) profiles were analysed by Fourier analysis (Statgraphics package). This procedure decomposes the variance of the data into contributions over a range of frequencies. The data were analysed using the integrated periodogram technique (Statgraphics) to verify non-random variation in the time series.

3.3 Results

The PTH (1-84) concentrations found in the normal subjects and patients with primary hyperparathyroidism are shown in Table 5.5.

In three of the the normal subjects (DOR, FCL, WDF) the PTH (1-84) concentrations were within the reference range (1-5 pmol/l) in both basal periods although in two subjects (WDF, FCL) the mean PTH (1-84) concentration was significantly lower in the second basal period (3.6 v 2.3 and 4.4 v 2.3 pmol/l respectively; $p<0.05$)). The PTH (1-84) concentration in the fourth subject (GHB) was at the upper end of the reference range (5.5 pmol/l) in the first basal study and clearly elevated (8.8 pmol/l) in the second basal period. This has been ascribed to a severe dietary regime undertaken by the subject prior to the study and is consistent with the elevated PTH (1-84) concentrations observed in fasting subjects (Section 2.7 above).

In response to the EDTA infusion the PTH (1-84) concentrations increased in all subjects by a mean factor of 5.3 (range 2.7-7.6). The basal PTH (1-84) concentrations in the hyperparathyroid patients were in the range 3.5-11.2 pmol/l which increased following EDTA by a similar factor to that observed in the normal subjects (mean 3.9; range 2.7-7.6).

The statistical analysis, using the integrated periodogram technique, confirmed the presence of non-random variation in the PTH (1-84) profiles. Figures 5.13-5.16 show for each individual normal subject a plot of the PTH (1-84) concentrations (smoothed using a three sample moving average technique to minimise effects of assay noise) during the initial basal period, the basal pre EDTA period and the EDTA infusion. Associated with each of these graphs is a plot of the Fourier analysis of the profile. The Fourier analysis estimates the relative contributions to the profile from pulsatility across a range of frequencies from which the plot of the squared

Table 5.5

PTH(1-84) concentrations in normal subjects and patients with primary hyperparathyroidism before and during EDTA infusion.

	Basal 1 pmol/l (sd)	Basal pre EDTA pmol/l (sd)	EDTA pmol/l (sd)	Ratio EDTA/basal
Normal Subjects:				
1. DOR	3.7 (0.39)	3.5 (0.71)	13.6 (1.5)	3.9
2. WDF	3.6 (0.61)	2.3 (0.54)	17.4 (2.1)	7.6
3. FCL	4.4 (0.42)	2.3 (0.52)	16.1 (1.4)	7.0
4. GHB	5.5 (0.43)	8.3 (0.94)	22.5 (1.4)	2.7
Hyperparathyroid Patients:				
1. GR		3.5 (1.2)	26.5 (5.3)	7.6
2. ME		11.2 (2.1)	33.4 (2.2)	3.0
3. DW		10.6 (1.3)	25.7 (4.1)	2.4
4. MC		7.2 (0.8)	19.5 (2.3)	2.7

amplitude of the component sinusoids against frequency is derived.

The PTH (1-84) concentrations in the first basal profile in subject 1 (DOR) show a pronounced pulsatile pattern with the appearance of pulses at approximately five minute intervals. The Fourier analysis confirms the dominant contribution to this profile is the sinusoid with a frequency of 12 pulses/h (Figure 5.13). The profile for the second basal pre EDTA also shows pulsatile pattern although the frequency of pulses is reduced. This is reflected in the Fourier analysis in which shows once again a single dominant peak but with a frequency of 6 pulses/h. In addition there are also small contributions from higher frequency rhythms. During the EDTA infusion the the Fourier analysis indicates a major dominant peak with frequency of 6 pulses/h similar to that in the pre EDTA basal but with increased amplitude. There are also contributions from higher frequencies.

In subject 2 (WDF) during the initial basal profile a dominant peak with a frequency of 12 pulses/ h is observed. There are also contributions from lower frequencies with periods in the range 3 to 6 pulses/h (Figure 5.14). The analysis of the pre EDTA basal period shows a qualitatively similar pattern but with a shift to higher frequencies. The major peak is observed at 24 pulses/h together with lower frequencies in the range 6 to 12 pulses/h. There is also a component of higher frequencies around 48 pulses/h. During EDTA a qualitatively similar pattern to that obtained in the pre EDTA basal is observed with a peak at period 24 pulses/h but with increased contribution from the other lower (around 6 pulses/h) and higher (around 48 pulses/h) frequencies.

The analysis of the data from subject 3 (FCL) (Figure 5.15) shows the main contribution to initial basal profile to be from peaks with periods at 3 to 6 pulses/h but there is also a component at 12 pulses/h. The basal pre EDTA shows a similar pattern but with additional higher frequency components. During the EDTA infusion the pre EDTA pattern is substantially similar but with increased amplitude.

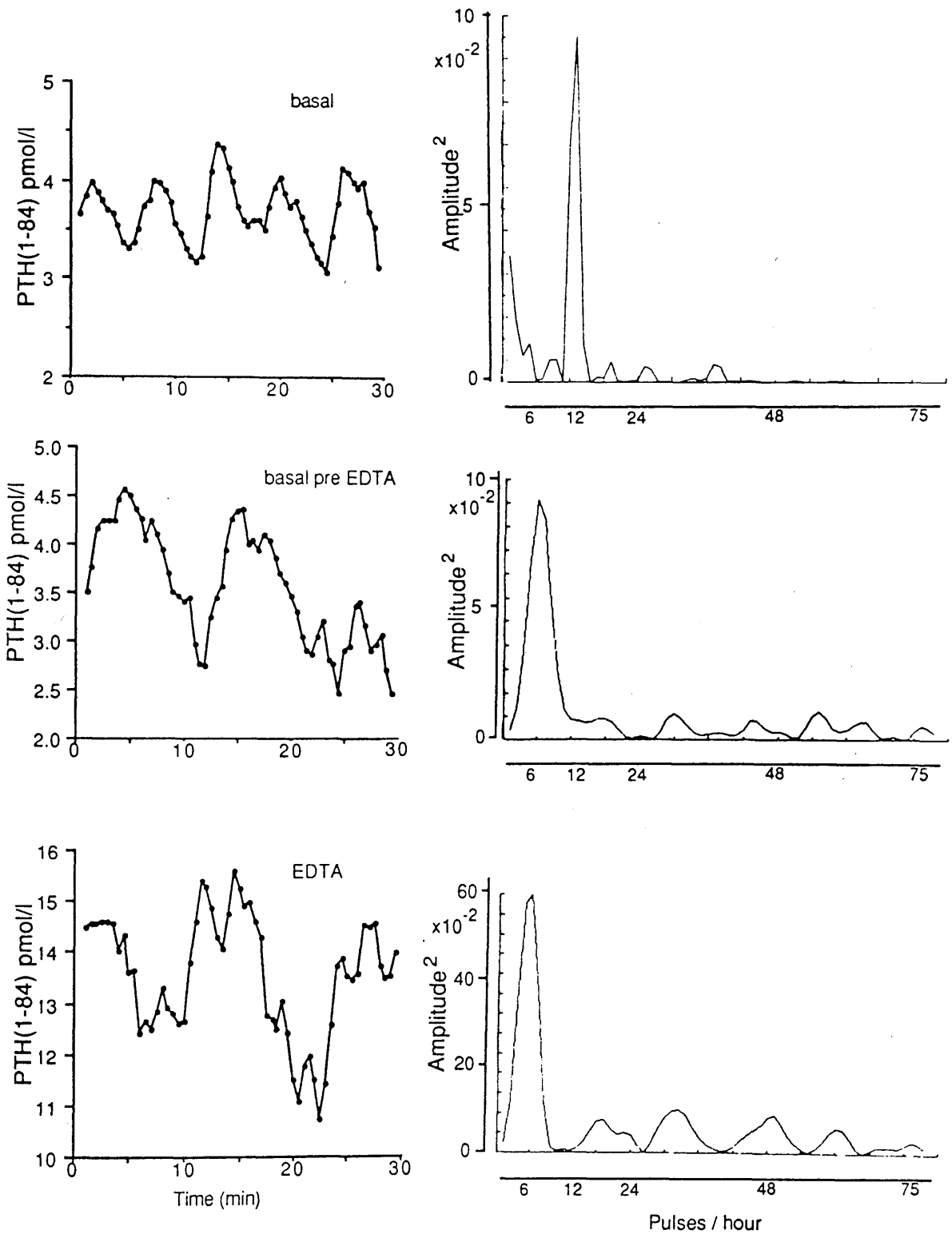


Figure 5.13

PTH (1-84) concentrations (smoothed using a three sample moving average) in subject 1 (DOR) during the initial basal period, the basal period prior to EDTA (1 year later) and during the EDTA infusion. Associated with each profile is the Fourier analysis of the data.

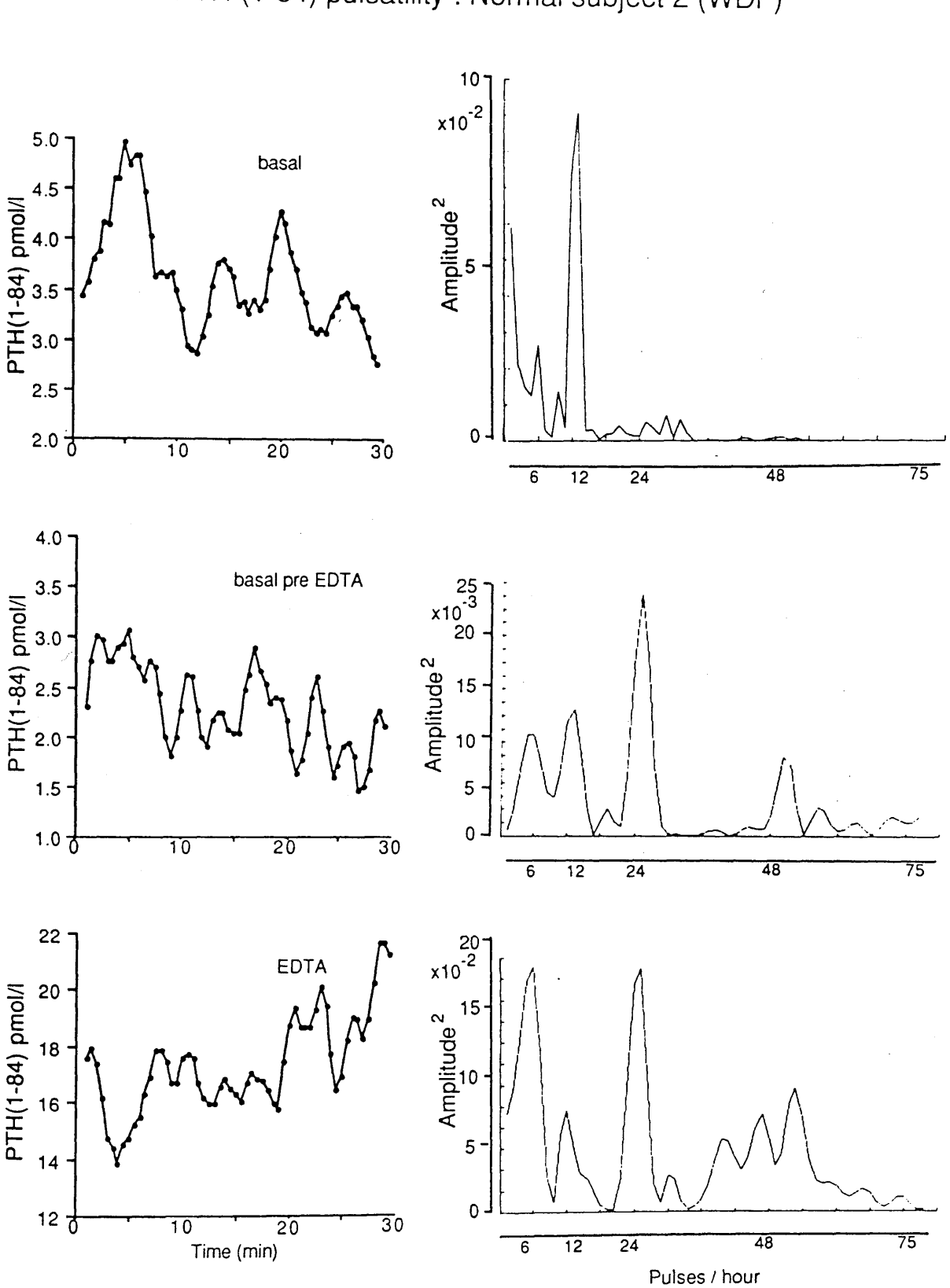


Figure 5.14

PTH (1-84) concentrations (smoothed using a three sample moving average) in subject 2 (WDF) during the initial basal period, the basal period prior to EDTA (1 year later) and during the EDTA infusion. Associated with each profile is the Fourier analysis of the data.

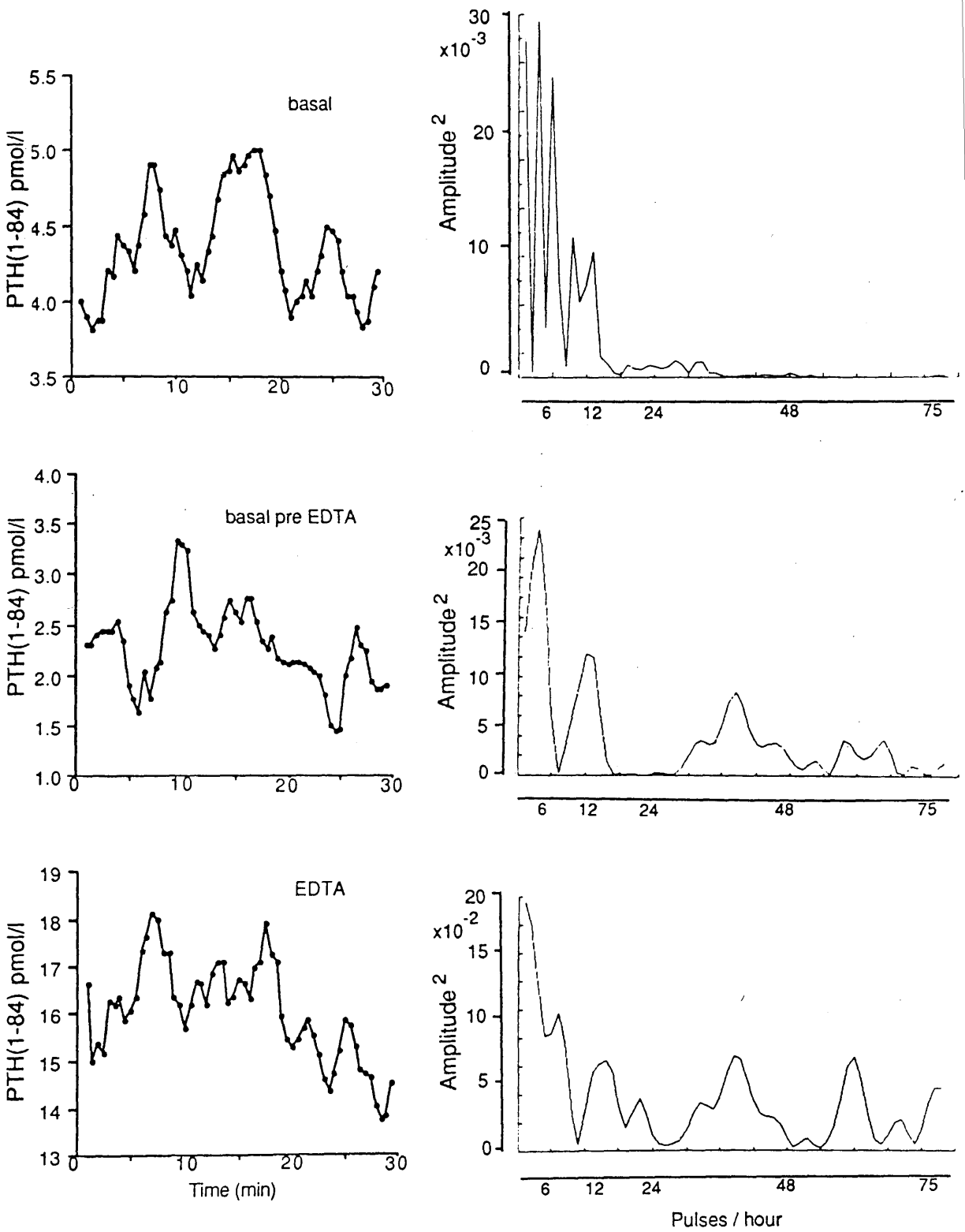


Figure 5.15

PTH (1-84) concentrations (smoothed using a three sample moving average) in subject 3 (FCL) during the initial basal period, the basal period prior to EDTA (1 year later) and during the EDTA infusion. Associated with each profile is the Fourier analysis of the data.

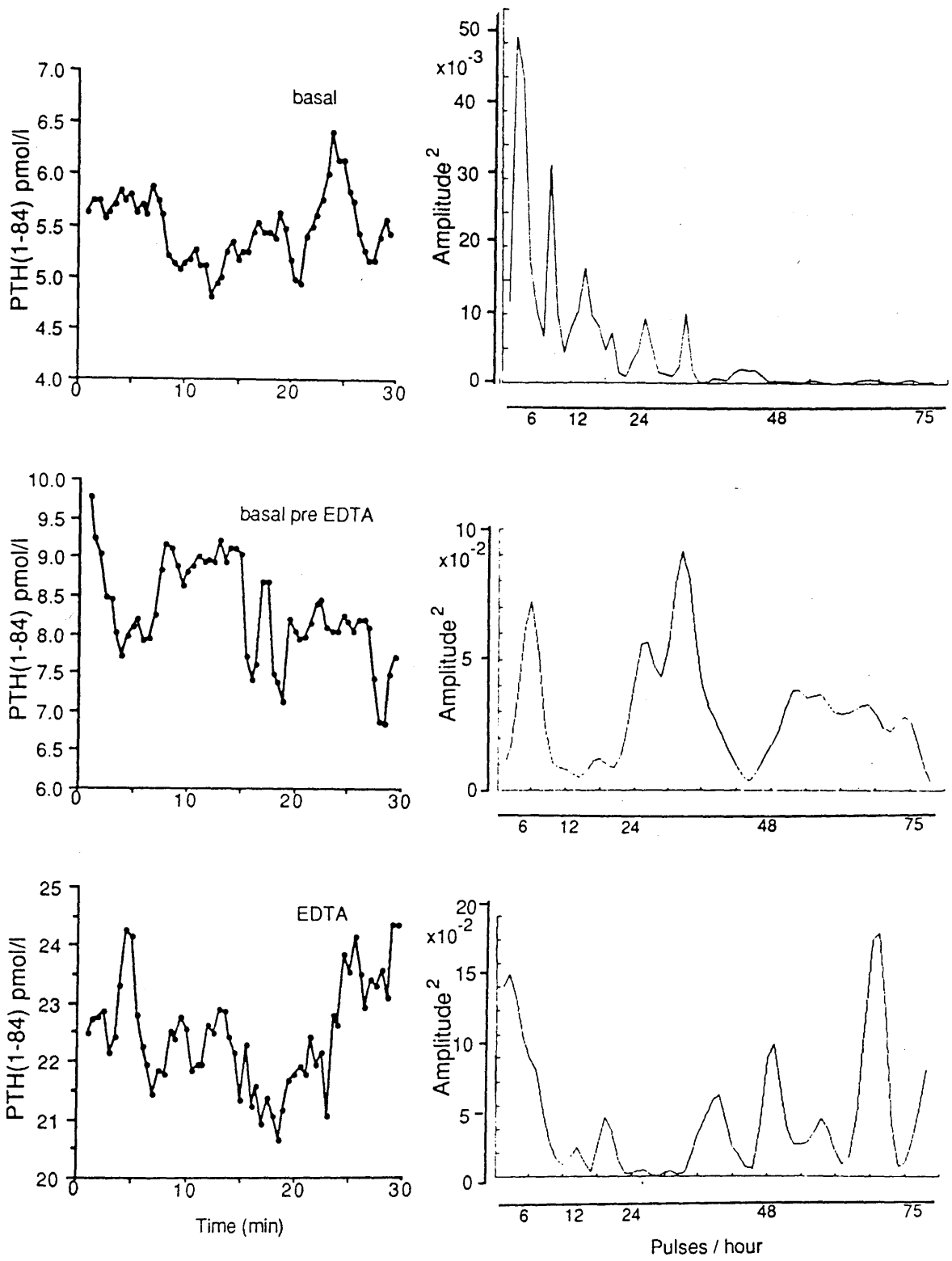


Figure 5.16

PTH (1-84) concentrations (smoothed using a three sample moving average) in subject 4 (GHB) during the initial basal period, the basal period prior to EDTA (1 year later) and during the EDTA infusion. Associated with each profile is the Fourier analysis of the data.

The analysis of the basal profile in subject 4 (GHB) (Figure 5.16) shows a major contribution from the peak with in the range 4 to 6 pulses/h. There are however additional components across a wide range of frequencies (12-30 pulses/h). The pre EDTA profile in this subject shows a complex composition with, in addition to a peak at 6 pulses/h, major contributions from a broad range of higher frequencies (24 to 75 pulses/h). This may to be related to the dietary regime undertaken by this subject. During EDTA fusion the pattern is similar to those observed in the other subjects with contributions from frequencies in the range 3 to 6 pulses/h and from higher frequency components.

By comparison with the normal subjects the analysis of the pre EDTA basal profiles in two of the the hyperparathyroid patients (GR, ME) showed a complex mixture of frequencies in the range 3 to 30 pulses/h (Figures 5.17 & 5.18). During EDTA infusion in these two patients a similar complex mixture of frequencies was observed but with increased amplitude. However the data from the other two hyperparathyroid patients (MC, DW) showed patterns similar to that of the normal subjects with single dominant peaks with period in the range 6 to 12 pulses/h (Figures 5.19 & 5.20). During EDTA infusion in these patients an increase in amplitude and higher frequency components was observed again similar to the effects seen in the normal subjects.

3.4 Discussion

The results of this study confirm pulsatile variation in the circulating concentrations of PTH (1-84) in normal subjects. The observation of pulses occurring at 10-15 minute intervals (4 to 6 pulses/h) as a component common to the profiles from all the normal subjects under basal conditions is consistent with previous data on bioactive PTH (Dixit et al, 1987) and PTH (1-84) (Harms et al, 1989). However the estimate of the frequencies of the components of pulsatility varied both between individuals and also within an individual between the two basal profiles. It is notable however

Figure 5.17

PTH (1-84) concentrations (smoothed using a three sample moving average) in hyperparathyroid patient 1 (GR) during the basal period prior to EDTA , and during the EDTA infusion. Associated with each profile is the Fourier analysis of the data.

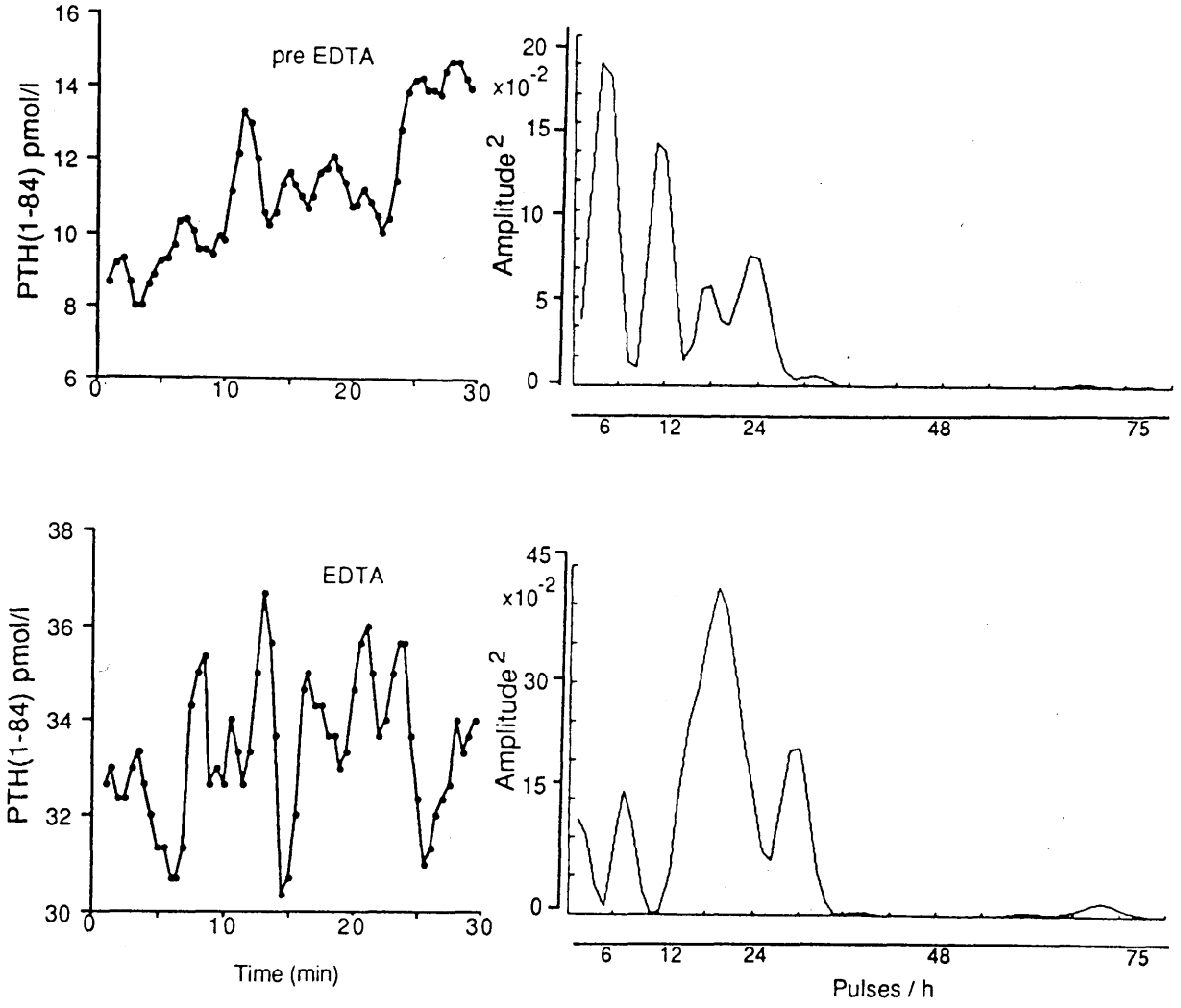


Figure 5.18

PTH (1-84) concentrations (smoothed using a three sample moving average) in hyperparathyroid patient 2 (ME) during the basal period prior to EDTA and during the EDTA infusion. Associated with each profile is the Fourier analysis of the data.

Figure 5.19

PTH (1-84) concentrations (smoothed using a three sample moving average) in hyperparathyroid patient 3 (MC) during the basal period prior to EDTA and during the EDTA infusion. Associated with each profile is the Fourier analysis of the data.

Figure 5.20

PTH (1-84) concentrations (smoothed using a three sample moving average) in hyperparathyroid patient 4 (DW) during the basal period prior to EDTA and during the EDTA infusion. Associated with each profile is the Fourier analysis of the data.

that the qualitative pattern of pulsatility was consistent within an individual subject. Thus the subjects with pronounced pulsatility (i.e. showing a major dominant peak on Fourier analysis) in the initial profile maintained this pattern throughout the study despite a shift in the estimate of pulse frequency. Similarly subjects who showed more complex contributions to the pulsatile profile also maintained this qualitative pattern. There was however good agreement in the estimate of pulse frequencies between the pre EDTA basal and the profile during EDTA within individual normal subjects. The estimate of pulse frequency therefore shows a close relationship within a day but is significantly different between the basal profiles carried out a year apart. This shift in pulse frequency may reflect changes in physiological parameters within an individual.

The EDTA infusion increased PTH (1-84) concentrations in the normal subjects in agreement with previous studies (Hackeng et al, 1986). The effect of EDTA on pulsatility appeared to be an amplification of the components present in the pre-EDTA profile resulting an increase the amplitudes of the components and in the contributions from high frequency rhythms. Thus the response to an EDTA infusion in normal subjects involves an elevation in PTH (1-84) concentrations and modulation of the amplitude and frequency of pulses.

The pattern of pulsatility in the hyperparathyroid patients during the basal profile was not consistent in that two of the patients showed a complex range of component frequencies of increased amplitude relative to the normal subjects while the other two patients showed pronounced pulsatility with a single dominant peak similar to the normal subjects. The patterns were not related simply to either PTH (1-84) or calcium concentration but appeared, like those of the normal subjects, consistent within an individual patient. The response to EDTA was similar to that observed in normal subjects both with respect to the increase in PTH (1-84) and the modulation of pulse amplitude and frequency. This is consistent with the concept of an error in the set point for calcium in primary hyperparathyroidism in that despite

hypercalcaemia the lowering of serum calcium concentration by EDTA infusion produces a response similar to that of observed in normal subjects (Gardin et al, 1988).

The release of a hormone in a pulsatile manner is thought to maximise the efficiency of hormone release and the information content of the signal. In the case of the hormones of the hypothalamic pituitary axis, where continued stimulation of the secretory cells causes desensitization, the cells are activated to release hormone in 'bursts'. The silent periods in between the bursts of activity avoids desensitization of the secretory cell and provides maximum hormone output (Leng 1988a). The bursts of activity can be either limited to within groups of secretory cells producing continuous release of hormone as in the case of vasopressin or synchronised across the gland producing pulsatile release of hormone as for LH (Leng 1988b).

The pulse generator is thought to be an integral part of the physiology of the hypothalamic-pituitary axis acting centrally via the hypothalamus (Lincoln 1988) although the pituitary itself has been shown to have intrinsic pulsatility (Robinson and Dyer 1988). There is no data on the pulse generator for PTH (1-84) secretion. Harms et al, (1989)haveshown the pulsatile variation in circulating concentrations of PTH (1-84) to be independent of changes in serum calcium. The parathyroids are known to respond to adrenergic stimulii (Heath 1980) but further studies would be required to confirm a possible role in pulse generation. Catastrophe theory has shown that combinations of simple linear inputs to a feedback control system or simple chemical reactions can produce oscillations and provide the basis for pulse generation (Leng 1988a). Both cAMP and intracellular calcium have been shown to be capable of producing endogenous oscillations in response to neuromodulatory agents in cell systems (Gillette 1988). Thus it is also possible that the pulsatility is a property either inherent to the cells of the parathyroid glands or arises from the feedback control of PTH (1-84) secretion.

The pulsatile release of a hormone is an important principle in dynamic hormone-receptor interactions with the modulation of pulse amplitude and frequency being major determinants of end organ response (Spratt et al, 1987, Gross et al, 1987). The manipulation of gonadotrophin secretion by GnRH pulse amplitude and frequency modulation has been shown to shift testicular function either to hormone secretion or spermatogenesis. The underlying mechanism is the preferential activation of either the type 1 (cAMP mediated) receptors involved in hormone secretion or the type 11 receptors (inositol phosphate mediated) of spermatogenesis (Spratt et al, 1987). By analogy it has been suggested that pulse amplitude and frequency modulation of PTH (1-84) concentrations governed the expression of either type 1 or type 11 receptors and ultimately PTH biological activity. Following the finding of reduced pulsatility in patients with osteoporosis disturbed glandular PTH (1-84) secretion has been implicated in the pathogenesis of osteoporosis (Hesch et al, 1988).

The concept of modulation of pulsatility as a control system is further supported by the finding of a loss of circadian rhythm but increased pulsatility in fasting subjects (Section 2.7 above) and also by the complex pulsatility found in the subject in this study undertaking a severe dietary regime. It is known that fasting increases the complexity of the pulsatility of growth hormone secretion which is thought to act in mediating the switch from the predominantly gluconeogenic to a lipolytic source of metabolic fuel in prolonged fasting (Ho et al, 1989). Similarly the increased pulsatility may be involved in the mobilisation of calcium from bone during a fast.

In conclusion it seems clear that in common with many other hormones PTH (1-84) is secreted in a pulsatile manner with modulations of amplitude and frequency of pulses being an integral part of the control of PTH biological activity. The assessment of the components of pulsatility in individuals has indicated that while the tendency to either pronounced pulses or a more complex harmonics is characteristic

of the individual the estimate of the component pulse frequency is not consistent between experiments. It remains to be established whether the alterations in amplitude and frequency of pulses seen in the hyperparathyroid patients contribute to or result from the pathogenesis of hyperparathyroidism. Further studies are required to elucidate the role of pulsatility of PTH (1-84) secretion in normal physiology and disease states.

4 STUDIES ON THE PTH (1-84) RESPONSE TO PAMIDRONATE THERAPY IN PATIENTS WITH PAGET'S DISEASE OF BONE AND HYPERCALCAEMIA OF MALIGNANCY

4.1 Introduction

The bisphosphonates,a group of pyrophosphate analogues, are potent inhibitors of osteoclast-mediated bone resorption. Bisphosphonates are used in the treatment of diseases associated with increased bone resorption such as Paget's disease (Russel et al 1974, Frijlink et al, 1979, Adami et al, 1986, Gray et al, 1987), hypercalcaemia associated with malignancy (HCM) (Sleeboom et al, 1983, Singer and Fernandez 1987, Van Breukelen et al, 1982, Ralston et al, 1985, Cantwell and Harris 1987) and involutional osteoporosis (Huaux et al, 1985). Pamidronate (3-amino-1-hydroypropylidene-1,1-bisphosphonate, APD) is one of the more effective bisphosphonates in the treatment of Paget's disease and HCM and the effects of APD treatment on calcium and phosphate homeostasis have been well documented (Van Breukelen et al, 1982, Sleeboom et al, 1985, Singer and Fernandez 1987, Ralston et al, 1988, McCloskey et al, 1988).

APD treatment in patients with Paget's disease or HCM has provided the opportunity to study PTH secretion from normal or suppressed parathyroid glands in response to the lowering of serum calcium (Adami et al, 1982, Sleeboom et al, 1983, Papapoulos 1986). To date however studies on the response of PTH to APD therapy have been limited by PTH assay technology. While responses have been observed with N and

C-terminal immunoassays the results have been inconsistent (Adami et al, 1982, Sleeboom et al, 1983). More recently, using homologous immunoradiometric assays specific for either the N or C terminal, increases in PTH were observed only when the patients became hypocalcaemic (Papapoulos et al, 1986). The development of sensitive and specific two-site immunometric assays for PTH (1-84) has allowed changes within the reference interval in normal subjects to be demonstrated (Logue et al, 1989).

We therefore re-examined the PTH response to APD therapy in Paget's disease and HCM. Also included in the study are two indirect measurements of PTH secretion, the maximum tubular reabsorption of phosphate per litre glomerular filtrate (TmPO4/GFR) (Bijvoet 1977) and nephrogenous cyclic adenosine monophosphate (NcAMP) (Broadus et al 1977). Through the comparison of these indices with the PTH (1-84) it was hoped to identify the role, if any, of PTHrP in the response to APD in HCM.

4.2 Patients and Methods

Twelve patients with Paget's disease (4 M; 8 F; aged 51-63 yrs) were included in the study. All had their bone disease confirmed by standard biochemical, radiological methods and where appropriate by histomorphometry. All were studied prior to and following a single intravenous dose of 45 mg Pamidronate, which was given over 4 hours in 0.5 litres of 0.9% NaCl.

Twelve patients with HCM (7 M; 5 F; aged 48-78 yrs) were also studied. The origin of the primary tumour in these patients was breast (3), myeloma (2), lung (4); lymphoma (2) and genito-urinary tract (1). Standard radionuclide bone scan was used to assess the presence and extent of metastatic bone disease. Eight of the twelve patients had metastatic bone disease, and the tumour load was estimated as being heavy in two and light in six patients. All patients were rehydrated over a 24 hr period with a minimum of 2 litres of intravenous 0.9% NaCl prior to the

administration of a single intravenous dose of 30 mg Pamidronate given over 4 hours in 0.5 litres of 0.9% NaCl. Serum creatinine concentrations were within the reference range (60-130 μmol/l) in ten patients and elevated in two (150, 180 μmol/l) following rehydration. No patient developed renal failure during the study.

In both groups of patients blood samples were obtained by venesection prior to Pamidronate (day 0) and daily thereafter for seven days. All blood specimens were collected between 10.00-11.00 hrs, and a timed (2 hour) fasting urine was obtained after discarding the first morning urine specimen. Serum, K-EDTA plasma and urine were stored at -20°C prior to analysis.

Serum and urinary calcium, albumin, phosphate and creatinine were measured using standard automated methods. Serum calcium was adjusted for variations in albumin concentration, as describedpreviously(Gardner et al, 1981). Intact PTH (1-84) was measured in serum using an in-house immunoradiometric assay (Logue et al, 1990a). $TmPO_4/GFR$ was determined according to the method of Bijvoet (1977). Plasma cAMP was measured with a commercially available kit (Amersham International plc, Aylesbury, Bucks, UK); urinary cAMP with an in-house radioimmunoassay (O'Reilly et al, 1986) and nephrogenous cAMP calculated by the method of Broadus et al (1977). Each series of samples for an individual patient was analysed within a single batch.

Statistical analysis was performed using a paired difference t test to compare each sample point with the appropriate day zero result for the individual patient.

4.3 Results

The mean (± SEM) results for the parameters measured in the patients with Paget's disease are displayed in Figure 5.21. Prior to therapy, the mean concentration of all four parameters was within the appropriate reference interval: adjusted calcium 2.20-2.60 mmol/L; PTH (1-84) 1.0-5.0 pmol/L; $TmPO_4/GFR$ 0.80-1.35 mmol/L;

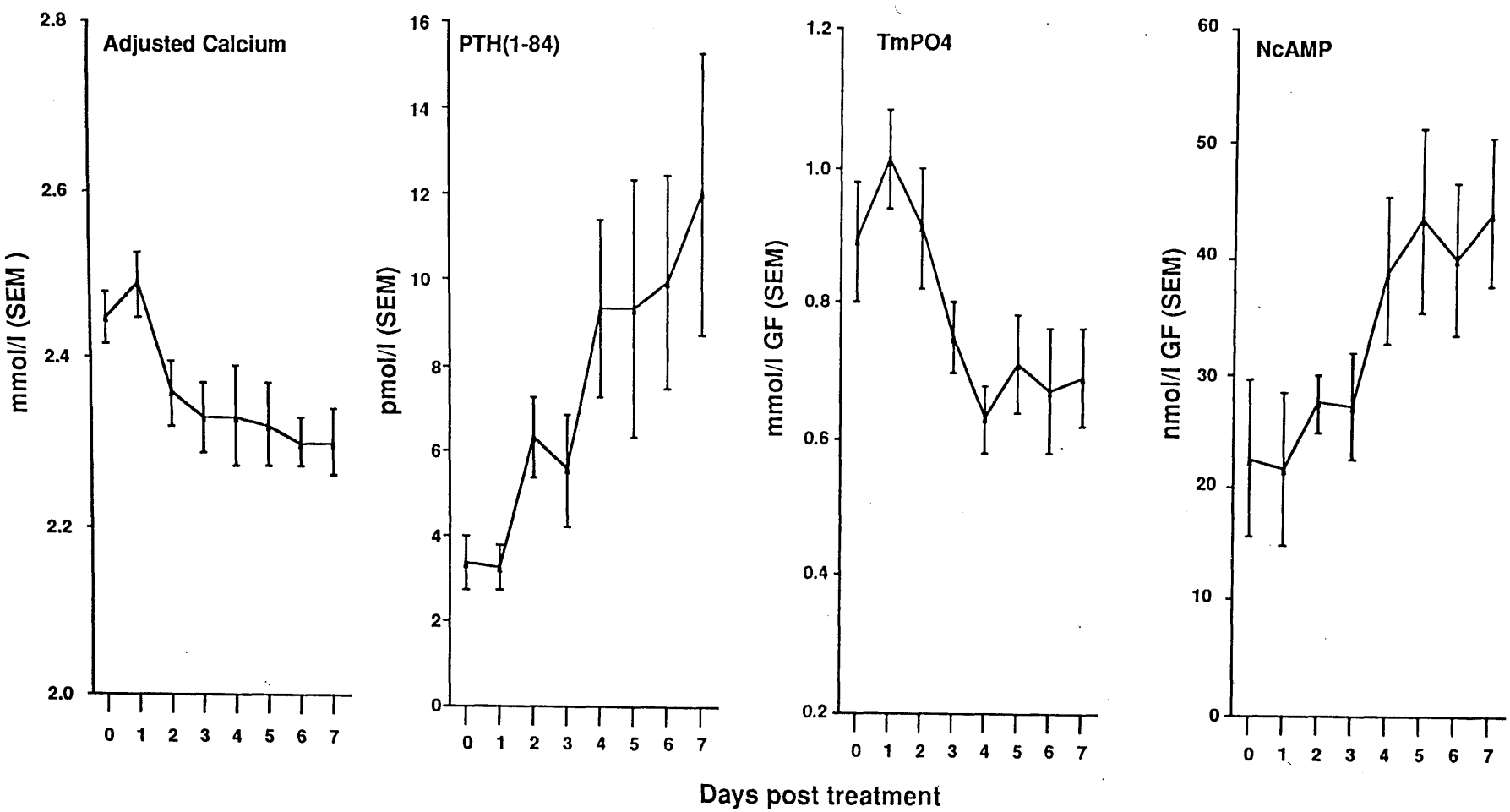


Figure 5.21

Adjusted calcium, PTH (1-84), $TmPO_4/GFR$ and NcAMP in patients with Paget's Disease of Bone following Pamidronate treatment.

NcAMP 8-28 nmol/L. Following Pamidronate, there was a small but significant fall in serum adjusted calcium, within the reference range, on day 2 ($p < 0.01$) which was sustained throughout the remainder of the study. A significant increase in PTH (1-84) occurred on day 2 ($p < 0.05$). The serum PTH (1-84) concentration continued to increase throughout the period of the study, reaching values of more than twice the upper limit of the reference interval. The $TmPO_4/GFR$ fell gradually to a trough on day 4 ($p < 0.001$), which persisted thereafter. In keeping with the rise in PTH (1-84) NcAMP rose to a peak on day 4 ($p < 0.01$), which was also sustained. These results are consistent with a normal PTH response to a Pamidronate induced reduction of serum calcium.

The mean (± SEM) results for the parameters measured in the patients with HCM are displayed in Figure 5.22. Following rehydration, but prior to Pamidronate, all subjects had elevated concentrations of serum adjusted calcium (range 2.85-3.95 mmol/L). Nine patients had PTH (1-84) concentrations below the assay detection limit (0.5 pmol/L), whilst the other three patients had concentrations close to the low end of the reference interval (0.9-1.1 pmol/L). Despite this suppression of PTH (1-84), the mean $TmPO_4/GFR$ concentration was below the reference interval and the mean NcAMP concentration was significantly elevated.

Following Pamidronate therapy, there was a rapid and continuing fall in the serum adjusted calcium in all subjects, with the mean concentration reaching the upper limit of the reference interval on day 5. PTH (1-84) concentrations remained suppressed for days 1-3 but rose significantly on day 4 ($p < 0.05$) and continued to rise, such that the mean concentration was above the reference interval at the end of the study. The $TmPO_4/GFR$ and NcAMP responses to Pamidronate therapy were complex. Thus, the initially subnormal $TmPO_4/GFR$ fell significantly, reaching a nadir on day 5 ($p < 0.001$) before rising significantly on the final day of the study ($p < 0.01$). NcAMP concentrations showed the reverse effect, with a significant rise from the elevated basal concentration to a peak on day 5 ($p < 0.01$) which fell significantly on

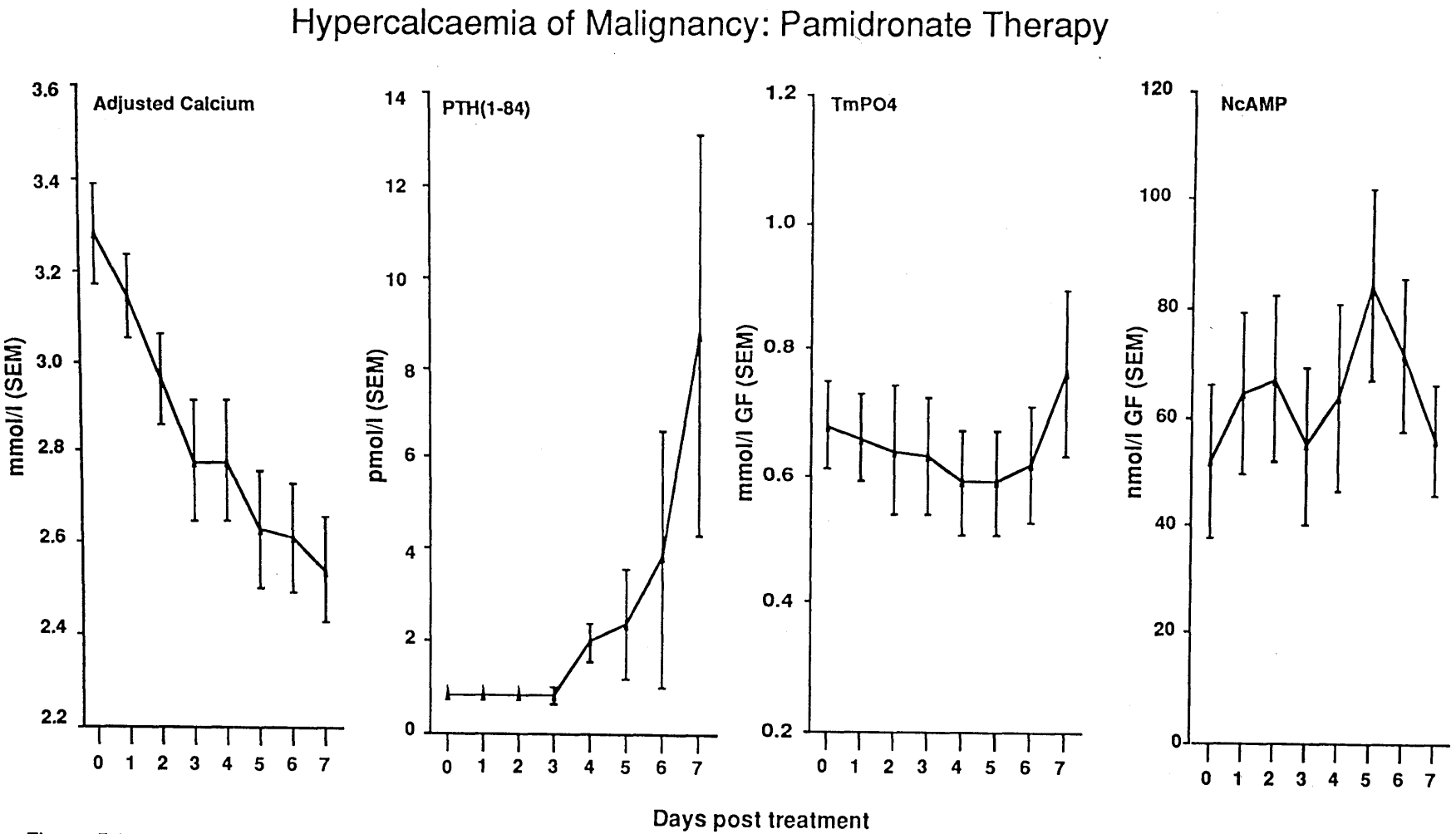


Figure 5.22

Adjusted calcium, PTH (1-84), $TmPO_4/GFR$ and NcAMP in patients with hypercalcaemia of malignancy following Pamidronate therapy.

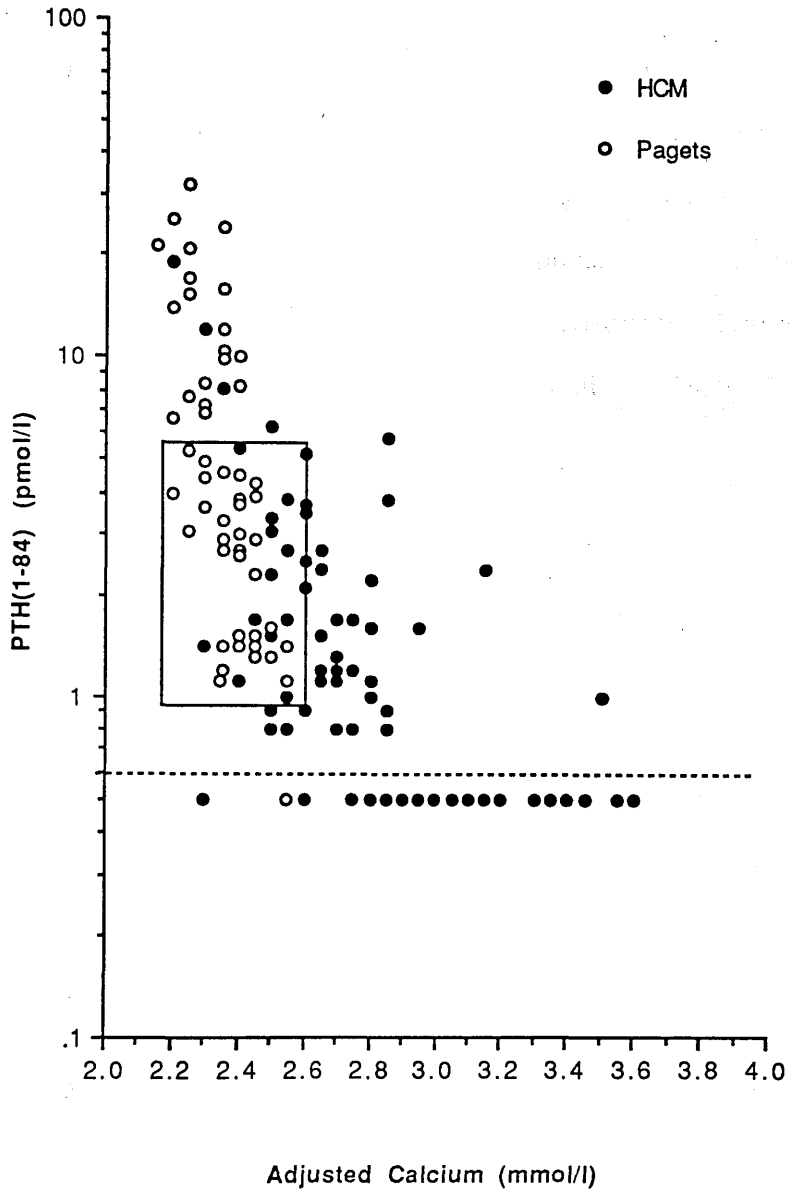


Figure 5.23

PTH (1-84) values corresponding to the Adjusted calcium value obtained on the same sample in Paget's Disease of bone and hypercalcaemia of malignancy. Enclosed rectangle represents values within reference ranges, dotted line limit of detection for PTH (1-84).

possible to demonstrate a significant increase in PTH (1-84) secretion coincident with the fall in serum calcium. The concentration of PTH (1-84) rose progressively during APD therapy despite the fact that the serum calcium did not continue to fall after day 2 and despite the fact that no patient became frankly hypocalcaemic.

The increased secretion of PTH (1-84) following APD therapy in Paget's disease could also be detected by measurement of the indirect parameters of PTH (1-84) action. PTH acts via renal tubular receptors and cyclic AMP second messenger to reduce the maximal tubular reabsorption of phosphate. Thus the findings of decreased serum phosphate, decreased TmPO4 and increased NcAMP following APD therapy are consistent with the known biological effects of increased PTH (1-84) secretion (Bijvoet 1977, Broadus et al, 1977).

The patients with HCM showed the expected combination of hypercalcaemia and suppressed serum PTH (1-84) concentrations prior to therapy with APD. However the pretreatment concentrations of $TmPO_4$ and NcAMP were decreased and increased respectively. These observations are consistent with the recognised existence, in a proportion of these patients, of PTHrP a molecule which is structurally distinct from PTH (1-84) but which contains striking homology in the N terminal amino acid sequence and so binds the renal tubular PTH receptor (Stewart et al, 1983, Ralston et al, 1984, Suva et al, 1987).

APD therapy to the patients with HCM produced the expected sharp and prolonged decrease in serum adjusted calcium (Van Breukelen et al, 1982, Sleeboom et al 1985, Singer and Fernandez 1987, Ralston et al, 1988, McCloskey et al, 1988). At the end of the study the mean adjusted calcium was within the reference intervals with a range 2.25 to 2.80 mmol/l. This study therefore confirms the effectiveness of APD as a therapeutic agent in HCM. Of particular interest however, were the PTH (1-84) results for these showed a significant rise on day 4 and continued to increase to a mean concentration above the reference interval. The initial increase in PTH (1-84)

occurred at a time when the mean serum adjusted calcium was above the reference interval, and the elevated PTH (1-84) concentrations occurred when the mean adjusted calcium was within the reference interval with no patient hypocalcaemic. These observations contrast with previous reports that the PTH secretion occurs only as patients become hypocalcaemic (Papapoulos et al 1986) and strongly suggest a resetting of the 'trigger point' for PTH secretion in patients with HCM. The concept of an altered 'trigger point' for PTH (1-84) secretion may explain a number of conditions related to altered calcium homeostasis (Parfitt 1979). Previous studies using acute changes in calcium have shown both rate of fall and direction of change to be important determinants of the PTH response in normal subjects and patients with secondary hyperparathyroidism (Adami et al, 1982, Cunningham et al, 1989). However, the data from this study indicate that in HCM the absolute calcium concentration is more important in 'triggering' PTH (1-84) secretion than the rate of fall in calcium that can be obtained following Pamidronate therapy.

The clinical implication of these results is related to the interpretation of PTH (1-84) concentrations in patients receiving APD therapy. Confusion between the two major causes of hypercalcaemia (primary hyperparathyroidism or malignancy) may occur in HCM if a sample is obtained after treatment with APD as in some cases a modest lowering of the serum calcium results in detectable PTH (1-84) secretion.

The observed changes in TmPO4/GFR and NcAMP following treatment of HCM with APD require careful interpretation. If PTHrP is responsible for the abnormal concentrations of these parameters pretreatment in some patients then the exacerbation of the abnormality (decreasing TmPO4/GFR increasing NcAMP), before a significant rise in PTH (1-84), from 2 to 5 days after APD may suggest increased secretion of PTHrP at this time, either as a direct consequence of the therapy or as a manifestation of the pathophysiology of the disease. The paradoxical increase in TmPO4/GFR and decrease in NcAMP on day 7 is also interesting for it occurs at a time of increasing PTH (1-84) secretion and yet is suggestive of

decreased stimulation of the renal tubular PTH receptor. The explanation of this phenomenon must await validated assays for PTHrP as the assays currently available give conflicting results in some patient groups depending on methodology and specificity (Henderson et al, 1990, Burtis et al, 1990).

5 TREATMENT OF SECONDARY HYPERPARATHYROIDISM IN HAEMODIALYSIS PATIENTS

5.1 Introduction

Secondary hyperparathyroidism is a consistent feature of advanced renal failure. Plasma concentrations of parathyroid hormone become elevated early in renal disease and often continue to increase as renal function declines (Reiss et al, 1968, Arnaud 1973). Nearly all patients with renal failure show some degree of secondary hyperparathyroidism and this is thought to be an adaptive response to hypocalcaemia which occurs because of hyperphosphataemia and reduced calcium absorption (Fuss et al, 1976, Avram et al, 1978, Bricker 1972). In addition, skeletal resistance to the effects of PTH, and altered sensitivity of the parathyroid glands to feedback regulation by calcium, may both play a role (Llach et al, 1975, Brown et al, 1982).

More recently it has been suggested that reduced production of $1,25(OH)_2D_3$ in renal failure may lead to hyperparathyroidism by a direct effect on the parathyroid gland. $1,25(OD)_2D_3$ binds to specific receptors on parathyroid tissue and suppresses the levels of m-RNA for the synthesis of pre-pro PTH, the biosynthetic precursor of PTH (Karmall et al, 1989). Through this mechanism the low plasma concentrations of $1,25(OH)_2D_3$ in advanced chronic renal failure are thought to be an additional important cause of PTH hypersecretion (Slatopolsky et al, 1984, Korkor 1987, Sherwood 1987, Feinfield and Sherwood 1988, Slatopolsky et al, 1988).

Calcitriol ($1,25(OH)_2D_3$) is effective in the prevention and treatment of secondary hyperparathyroidism in uraemic patients undergoing dialysis (Coburn et al, 1977, Mason et al, 1980, Baker et al, 1986). However, the occurrence of hypercalcaemia limits the dose tolerated in the long term to 0.5 mg/day or less (Coburn et al, 1977, Brickman et al, 1980). Furthermore the $1,25(OH)_2D_3$ plasma concentrations achieved by these doses are likely to be low because of the significant degree of Calcitriol metabolism in the gut (Napoli et al, 1983).

Even with optimal serum calcium concentrations many patients treated with Calcitriol develop progressive secondary hyperparathyroidism and eventually require subtotal parathyroidectomy (Kanis et al, 1976, Shimamatsu et al, 1987, Sharman et al, 1982). Recently Slatopolsky and co-workers have tested the hypothesis that the administration of Calcitriol intravenously might achieve better PTH suppression (Slatopolsky et al, 1984). They showed that excellent control of PTH secretion could be achieved by a thrice-weekly injection of Calcitriol. High doses were tolerated without hypercalcaemia occurring and higher plasma concentrations of $1,25(OH)_2D_3$ were achieved with intravenous compared with oral administration.

In this study we attempted to suppress secondary hyperparathyroidism with high doses of oral Calcitriol since the intravenous preparation is generally unavailable, and we prevented hypercalcaemia occurring by using a low calcium dialysate. In addition we compared the effects of daily vs thrice-weekly administration of Calcitriol, since it might be the intermittent dosage regimen rather than the intravenous route of administration which led to the better suppression of hyperparathyroidism in the study of Slatopolsky et al (1984).

5.2 Patients and Methods

Nineteen patients undergoing regular haemodialysis gave written informed consent to enter the study which was approved by the local ethical committees. There were 13

men and 6 women of mean age 40 years (range 19-67 years). These patients were dialysed for 4-5 hours thrice-weekly and the mean duration of haemodialysis was 39 months (range 3-96 months). The original diagnoses were glomerulonephritis in 6, the urological complications of spina bifida in 3, Alport's syndrome in 2, reflux nephropathy in 2, renovascular disease in 2, and mega-ureter, amyloidosis, Fabry's disease and polycystic kidneys in one each. Seven patients were receiving vitamin D analogues and five calcium carbonate, but these were stopped prior to the study. Twelve patients were receiving aluminium hydroxide in a mean dose of 2400 mg/day and this was continued during the study.

All patients had biochemical evidence of secondary hyperparathyroidism with a mean PTH (1-84) plasma concentration of 62 pmol/l (range 15-125),the normal range being 1-5 pmol/l. Nine patients had radiological evidence of renal osteodystrophy and 9 had elevated serum alkaline phosphatase concentrations (ALP greater than 110 IU).

Patients were randomly assigned to receive daily (conventional), or thrice-weekly (intermittent) therapy with Calcitriol. For 2 weeks (control period) all patients were dialysed using a dialysate containing 1.0 mmol/l of calcium instead of one containing 1.55 mmol/l. For 12 weeks (treatment period) patients received oral Calcitriol and continued to dialyse with low calcium dialysate. The conventional group received 0.25 microgrammes as the single morning oral daily dose with a dosage increment of 0.25 microgrammes at the beginning of each subsequent week until the serum calcium concentration, corrected for albumin, was 2.6-2.7 mmol/l after which the dose was maintained at that level. If the serum calcium exceeded 2.7 mmol/l Calcitriol was withheld until it had fallen to the desired range and it was then recommenced at 0.25 microgrammes less than the previous dose. The intermittent group received 0.5 microgrammes orally thrice weekly at the end of each dialysis with a dosage increment of 0.5 microgrammes at the end of each subsequent week until the serum calcium concentration was in the range 2.6-2.7 mmol/l. If the serum

calcium exceeded 2.7 mmol/l, Calcitriol was withheld until the calcium fell to the desired range, after which it was recommenced at 0.5 microgrammes less than the previous dose. Calcium carbonate (Titralac) was prescribed if there was no rise in serum calcium after several weeks of Calcitriol therapy.

Predialysis serum calcium, phosphate, albumin and alkaline phosphatase were measured twice weekly. During the control period and during the last 2 weeks of therapy serum aluminium and magnesium, plasma PTH (1-84) and serum $1,25(OH)_2D_3$ were measured twice weekly and for the rest of the study once weekly. All bloods were taken pre-dialysis which meant that $1,25(OH)_2D_3$ concentrations were measured 1-6 hours after the morning Calcitriol dose in the conventional group and 44-68 hours after the last post-dialysis Calcitriol dose in the intermittent group.

Analytical Methods

Calcium, phosphate, alkaline phosphatase (ALP) and albumin were measured by standard automated techniques. Magnesium and aluminium were measured by atomic absorption spectroscopy.

Intact PTH (1-84) was measured using the two-site immunometric assay with a sensitivity of 0.5 pmol/l and a range of 1.5-250 pmol/l. It has an intra-assay coefficient of variation less than 10%. The normal range is 1-5 pmol/l and the assay is unaffected by a 200 molar excess of C-terminal fragments (Logue et al, 1990a).

$1,25(OH)_2D_3$ was measured using the method of Rheinhardt et al (1984) but using high performance liquid chromatography (HPLC) in the final purification of vitamin D metabolite from serum. The intra-assay coefficient of variation is less than 10% and the normal range 30-100 pmol/l. The sensitivity of the assay is 5 pmol/l and for statistical purposes concentrations lower than this have been arbitrarily assigned at 4 pmol/l.

Statistics

Paired pre- and post-treatment changes in PTH, $1,25(OH)_2D_3$, calcium and alkaline phosphatase were assessed by the Wilcoxon Signed Rank Test. The relative effects of the 2 different dosage regimens on these parameters were compared using the Wilcoxon Rank Sum Test.

5.3 Results

The treatment was well tolerated, but one patient in the intermittent group complained of gastric fullness and dyspepsia for 24 hrs after each dose of Calcitriol and he was withdrawn from the study after 11 weeks. Two other patients were withdrawn from the study after 12 weeks following renal transplantation and urological surgery. The results of all patients are included in the analysis.

All but three patients achieved a net rise in serum calcium and all but one patient achieved a net fall in PTH (1-84). Nine patients had their dose of Calcitriol reduced because of hypercalcaemia (adjusted serum calcium greater than 2.7 mmol/l), but in no patient was this associated with symptoms and in all cases it resolved within 2-4 days. Seven patients (4 conventional, 3 intermittent) required calcium carbonate supplements to facilitate a rise in serum calcium.

The mean dose of Calcitriol at the end of the study was 2.0 microgrammes/day (range 1-3 microgrammes/day) in the conventional group and 2.6 microgrammes post-dialysis (range 1-4 microgrammes post-dialysis) in the intermittent group.

The biochemical results are displayed in Figures 5.24 and 5.25 and Tables 5.6 and 5.7. The mean plasma PTH (1-84) concentration fell from 62 pmol/l (range 15-125 pmol/l) during the control period to 39 pmol/l (range 5-120 pmol/l) in the first month of treatment ($p < 0.01$). The mean PTH (1-84) concentration then fell further to 22 pmol/l (range 1-70 pmol/l) at the end of the treatment period (Figure 5.24). The

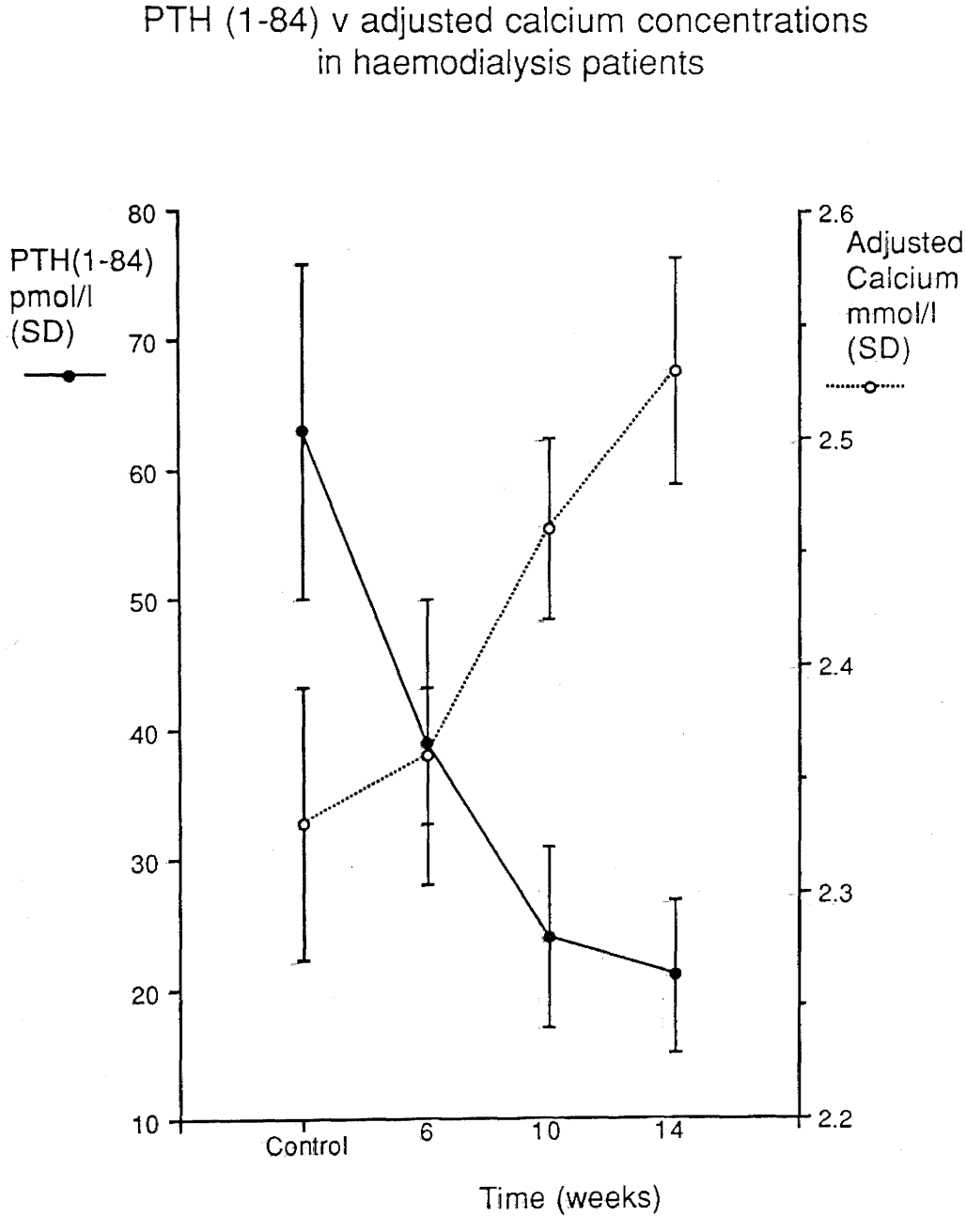


Figure 5.24

Mean adjusted serum calcium and serum PTH (1-84) concentrations at 4 week intervals.

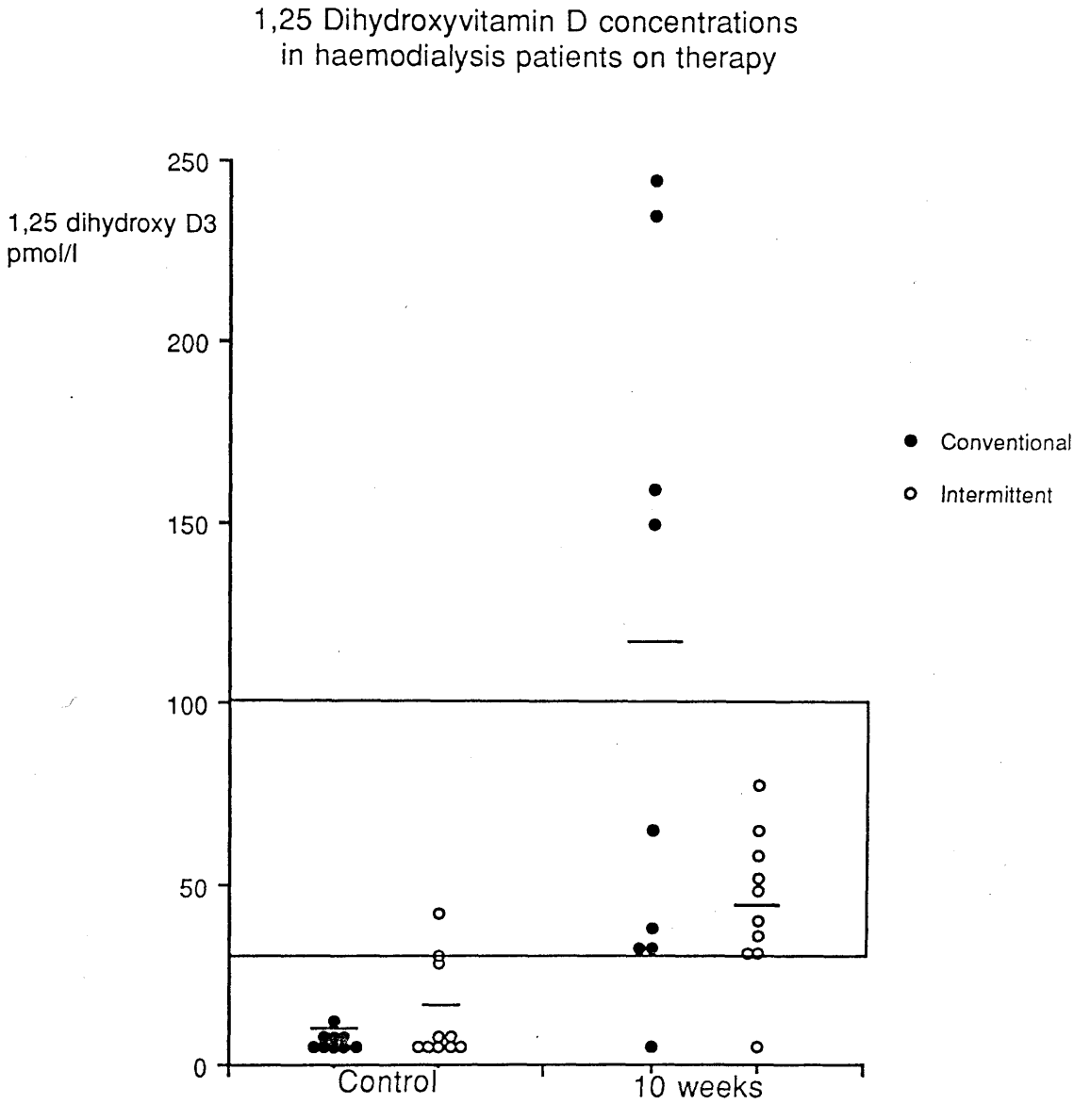


Figure 5.25

$1,25(OH)_2D_3$ serum concentrations during the control period and at 10 weeks in the conventional and intermittent groups and mean concentrations (——). The serum concentrations for the conventional group are (1-6) hours post-dose, whereas those for the intermittent group are (44-68) hours post-dose. The boxed area is the $1,25(OH)_2D_3$ normal range for healthy individuals.

Table 5.6

Comparison between control and final mean values for phosphate, ALP, aluminium and magnesium

	CONTROL	FINAL	
Phosphate (mmol/l)	2.11 (1.17-3.47)	2.22 (1.33-3.26)	N.S.
ALP (IU)	144 (48-461)	123 (61-346)	$p < 0.05$
Aluminium (µmol/l)	1.45 (0.8-2.9)	1.51 (0.4-2.6)	N.S.
Magnesium (mmol/l)	1.07 (0.62-1.38)	1.11 (0.78-1.81)	N.S.

Table 5.7

Comparison between initial and final mean values for adjusted calcium, PTH (1-84) and ALP in the conventional and intermittent therapy groups

		INITIAL	FINAL
Adjusted calcium (mmol/l)	conventional	2.33 (2.19-2.45)	2.45 (2.26-2.62)
	intermittent	2.33 (2.05-2.55)	2.58 (2.34-2.62)
PTH (1-84) (pmol/l)	conventional	64 (15-125)	26 (1-70)
	intermittent	61 (20-125)	18 (4-41)
ALP (iu/l)	conventional	163 (48-461)	138 (61-346)
	intermittent	128 (70-245)	108 (72-155)

mean serum calcium concentration during the control period was 2.33 mmol/l (range 2.05-2.55 mmol/l) and after one month of Calcitriol was 2.36 mmol/l (range 2.09-2.59 mmol/l). At the end of the treatment period the mean serum calcium concentration had risen to 2.52 mmol/l (range 2.26-2.67 mmol/l) ($p < 0.01$ (Figure 5.24)). The mean serum alkaline phosphatase concentration fell from 144 IU/l (range 48-461IU/l) to 123 IU/l (range 61-346 IU/l) at the end of the treatment period ($p < 0.05$) (Table 5.6). There were no significant changes in the mean serum phosphate, magnesium or aluminium concentrations during the study (Table 5.6). Sixteen patients were taking aluminium hydroxide at the end of the study in a mean dose of 2500 mg/day.

Ten patients were entered in the intermittent group and 9 patients in the conventional group. There were no significant differences in the biochemical changes between the two treatment regimens (Table 5.7), apart from serum $1,25(OH)_2D_3$ concentrations. Mean $1,25(OH)_2D_3$ concentrations in the control period were low in both patient groups (Figure 5.25). The 1,25(OH)2D3 concentrations measured 1-6 hours after Calcitriol increased from a mean of 5 pmol/l (<5-17) in the control period to 114 pmol/l (<5-225) at week 10 in the conventional group ($p < 0.01$). The mean serum concentrations measured 44-68 hours post-dose in the intermittent group rose from 13 pmol/l (<5-43) in the control period to 48 pmol/l (<5.-71) at week 10 ($p < 0.01$) (Figure 5.25).

5.4 Discussion

This study shows that in patients with moderately severe secondary hyperparathyroidism, even after many years of renal replacement therapy, medical treatment can achieve marked parathyroid suppression. The combination of high oral doses of Calcitriol and low calcium dialysate was well tolerated and achieved much higher serum concentrations of $1,25(OH)_2D_3$ without causing hypercalcaemia than oral Calcitriol therapy alone (Slatopolsky et al, 1984). There was strong evidence of

a direct effect of $1,25(OH)_2D_3$ on the parathyroid gland since PTH (1-84) concentrations fell significantly during the first month of active treatment despite static serum calcium concentrations. An additive suppressive effect of PTH (1-84) secretion was observed when the serum calcium had increased in the latter two months with higher doses of Calcitriol.

There was no difference in the degree of PTH (1-84) suppression between patients receiving daily Calcitriol and those receiving it thrice-weekly. Studies in the rat demonstrate that maximal inhibition of PTH m-RNA synthesis occurs at concentrations of 1,25(OH) Vit D around 50-100 pmol/l (Silver et al, 1986) which is similar to the concentrations measured 44-68 hrs after Calcitriol at the end of this study. A 'therapeutic range' for $1,25(OH)_2D_3$ concentrations in chronic renal failure in man has not yet been determined nor have the relative importance of high 'peak' or sustained 'trough' concentrations. However, we have clearly achieved concentrations sufficient for a good clinical response in both groups.

The serum $1,25(OH)_2D_3$ concentrations were low or undetectable at the beginning of the study and were significantly increased after 10 weeks in both treatment groups. The serum $1,25(OH)_2D_3$ concentrations were not directly comparable between the two groups because of the different times of sampling. However, serum concentrations measured 44-68 hrs after the last dose in patients in the intermittent group were well within the normal range and this demonstrates that the gut metabolism of Calcitriol after oral treatment is not a significant impediment to the attainment of physiological serum $1,25(OH)_2D_3$ concentrations (Napoli et al, 1983).

Calcitriol has been shown to increase phosphate as well as calcium absorption leading to hyperphosphataemia (Ramirez et al, 1986, Demantis et al, 1986, Fournier et al, 1987). Mean phosphate and aluminium concentrations did not rise during the study, but there was a modest increase in the requirement for aluminium containing phosphate binders. The overall control of phosphate in these patients was poor from

the beginning of the study, despite thrice-weekly dialysis and advice about dietary restriction and phosphate binding therapy. As a result the mean Ca/PO_4 product was high at 4.87 mmol/l (2.87-8.44) and increased with the rise in calcium to 5.56 mmol/l (3.48-8.45) at the end of the study. The risk of metastatic calcification is proportional to the level of Ca/PO_4 (Parfitt 1969b) and we would therefore not suggest attempting to increase the serum calcium level in longer term studies of PTH (1-84) suppression unless the serum phosphate was well controlled.

The mean calcium loss was calculated (by measuring pre- and post-dialyser dialysate calcium concentrations, dialysate flow rates and dialysis time) at 6.3 mmol/l (250 mg) per dialysis. Seven patients required supplementary oral calcium carbonate to facilitate a rise in serum calcium during the treatment period. In these patients the dietary calcium intake was presumably inadequate and a higher dialysate calcium could have been used without causing hypercalcaemia (Raman et al, 1976).

We have achieved a 65% reduction in PTH (1-84) levels in patients with moderately severe secondary hyperparathyroidism over 12 weeks. This is comparable to the effects of intravenous Calcitriol reported by Slatopolsky et al (1984). Regimens utilising high doses of Calcitriol may offer considerable clinical benefits if these effects can be shown to be sustained in longer term studies. The combination of high doses of oral Calcitriol and hypocalcaemia dialysate has advantages over intravenous Calcitriol in that it is more readily available, cheaper, and more applicable to patients treated by CAPD.

Furthermore, we have shown that thrice-weekly Calcitriol therapy is as effective as daily therapy. This may have implications for simplifying treatment regimens and suggests that even wider spacing of doses should be investigated.

CHAPTER 6

GENERAL DISCUSSION

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The production of antibodies useful in diagnostic PTH assays has traditionally been difficult. The immune response to PTH (1-34) in mice has been shown to be under genetic control (Nussbaum et al, 1985) and the antibody responses to PTH (1-34) obtained in this study are consistent with a species and strain specific response. In this project the strategy of using chemically synthesised peptides to generate site specific monoclonal antibodies was successful in producing N-terminal, but not C-terminal monoclonal antibodies. It is interesting that other groups produced by similar strategies C-terminal, but not N-terminal monoclonal antibodies. This may reflect in part differences in the selection and preparation of the immunogens and in immunisation schedules. The production of monoclonal antibodies is totally dependent, however, on the selection procedure and as such the antibody screening assay is central to the process.

As radiolabelled (hence oxidised) PTH (1-34) was employed in the antibody screening assay in this study, it is therefore perhaps not surprising that monoclonal antibodies which recognised oxidised PTH (1-34) preferentially were selected. It may also contribute to the failure to detect a response in the CBA mouse strain which had been identified as a high responder to PTH (1-34) (Nussbaum et al, 1985). Similarly the screening assay, using radiolabelled PTH (53-84), may have played a part in the failure to detect C-terminal monoclonal antibodies. Thus it is possible that the successful production of either C-terminal or N-terminal antibodies may be a consequence of the assay methods used for antibody screening by the various groups.

Nevertheless by combining the monoclonal antibodies produced the primary aim of the work of this thesis, the development of a two-site immunometric assay specific for intact PTH (1-84), has been successfully achieved. In addition the original design specifications, that the assay should measure PTH (1-84) in all normal

subjects and be free of interference from PTH fragments, have also been met. The performance of the PTH (1-84) assay developed, in terms of limit of detection and assay precision, compares favourably with other methods (Nussbaum et al, 1987, Brown et al, 1987).

However the loading of the assay with C-terminal monoclonal antibody to avoid interference from C-terminal fragments requires the large scale production of this antibody - in this laboratory alone, on present usage, 1 g of purified monoclonal antibody is required per year. This is has significant cost implications. It is possible with alternative assay design in two-site IRMA methods to avoid the requirement for a gross excess of C-terminal monoclonal antibody by employing N-terminal 'capture' followed by a wash step to remove the C-terminal fragments before the addition of the radiolabelled antibody. The assay development studies while identifying the best combination of monoclonal antibodies to be 3B3 and ESQ1, also showed however that these antibodies did not function as well in this preferred configuration. Subsequently a two-site IRMA for PTH (1-84) in the preferred configuration of N-terminal capture, to avoid interference from C-terminal fragments, has been developed using the 6E3 monoclonal antibody, also produced in this project, in combination with an affinity purified polyclonal antibody to C-terminal PTH. This method, while involving an extra wash step, substantially reduces the volumes of reagents required with comparable assay performance (Greig et al, 1990).

This preferred configuration relies on the current evidence from clearance studies that N-terminal fragments are not produced in excess relative to intact PTH (1-84) (Bringhurst et al, 1988). There is some evidence, however, that significant amounts of N-terminal fragments are produced in some pathological situations, particularly end stage renal failure (Goltzman 1986). The monoclonal antibody 4G3 produced in this project was shown to be specific for PTH (1-34) in that it recognises PTH (1-84) poorly. It has been possible to develop a two-site IRMA for PTH (1-34) using 4G3 in combination with radiolabelled 3B3. The assay measures low picomolar

concentrations of PTH (1-34) and shows less than 0.2% cross reactivity with PTH (1-84). Studies are now in progress to identify whether significant amounts of N-terminal fragments may be produced *in vitro* or appear in the circulation *in vivo*.

The second aim of the work of this thesis was the application of the assay to the physiology and pathophysiology of calcium homeostasis. The main clinical application of PTH (1-84) assays is in the differential diagnosis of hypercalcaemia. The PTH (1-84) results from this assay allow good discrimination between normal subjects and patients with primary hyperparathyroidism. The potential for overlap between these groups noted for this assay is similar to results obtained with published PTH (1-84) assays (Nussbaum et al, 1987, Brown et al, 1987, Frolich et al, 1990). The studies on the circadian secretion of PTH (1-84) have described a defined rhythm in normal subjects with PTH (1-84) rising both in the early evening and in a broad peak through the night. There was considerable variation in the return to baseline concentrations between subjects with the greatest variability between subjects was in the period from 0600 to 1000 h. Thus, PTH (1-84) concentrations in early morning samples, such as those typically collected from in-patients, may therefore appear to be elevated spuriously. An important observation from these studies is that the discrimination between normal subjects and patients with primary hyperparathyroidism is time dependent. Thus for optimal discrimination based on PTH (1-84) concentrations samples should be taken, and reference ranges established, between 1000 and 1600 h. Further, while the synchronised circadian rhythm is lost in patients with primary hyperparathyroidism the PTH (1-84) concentrations vary markedly about the patient's 24 h mean concentration. Thus potential for overlap between these groups probably accurately reflects the pathophysiology of this condition such that patients with mild primary hyperparathyroidism may have PTH (1-84) concentrations within the reference range (Logue et al, 1990b).

The PTH (1-84) concentrations in patients with hypercalcaemia of malignancy were well separated from those in patients with primary hyperparathyroidism with most HCM patients having undetectable PTH (1-84) concentrations. Studies using intact PTH (1-84) assays have shown a higher proportion of HCM patients to have detectable PTH (1-84) concentrations (Nussbaum et al, 1987, Brown et al, 1987) than observed with the assay developed in this project. The most probable explanation for this observation lies in the selection of the HCM patients. The APD study has shown that in treated HCM patients a relatively small decrease in serum calcium can produce a rise in PTH (1-84) even though the patient remains hypercalcaemic (Fraser et al, 1990). Thus the finding of detectable PTH (1-84) in HCM patients may reflect decreases in calcium due to either a response to the initiation of treatment or perhaps fluctuations in the course of the underlying malignant condition. PTH (1-84) concentrations in samples collected after the initiation of treatment, such as rehydration or APD, should therefore be interpreted with caution in the light of these observations.

It was not possible to make a direct comparison of the performance of the PTH (1-84) IRMA with that of the N-terminal specific assay previously employed in this laboratory due to lack of reagents for the latter method. However, recent studies comparing the performance of intact PTH (1-84) IRMA with that of the best 'research' mid-molecule RIA methods have reported either equivalent (Endres et al, 1989) or superior performance of the intact PTH (1-84) methods (Wheeler et al, 1990) in the differential diagnosis of hypercalcaemia. In addition, given that the interpretation of mid-molecule assay results is difficult in renal failure, the ability of the PTH (1-84) IRMA to follow the suppression of secondary hyperparathyroidism as seen in the study in haemodialysis patients (Van Der Merwe et al, 1990) makes the PTH (1-84) IRMA the method of choice for the laboratory assessment of patients with disorders of calcium metabolism.

In common with most other hormones PTH (1-84) concentrations in the circulation vary in a manner consistent with pulsatile secretion. The pulsatile release of hormones is an important principle in dynamic hormone-receptor interaction with the the modulation of pulse amplitude and frequency being major determinants of end organ response.

The information that can be obtained from studies on the dynamics of hormone secretion is directly related to the frequency of sampling. A sampling frequency of four to five samples for every cycle to be observed and a sampling period sufficient to observe four to five cycles has been recommended to avoid problems with interpretation due to aliasing phenomena (Mathews 1988). Obviously an overriding consideration is that blood-loss in the subjects should be kept within ethical limits. The sampling period and frequency employed in this study were chosen specifically, with these considerations in mind, in the light of a previous report on the pulsatile nature of bioactive PTH (Dixit et al, 1987). The results confirmed a similar frequency of pulsatility, with pulses every 10-15 minutes, between intact PTH (1-84) and that reported for bioactive PTH. This supports the concept of PTH (1-84) being the major bioactive form of PTH in the circulation. Nevertheless more extensive sampling periods with appropriate sampling frequencies would be required both to establish the variation in pulsatility over a 24 h period and to investigate pulsatility at lower frequencies, for example, in the range of 2-4 h which have been demonstrated for other hormones.

There is general agreement that some form of objective analysis of the hormone profiles generated is required to enable valid comparison of the pulsatile secretion of hormones. The method chosen for this study is a standard mathematical technique in the analysis of time-series data. The method provides a good estimate of the dominant harmonics present taking account of the variance of the data. However analysis techniques specifically designed for the investigation of hormone pulsatility

have been developed(Urban et al, 1988). The use of such techniques would provide a more rigourous analysis of the data generated in this study as parameters of hormone secretion (half-life, clearance) and assay performance (dose-related precision) would also taken into account.

The significance of the study of patterns of hormone pulsatility lies in the observations that modulation of amplitude and frequency can have dramatic effects on end organ response. Such changes may be seen in response to a change in metabolic state or in disease states. In this study the PTH (1-84) pulsatility, at the frequencies studied, appeared to be characteristic of the individual and did not correlate with the presence of primary hyperparathyroidism. On the other hand, in the investigation of the circadian secretion of PTH (1-84), where the sampling period was 24 h and sampling frequency 0.5 h, major changes were seen in the pattern of secretion in normal subjects on fasting. This pattern was qualitatively similar to that observed in hyperparathyroid subjects in whom the circadian rhythm of PTH (1-84) is absent. This may indicate that pulsatility with frequencies in the range 2-4 h may play a part in the response to metabolic changes and be associated with primary hyperparathyroidism. Studies are underway on the analysis of the underlying pulsatility in the data from the circadian studies.

It has been shown that the profile of the circadian secretion hormones, such as ACTH and TSH, are generated by modulation of the amplitude and frequency of the underlying pulsatile secretion (Veldhuis et al, 1990, Rossmanith et al, 1988). These observations further emphasise the inter-relationship between pulsatile and circadian secretion of hormones.

The most significant finding of the circadian studies is that the PTH (1-84) concentrations in normal subjects over a 24 h period show a stronger temporal correlation with other circadian hormones, notably prolactin, than with adjusted calcium concentrations. This both challenges the view that PTH (1-84) and plasma

ionised calcium concentrations are inversely related and also suggests that other factors may be implicated in the tonic control of PTH (1-84) secretion. However, while the secretion of PTH (1-84) and prolactin follow a well defined circadian pattern such that there is a strong correlation between these hormones over a 24 h period the sleep-shift study indicated that the mechanism by which they are entrained to the biologically clock are fundamentally different. Nevertheless the evidence suggests that the control of PTH (1-84) secretion has a neuroendocrine component. In addition it was notable that the NcAMP output of the subjects in the sleep-shift experiment suggested decreased PTH bioactivity. PTH (1-84) concentrations in normal subjects are maintained in a distinct phase relationship with those of prolactin and other circadian hormones over a 24 h period. It may be that this high degree of temporal organisation allows concerted metabolic effects between these hormones especially through the night.

The most consistent relationship throughout the circadian studies is that between PTH (1-84) and serum phosphate concentrations. The rhythm in serum phosphate is qualitatively similar and coincident with that of PTH (1-84). In the fasting study both rhythms were markedly altered. Thus a role for serum phosphate in the generation of the circadian profile of PTH (1-84) could not be excluded, as had been done previously, based on the dissociation of these rhythms during fasting.

From the observation that continuous infusions of PTH are not effective in promoting bone growth and that increased bone formation requires a regimen resulting in transient rises in PTH by daily injections (Podsbeck et al, 1983, Malluche et al, 1982) it has been postulated that the nocturnal rise in PTH (1-84) is an important factor in normal bone physiology. Further it has been suggested that abnormalities of the PTH (1-84) secretion rhythm may play a part in the aetiology of osteoporosis. This concept has been strengthened by the finding of reduced pulsatility of PTH (1-84) secretion in osteoporotic patients (Harms et al, 1989). It is of note also that abnormalities have been demonstrated in osteoporotic compared to

normal elderly women in the response of PTH and 1,25 dihydroxyvitamin D to provocative testing with a phosphate challenge (Silverberg et al, 1989). In addition a survey of the effect of ageing on parameters of bone metabolism in a healthy elderly population revealed that the only major difference between males and females was in the regulation of the phosphate set-points with age (Sherman et al, 1990). Thus it would be possible to postulate a role in the bone loss associated with osteoporosis for alterations in the PTH (1-84) secretion rhythm which may be either directly related to, or merely reflected by, abnormal phosphate metabolism.

Proposals for future work

1. The original aims of this thesis in terms of the performance of the PTH (1-84) assay developed have been successful. The assay sensitivity may potentially be improved by the development of a non-isotopic label system. Whilst the clinical studies have shown that the sensitivity of the assay, using the iodine^{125} radiolabel, has not been been limiting the development of a more sensitive assay will allow equivalent performance to be attained with a smaller sample volume - thus enabling greater sampling frequencies to be employed in studies of the dynamics of PTH (1-84) secretion.

2. The development of an assay system for parathyroid hormone related peptide (by the application of a similar strategy that employed for PTH (1-84)) will allow the elucidation of the relative roles of PTH (1-84) and PTHrP in the aetiology and treatment of hypercalcaemia of malignancy and permit an understanding of the role of PTHrP in normal physiology.

3. Further work is required to evaluate the relative contributions of ionised calcium, phosphate and neuroendocrine factors to the control of the circadian rhythm of PTH (1-84) secretion.

4. It is increasingly possible that subtle alterations in the fine control of PTH (1-84) contribute to the aetiology of both type 1 and 11 osteoporosis. In order to examine this possibility it will be necessary to assess both the pulsatility and the circadian rhythm of PTH (1-84) secretion in well defined patient groups and in control subjects matched for age, sex and dietary intake.

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